DFID

Applied studies on epizootic ulcerative syndrome

Final Report

February 2001

Institute of Aquaculture
University of Stirling, Scotland

Aquatic Animal Health Research Institute
Department of Fisheries, Thailand

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Project R6979 of the Aquaculture Research Programme
of the Department for International Development of the United Kingdom

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Department of Fisheries, Thailand

PROJECT COMPLETION SUMMARY SHEET

Sheet Completed 28 February 2001

TITLE OF PROJECT: Applied studies on EUS - The ecology, immunogenicity and treatment of *Aphanomyces invadans*

R NUMBER: R6979

PROGRAMME: Aquaculture Research Programme (ARP)

PROGRAMME MANAGER (INSTITUTION): Prof J.F. Muir

PROGRAMME PURPOSE: Productive benefits of aquatic resources for poor people generated through improved knowledge of aquaculture processes and their management

PRODUCTION SYSTEM: Land/Water Interface

BENEFICIARIES: Asian freshwater fish farmers

TARGET INSTITUTIONS: AAHRI, FRI, BAU, CARE-LIFE, CIFA, BFAR, FDD, FRTI, RIA1

GEOGRAPHIC FOCUS: Southeast and South Asia

	Planned	**Actual**
START DATE:	1 July 1997	1 July 1997
FINISH DATE:	31 December 2000	28 February 2001
TOTAL COST:	£250,613	£248,613

1. Project purpose:

To generate the information needed for the formulation of strategies to contain EUS; and to develop and introduce improved prophylactic and therapeutic treatments to provide fish farmers with a means of reducing losses due to EUS.

2. Outputs:

Some modifications were made to the original logical framework in January 1999. This was partly due to problems that were encountered in developing an environmental DNA probe for *Aphanomyces invadans*, but the change meant that the project research could be redirected towards having a greater immediate impact for small-scale fish farmers. The studies that aimed to develop the DNA probe for environmental testing were scaled down and greater emphasis was placed on field studies, particularly pond and farm trials, and epidemiological surveys in Bangladesh and Nepal. The outputs stated below in inverted commas are those listed in the January 1999 logframe, along some other outputs given in the original July 1997 logframe.

2.1 DIAGNOSIS OF EUS

"Improved diagnostic procedures. Development of immunological tools for diagnosis of EUS " Jan 99.

A DNA probe procedure was developed that enables identification of cultures of *A. invadans*. A detailed description of the molecular characterisation of *A. invadans* was prepared and submitted for publication. A variety of histological stains were compared and the fluorescent stain, Uvitex, was considered the most practical and useful for the diagnosis of EUS. Further procedures were tested using the *A. invadans*-specific DNA sequence (e.g. *in situ* hybridisation, PCR of DNA extracted from paraffin embedded tissues) and monoclonal antibodies (e.g. immunohistochemistry, immunocytochemistry) but these did not prove robust enough techniques for the diagnosis of EUS.

2.2 ECOLOGY & GEOGRAPHICAL DISTRIBUTION OF *A. INVADANS*

"Better understanding of the geographical distribution of *A. invadans* and identification of areas at risk." Jan 99

An effective environmental probe could not be developed using the available DNA sequence. However, through contact with other researchers, a better understanding of the distribution of EUS was achieved. Reports of outbreaks of EUS in the region were monitored, and samples were obtained and processed at AAHRI. The project also identified an isolate of *A. invadans* from USA, confirming that the EUS fungus is involved in outbreaks of “ulcerative mycosis” in the USA. Surveys showed that *A. invadans* is endemic in natural water bodies in Bangladesh. A technique for identifying the presence of *A. invadans* by studying the serum of snakehead fish was tested in the Philippines and Thailand.

2.3 ZOOSPORE PHYSIOLOGY

"Based on *in vitro* studies, an understanding of developmental morphology of zoospores, optimal and lethal conditions for zoospore production and motility" July 97

Zoospore physiology studies provided information that enabled management recommendations to be made on the exclusion of *A. invadans* from fish farms.

2.4 TANK STUDIES

"A challenge system tested and available for further studies, including fungal pathogenicity in relation to other disease agents." July 97

Challenge systems were developed in which snakehead developed EUS after abrasion or being placed in pH5 water for 30 mins, and then being bathed in *A. invadans* zoospores. The systems were then used in pond trials to assess the ability of various treatments to prevent EUS in snakehead and mrigal. Some challenge work was undertaken using bacterial pathogens in Bangladesh and India. Challenge studies also investigated the susceptibility of various non-Asian fish species to *A. invadans* infection.

2.5 TREATMENT STUDIES

"screening of candidate fungicides" July 97

"Pond-tested treatments and interventions that prevent, or reduce the risk of, EUS outbreaks." Jan 99

Forty-nine candidate treatments were screened *in vitro* for activity against *A. invadans*. Some of these were tested in toxicity trials using silver barbs. Several treatments that showed promise were assessed further in pond trials in Thailand, Bangladesh and India. Acceptance and uptake of the treatments by farmers in Bangladesh were assessed using farmer field trials in collaboration with the CARE-LIFE project. Tank trials testing therapeutic treatments on naturally infected snakeheads and climbing perch showed that the chemicals were not effective against the fungus once it had already infected the fish.

2.6 IMMUNOLOGY

"An understanding of the potential for inducing EUS-resistance in susceptible fish. Demonstration of immunoprevalence of EUS." Jan 99

A comparison of the immune reactions of various fish species revealed differences in the effectiveness of the immune mechanisms of particular susceptible and resistant fish. In tank trials, particular immunostimulants were shown to be effective in reducing mortality of snakeheads challenged with *A. invadans.* A tank challenge study showed that serum from

snakeheads from endemic areas conferred a degree of protective immunity on naïve snakeheads. These studies demonstrate that protective antibodies against *A. invadans* are produced in fish, and that there is potential for vaccine development.

2.7 EPIDEMIOLOGY

"Identification of factors that increase the risk of EUS in Bangladesh" Jan 99

Cross-sectional studies in Bangladesh and Nepal successfully identified risk factors regarding pond management that significantly increase the chance of EUS outbreaks in ponds. Case-control studies in Mymensingh, Bangladesh identified pond water variables and aspects of fish biology that are risk factors for EUS.

2.8 DISSEMINATION

"Dissemination of information on current understanding of EUS causation, and methods for diagnosis and control, to fisheries officers and scientists." Jan 99

An EUS manual was produced and over 800 copies distributed, mainly on request. Various workshops and seminars were conducted in Thailand, Pakistan, Bangladesh, Philippines, Nepal, India, Sri Lanka and Vietnam (see Section 6). A leaflet describing control strategies for EUS was also produced and disseminated.

3. Contribution of outputs to project goal:

Various management techniques and preventative pond treatments have been identified that contribute to the goal of "reducing the impact of diseases on aquaculture production in Asia". Some treatments were assessed by farmers participating in the CARE-LIFE EUS study. Satisfaction with the treatments and uptake in the subsequent season was high among these farmers, indicating that the treatments were suitable for the farmers. Higher-cost treatments identified by the project (e.g. immunostimulants and surfactants) may be suitable for use by commercial farmers.

Dissemination activities have resulted in an increased consensus among fisheries researchers in the region about EUS causation, as demonstrated by the studies reported in regional conferences. It is hoped that this will help focus research away from the study of particular pathogens and towards work on treatments for the disease, which would have greater immediate impact on farmers. Increased acceptance of a definition for EUS, and improved diagnostic techniques, will also enable laboratories to monitor outbreaks and help prevent further spread to unaffected regions.

4 Peer reviewed publications:

1998

- Khan, M.H., Marshall, L., Thompson, K.D., Campbell, R.E., and Lilley, J.H. (1998) Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the Oomycete fish pathogen, *Aphanomyces invadans*. Bulletin of the European Association of Fish Pathologists 18(6), 192-197.

1999

- Blazer, V.S., Vogelbein, W.K., Densmore, C.L., May, E.B., Lilley, J.H., and Zwerner, D.E. (1999) *Aphanomyces* as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. Journal of Aquatic Animal Health 11, 340-349. (Nominated for American Fisheries Society "Best Paper Award")

- Thompson, K.D., Lilley, J.H., Chen, S.-C., Adams, A., and Richards, R.H. (1999) The immune response of rainbow trout (*Oncorhynchus mykiss*) against *Aphanomyces invadans*. Fish and Shellfish Immunology 9(3), 195-210.

2000

- Blazer V., Vogelbein W.K., Densmore C., Kator H., Zwerner D., and Lilley J. (2000) Etiology and pathogenesis of skin ulcers in menhaden, *Brevoortia tyrannis*: does *Pfiesteria piscicida* play a role? Marine Environmental Research 50(1), 487-488.
- Lilley, J.H., and Chinabut, S. (2000) DNA-based studies on *Aphanomyces invadans,* the fungal pathogen of epizootic ulcerative syndrome (EUS). In: Walker, P. and Subasinghe, R. (Eds), DNA-based Molecular Diagnostic Techniques: Research Needs for Standardization and Validation of the Detection of Aquatic Animal Pathogens and Diseases. Pp. 83-87 FAO/NACA/ACIAR/CSIRO/DFID, Bangkok, Thailand.

2001

- Miles, D.J.C., Polchana, J., Lilley, J.H., Kanchanakhan, S., Thompson, K.D., and Adams, A. (2001) Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome (EUS). Aquaculture 195(1-2), 1-15.

in press:

- Campbell, R.E., Lilley, J.H., Taukhid, Panyawachira, V., and Kanchanakhan, S. (2001) *In vitro* screening of novel treatments for *Aphanomyces invadans*. Aquaculture Research
- Khan, M.H., Lilley, J.H., Majumder, B., Sarker, M.G.A., Alauddin, M., Hoque, A., Ahmed, G.U., and Chowdhury, M.B. (2001) Cross-sectional survey of epizootic ulcerative syndrome (EUS) cases in Bangladesh. Diseases in Asian Aquaculture IV
- Lilley, J.H., Beakes, G.W., and Hetherington, C.S. (2001) Characterization of *Aphanomyces invadans* using pyrolysis mass spectrometry (PyMS). Mycoses 3-4,
- Miles, D.J.C., Kanchanakhan, S., Lilley, J.H., Thompson, K.D., Chinabut, S., and Adams, A. (2001) Effect of macrophages and serum of fish susceptible or resistant to epizootic ulcerative syndrome (EUS) on the EUS pathogen, *Aphanomyces invadans*. Fish and Shellfish Immunology

5. Other publications

1998

- FRTI (1998) A report regarding visit of mission to Punjab in connection with epizootic ulcerative syndrome (EUS). 20–25 April 1998. 24 pp. Sponsored by Department for International Development (DFID), UK. Fisheries Research and Training Institute, Dept of Fisheries, Punjab, Lahore
- Lilley, J.H., Callinan, R.B., Chinabut, S., Kanchanakhan, S., MacRae, I.H., and Phillips, M.J. (1998). Epizootic ulcerative syndrome (EUS) technical handbook. Aquatic Animal Health Research Institute, Bangkok. 88pp.

1999

- Callinan, R.B., Chinabut, S., Mohan, C.V. and Lilley, J.H. (1999) Report of EUS Extension Visits to Nepal, India and Sri Lanka. 7-20 June 1999. ACIAR and DFID. 32 pp
- Lilley, J., Bangyeekhun, E., Panyawachira, V., and Cerenius, L. (1999) Zoospore physiology of *Aphanomyces invadans* 1. Polyplanetism. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok. 8(2), 6-8.
- Lilley, J.H. (1999) Bibliography of references on epizootic ulcerative syndrome (EUS) in Asia. Working document. Institute of Aquaculture, University of Stirling (510 references, 28 pages)

- Lilley, J.H. (1999) Bibliography of references on fungal pathogens of aquatic animals. Working document. Institute of Aquaculture, University of Stirling (885 references 42 pages)
- Miles, D.J.C. (1999) Lessons learned in macrophage culture. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok. 8(2), 9-12.

2000

- Lilley, J., and Panyawachira, W. (2000) Zoospore physiology of *Aphanomyces invadans* 2. Geotaxis. The AAHRI Newsletter, 9(1), Pp. 1-2. Aquatic Animal Health Research Institute, Bangkok.
- Lilley, J., Chinabut, S., and Khan, M. (2000) Current prevalence of epizootic ulcerative syndrome (EUS) and strategies for control. Aquaculture News, Institute of Aquaculture, University of Stirling 26, 13-16.

2001

in press:

- Khan, M.H., and Lilley, J.H. (2001) Risk factors and socio-economic impacts associated with epizootic ulcerative syndrome (EUS) in Bangladesh. In: Proceedings of DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999.
- Lilley, J.H., Callinan, R.B., and Khan, M.H. (2001) Social, Economic and Biodiversity Impacts of EUS. In: Proceedings of DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999.
- Lilley, J., Petchinda, T., and Panyawachira, W. (2001) Zoospore physiology of *Aphanomyces invadans* 3. Techniques for inducing sporulation. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok.
- Lilley, J., Petchinda, T., and Panyawachira, W. (2001) Zoospore physiology of *Aphanomyces invadans* 4. *In vitro* viability of cysts. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok.

6. Other dissemination of outputs

DFID Reports:

- Thirteen quarterly reports, three annual reports and eight trip reports.

Farmer meetings:

- During the course of research studies and diagnostic work, 114 Bangladeshi farmers, 64 Bangladeshi fishermen, 60 Nepali farmers and 40 Thai farmers have been interviewed, involving discussions of control strategies.
- The project supported a series of Farmer Science Congresses (FSC) and field days facilitated by CARE-LIFE staff (April-May 2000) for 567 farmers in Rajshahi and Kishorganj to discuss results of EUS treatments.

Websites:

- J. Lilley assisted in the design of the “Fungus” and “Syndrome” templates for the online FAO database “Aquatic Animal Pathogen and Quarantine Information System” (AAPQIS) <www.agri-aqua.ait.ac.th/aapqis/>. The webpages on EUS and *Aphanomyces invadans* will be kept up-to-date.

1998

Theses:

- Marshall, L. (1998). Histopathological assessment of an experimental challenge with *Aphanomyces invadans*, the necessary agent of epizootic ulcerative syndrome (EUS), in rainbow trout (*Oncorhynchus mykiss*) at different temperatures and in three native fish species: roach (*Rutilus rutilus*), stickleback (*Gasterosteus aculeatus*) and rainbow trout (*O. mykiss*). BSc thesis, University of Stirling, Scotland

Conference presentations:

- Campbell, R.E., Lilley, J.H., and Richards, R.H. (1998) *In vitro* assays to test novel treatments of *Aphanomyces invadans*. Oral presentation at the Third International Symposium on Aquatic Animal Health, Baltimore, USA, 30 August - 3 September 1998.
- Campbell, R.E., Lilley, J.H., and Richards, R.H. (1998) The use of natural products in the treatment of EUS (Epizootic Ulcerative Syndrome). Oral presentation at the Fifth Asian Fisheries Forum, Chiangmai, Thailand, 11-14 November 1998.
- Thompson, K.D., Lilley, J.H., Miles, D., Chinabut, S. and Adams, A. (1998) The immune response of rainbow trout *(Oncorhynchus mykiss)* and snakehead fish (*Channa striata*) against *Aphanomyces invadans.* Oral presentation at the Fifth Asian Fisheries Forum, Chiangmai, Thailand, 11-14 November 1998.

Workshops:

- J. Lilley, S. Chinabut and R. Campbell delivered an AAHRI workshop on EUS to 10 regional participants. Two reports were produced: (i) Lilley, J.H. (1998) Report of DFID/RCS visit to Thailand to deliver a Workshop on the Fungal Aetiology of EUS. 18-31 January 1998. University of Stirling. (ii) AAHRI (1998) Workbook for the Workshop on The Fungal Aetiology of EUS 35pp.
- S. Chinabut, J. Lilley and K. Morgan gave half-day workshops to about 50 staff at the Fisheries Research and Training Institute (FRTI), Lahore and 50 staff and farmers at the Sindh hatchery and training centre, (Pakistan April 1998). A pamphlet on EUS was produced in Urdu and is being distributed country-wide by the Office of the Fisheries Development Commissioner.

Interviews:

- Anon (1998) Prevention better than cure. [WWW document] New Agriculturist On-line. (Date posted: 4 August 1998) http://www.new-agri.co.uk/98-5/focuson/focuson8.html.

1999

Theses:

- Fairweather, D. J. (1999). Development of a bath challenge system to study component causes and preventative treatments of epizootic ulcerative syndrome (EUS) in snakehead fish (*Channa striata*). MSc thesis, Plymouth University
- Sihalath, S. (1999). Studies on zoospore physiology and chemotaxis of *Aphanomyces invadans*. MSc thesis, University of Stirling, Scotland
- Taukhid. (1999). *In vitro* testing of chemicals and natural products for treatment of epizootic ulcerative syndrome (EUS). MSc thesis, University of Stirling, Scotland

Conference presentations:

- Chinabut, S. (1999) The EUS disease of freshwater fish caused by *Aphanomyces invadans*. Oral presentation at: The 7th International Marine and Freshwater Mycology Symposium, City University, Hong Kong, 4-9 July 1999.
- Khan, M.H., Lilley, J.H., Majumder, B., Sarker, M.G.A., Alauddin, M., Hoque, A., Ahmed, G.U., and Chowdhury, M.B. (1999) Cross-sectional survey of epizootic ulcerative syndrome (EUS) cases in Bangladesh. Oral presentation at: Fourth Symposium on

Diseases in Asian Aquaculture. Cebu City, Philippines, 22-26 November 1999. Asian Fisheries Society, Philippines.

- Khan, M.H., and Lilley, J.H. (1999) Risk factors and socio-economic impacts associated with epizootic ulcerative syndrome (EUS) in Bangladesh. Oral presentation at: DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999.
- Khan, M.H., Marshall, L., Thompson, K.D., Campbell, R.E., and Lilley, J.H. (1999) Susceptibility of five fish species to epizootic ulcerative syndrome (EUS) following intramuscular injection with the Oomycete fish pathogen, *Aphanomyces invadans*. Poster presented at: Conference of the European Association of Fish Pathologists, Rhodes, Greece, 19-24th September 1999. P-222
- Lilley, J.H. (1999) DNA-based studies on *Aphanomyces invadans*, the causative fungus of epizootic ulcerative syndrome (EUS). Oral presentation at: Expert Consultation on the Research Needs for Standardization and Validation of DNA-based Molecular Diagnostic Techniques for the Detection of Aquatic Animal Pathogens and Diseases, Bangkok, 7-9 February 1999.
- Lilley, J.H., Callinan, R.B., and Khan, M.H. (1999) Social, Economic and Biodiversity Impacts of EUS. Oral presentation at: DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999.
- Miles, D.J.C., Seetubtim, P., Lilley, J.H., Kanchanakhan, S., Thompson, K.D., Chinabut, S., and Adams, A. (1999) The *in vitro* effect of macrophages and serum of EUS susceptible and non-susceptible fish species on the germination of *Aphanomyces invadans*. Oral presentation at: Fourth Symposium on Diseases in Asian Aquaculture. Cebu City, Philippines, 22-26 November 1999. Asian Fisheries Society, Philippines.
- Panyawachira, V., Lilley, J.H., Hart, D., and Kanchanakhan, S. (1999) A PCR-based technique for the identification of *Aphanomyces invadans*. Poster presentation at: Fourth Symposium on Diseases in Asian Aquaculture, Cebu City, Philippines, 22-26 November 1999. Asian Fisheries Society, Philippines.

Seminars:

- S. Chinabut, J. Lilley, R.B. Callinan and C.V. Mohan held one-day seminar and discussion sessions at the Fisheries Development Division, Ministry of Agriculture, Kathmandu, Nepal (60 participants from 22 districts); the Central Inland Capture Fisheries Research Institute (CICFRI), Barrackpore, India (20 participants); the Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India (15 participants); the Department of Aquatic Biology and Fisheries, University of Kerala, India (17 participants); and the National Aquatic Resources Research and Development Agency (NARA), Colombo, Sri Lanka (56 participants) (May 1999).
- J. Lilley gave a series of lectures and practicals to staff at the Research Institute for Aquaculture No.1 (RIA1) on EUS, fungal diseases of fish and fungal isolation and identification (June 1999)
- D. Miles addressed a meeting of about 50 fisheries extension officers in Mindanao, where EUS has recently been reported for the first time. (November 1999)

2000

Seminars:

- J. Lilley and M. Khan addressed about 80 Bangladesh Agriculture University (BAU) and Fisheries Research Institute (FRI) staff and student about EUS causation and control (March 2000).
- D. Miles and J. Lilley gave presentations on project results to staff of AAHRI and Kasetsart University (April 2000 and August 2000)

2001

Theses:

- Miles, D.J.C. (2001) Studies on host immune responses to *Aphanomyces invadans*. PhD thesis, University of Stirling, Scotland.
- Khan, M. H. (2001) Epidemiological studies of epizootic ulcerative syndrome (EUS) in Bangladesh. PhD thesis, University of Stirling, Scotland.

7. Follow-up indicated/planned:

7.1 No research projects are planned specifically on EUS, but it is envisaged that further research on diagnosis and treatment of EUS will be carried out at the institutes where the project has disseminated knowledge and research tools on EUS.

7.2 The CARE-LIFE farmer field trials on EUS will continue this season, and it is envisaged that, with input from ILEIA, Netherlands further studies will be conducted in the following season.

7.3 Further applications will be made for funds for EUS dissemination trips to areas that have not been effectively covered by the current project (e.g. Laos, Cambodia, southern Vietnam and southern China)

7.4 A further DFID-ARP project is being considered with the aim of promoting effective aquatic animal health services through identification of key institutional linkages and priority knowledge areas.

8. Name and signature of authors of this report:

J.H. LILLEY S. CHINABUT D.J.C. MILES

EXECUTIVE SUMMARY

This project was conceived to make applied use of the findings of previous DFID-funded projects which identified and characterised causal agents of epizootic ulcerative syndrome (EUS), in particular the fungal pathogen, *Aphanomyces invadans*. The tools and knowledge generated from these projects were utilised in a variety of research activities aimed at improving techniques for the diagnosis and control EUS. Research was conducted jointly with a number of regional institutes to encourage contact between researchers, and help build up a consensus among fisheries workers regarding EUS causation and control.

DNA probes, designed by previous projects, were developed for use in the diagnosis of EUS. It was not possible to develop a technique to identify *A. invadans* DNA in the environment, so studies concentrated on using histological diagnosis for disease confirmation. A comparative study of histological techniques identified Uvitex-H&E staining as the most rapid and reliable diagnostic technique. Contact with international scientists allowed verification of the presence of EUS in Nepal, Vietnam and Pakistan by histological diagnosis, and in USA by histology and genetic characterisation of *A. invadans.*

Studies on zoospore physiology demonstrated characteristics of the fungus relevant to the development of control strategies. *In vitro* studies on the fungus were also used to screen 50 compounds for antifungal activity. Candidate treatments were then tested in tank and pond trials.

Bath challenge models were developed for use in pond trials to test preventative treatments. These trials were conducted in Thailand, Bangladesh and India. A variety of low-cost inputs were found to reduce the number of fish affected and/or the severity of infections, but none were able to prevent infection completely. Farmer-based studies in collaboration with the CARE-LIFE project showed that farmers were keen to adopt treatments, and believed them to be successful.

Immunological studies demonstrated differences in the immune reaction of EUS-susceptible and EUS-resistant fish. Resistant fish tend to have a more effective cellular response to fungal infection. An immunostimulant trial indicated that Salar-bec is effective in boosting this non-specific response in susceptible snakeheads. Trials also showed that snakeheads from EUS-endemic areas have a strong serum response *to A. invadans* infection, and a passive immunisation trial showed that the antibodies involved are protective. This shows that there is potential for vaccine development, although this was not pursued further on this project.

Epidemiological studies in Bangladesh and Nepal identified a range of factors that increased the risk of EUS infection. Most significant of these was connection of ponds to natural water bodies and presence of wild fish in the pond. This was supported by prevalence data, which showed that EUS is endemic in natural water bodies in most of the areas examined. A range of management strategies to reduce the risk of EUS outbreaks was formulated.

Collaborative research, seminar tours and production and dissemination of manuals and leaflets ensured a widespread awareness of the project and its research outputs in South and Southeast Asia.

ABBREVIATIONS

AAHRI - Aquatic Animal Health Research Institute, Thailand
ACIAR - Australian Centre for International Agriculture Research
AFDO - Assistant Fisheries Development Officers, Nepal
APH3-APH4 - *Aphanomyces invadans* diagnostic DNA primers
APW - autoclaved pond water
ARP - Aquaculture Research Programme
ATCC - American Type Culture Collection
AVL - Aquatic Vaccines Ltd
BAU - Bangladesh Agricultural University
BFAR Bureau of Fisheries and Aquatic Resources
CICFRI - Central Inland Capture Fisheries Research Institute, India
CIFA - Central Institute of Freshwater Aquaculture, India
CSIRO - Commonwealth Scientific and Industrial Research Organisation, Australia
DAB - 3,3'Diaminobenzidine
DFID - Department for International Development, United Kingdom
DIG - digoxigenin
DoF - Department of Fisheries
ELISA - enzyme linked immunosorbent assay
EUS - epizootic ulcerative syndrome
FAO - Food and Agriculture Organisation of the United Nations
FDD - Fisheries Development Division
FITC - fluorescin isothiocyanate
FP1-FP2 - *Aphanomyces invadans* diagnostic DNA primers
FRI - Fisheries Research Institute, Bangladesh
FRTI - Fisheries Research & Training Institute, Pakistan
FSC - Farmer Science Congress
GMS - Grocott's methenamine silver
GP - glucose-peptone medium
GPY - glucose-peptone-yeast medium
H&E - haemotoxylin and eosin
ICLARM - International Center for Living Aquatic Resources Management
IFAT - indirect fluorescent antibody technique
IgG - immunoglobulin G
IHC – immunohistochemistry
ILEIA - Centre for Research and Information on Low External Input & Sustainable Agriculture, Netherlands
im - intra-muscular
IMI International Mycological Institute
IoA - Institute of Aquaculture, Stirling University
ITS1 internal transcribed spacer region
KW - Kruskal-Wallis
LIFE - Locally Intensified Farming Enterprises
m - million
MAb - monoclonal antibody
MG - mycotic granulomatosis
MSM - sporulating medium
NACA - Network of Aquaculture Centres in Asia, Bangkok
NARA - National Aquatic Resources Research and Development Agency, Sri Lanka
NGO - non-governmental organisation
NIFI - National Inland Fisheries Institute, Bangkok
NPV - Net Present Value
NSW - New South Wales
OIE - Office International des Epizooties
OR - odds ratio
PARG - participatory action research group
PAS - periodic acid - Schiffs

PBS - phosphate buffer saline
PCR - polymerase chain reaction
pdf - Adobe Acrobat file format
PET - paraffin embedded tissues
PG-1 - peptone-glucose-1 medium
pi - post-injection
ppm - parts per million
ppt - parts per thousand
PyMS - pyrolysis mass spectrometry
RAPD - random amplification of polymorphic DNA
RFLP - restriction fragment length polymorphism
RIA1 - Research Institute for Aquaculture No. 1, Hanoi, Vietnam
RR - relative risk
rRNA - ribosmal RNA
RSD - redspot disease
SDS-PAGE - sodium dodecyl sulphate polyacylamide gel electrophoresis
SEAADCP - Southeast Asian Aquatic Disease Control Project
SEAFDEC - South East Asian Fisheries Development Centre, Iloilo, Philippines
TCP - Technical Cooperation Project
UM - ulcerative mycosis (menhaden disease)
USGS - United States Geological Survey

CONTENTS

LIST OF APPENDICES

1. PAPER ONE - Lilley, J.H., Callinan, R.B., and Khan, M.H. (2001) Social, Economic and Biodiversity Impacts of EUS. In: Proceedings of DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999. (In press)

2. PAPER TWO - Lilley, J.H., and Chinabut, S. (2000) DNA-based studies on *Aphanomyces invadans,* the fungal pathogen of epizootic ulcerative syndrome (EUS). In: Walker, P. and Subasinghe, R. (Eds), DNA-based Molecular Diagnostic Techniques: Research Needs for Standardization and Validation of the Detection of Aquatic Animal Pathogens and Diseases. Pp. 83-87 FAO/NACA/ACIAR/CSIRO/DFID, Bangkok, Thailand.

3. Panyawachira, V., Lilley, J.H., Hart, D., and Kanchanakhan, S. (1999) A PCR-based technique for the identification of *Aphanomyces invadans*. Poster presentation at: Fourth Symposium on Diseases in Asian Aquaculture, Cebu City, Philippines, 22-26 November 1999. Asian Fisheries Society.

4. PAPER THREE - Lilley, J.H., Hart, D., Panyawachira, V., Kanchanakhan, S., Cerenius, L., and Söderhäll, K. (2001) Molecular characterization of the fish-pathogenic fungus *Aphanomyces invadans*. Mycological Research (draft, figures available from ARP Manager)

5. PAPER FOUR - Lilley, J.H., Beakes, G.W., and Hetherington, C.S. (2001) Characterization of *Aphanomyces invadans* using pyrolysis mass spectrometry (PyMS). Mycoses 3-4, (in press)

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28. Overview of Nepal cross-sectional study (hard copy of figures available from ARP Manager)

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30. Callinan, R.B., Chinabut, S., Mohan, C.V. and Lilley, J.H. (1999) Report of EUS Extension Visits to Nepal, India and Sri Lanka. 7-20 June 1999. ACIAR and DFID. 32 pp (pdf or hard copy available from ARP Manager)

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1. BACKGROUND

1.1 Epizootic ulcerative syndrome (EUS)

1.1.1 Background

Epizootic ulcerative syndrome (EUS) has been the cause of fish-kills in culture and capture fisheries in Southeast and South Asia for almost 20 years. Earlier outbreaks of mycotic granulomatosis (MG) in Japan and red spot disease (RSD) in Australia are now know to be the same as EUS. During serious outbreaks, the disease has had significant impacts on the livelihoods of rural fishermen and small-scale farmers in the region. The history of EUS is reviewed in detail in the final report of project R5997 (Lilley & Thompson, 1997) and in the handbook produced at the beginning of the present project (Lilley *et al*., 1998; Appendix 31). Information obtained early in the project on prevalence and impacts of the disease is reviewed by Lilley *et al*. (2001a, Appendix 1).

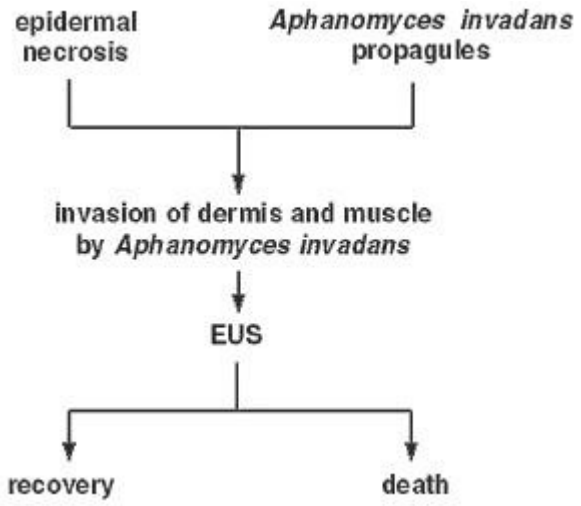
1.1.2 Case definition

An accepted case definition for EUS, and the definition used for the purposes of this project, is: "a fish with necrotising granulomatous dermatitis and myositis associated with hyphae of *Aphanomyces invadans*". This is a slightly modified definition of that given in Roberts *et al*. (1994), and requires histological examination of tissues to identify fungus and associated granulomas.

1.1.3 EUS causation

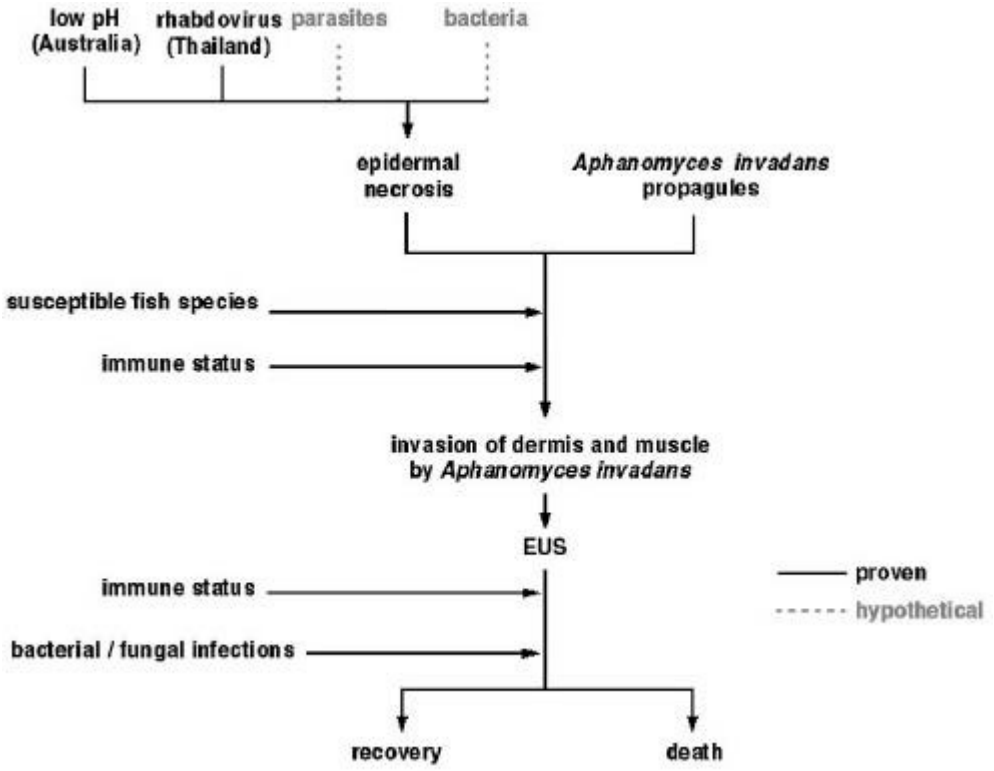
For most diseases, including EUS, there is strong evidence that outbreaks occur only when a number of causal factors combine. The causal factors for EUS that have been identified may be represented in a causal web (Figures 1.1). Two factors illustrated in Figure 1.1, (i) exposure of dermis and (ii) attachment and invasion by *A. invadans*, are considered 'necessary causes' for EUS to occur. These factors result in mycotic granulomatous dermatitis and myositis, which are, by definition, EUS.

Figure 1.1 Causal web showing necessary causes of EUS



It is important to recognise that, under different circumstances, different combinations of 'component causes' may lead to exposure of the dermis and infection by *A. invadans*. Figure 1.2 illustrates some proven and hypothesised component causes. During EUS outbreaks in Australia, fish are exposed to low pH water, in the presence of *A. invadans* propagules, and these conditions have been shown experimentally to reproduce EUS in susceptible fish (Callinan, 1997). However, in other areas where EUS occurs, the water is not low pH and other component causes (e.g. rhabdoviruses in Thailand) have been considered.

Figure 1.2 Causal web showing component causes of EUS



1.2 Previous DFID-funded EUS projects

1.2.1 Surveys

IoA involvement in EUS research started following a training workshop in Thailand, where workshop participants identified EUS as the most serious disease affecting Thai fisheries. The DoF and university scientists had already conducted a series of multidisciplinary studies into the disease and conducted meetings to discuss possible causes and control measures (Chulalongkorn University 1983; 1985; 1987; Ulcerative Fish Disease Committee, 1983). As disease outbreaks were known to be affecting neighbouring countries an FAO Technical Cooperation Project (TCP) was arranged to investigate affected areas. The survey team made histological, bacteriological, virological, and water quality investigations (Roberts *et al*., 1986). This helped to rule out several previously suspected causes such as agricultural run-off, as they were not present at all affected sites.

DFID-funded research into EUS started when outbreaks of the disease were reported from Bangladesh. A multidisciplinary team made detailed studies of the outbreaks (Roberts *et al.,* 1989). Further research priorities were identified, including mycological research as a fungal component was recognised in histological sections of affected fish.

1.2.2 Virology

The nature of the spread of EUS was thought to be indicative of a virus disease, and several early studies in Thailand had identified virus-like particles in EM sections (Wattanavijarn *et al.,* 1983; 1984), and virus-like activity in cell lines inoculated with infected material (Tangtrongpiros *et al.,* 1983). Rhabdoviruses were isolated from several affected sites (Frerichs *et al*., 1986) and birnaviruses (Hedrick *et al.,* 1986), a reovirus (Saitanu *et al.,* 1986) and a retrovirus (Frerichs *et al.,* 1991) were also implicated in the disease. Subsequent DFID-funded projects (R4222 and R5430) supported research at IoA, Glasgow University and DoF, Thailand. Frerichs (1995) reviewed the findings, stating that the heterogeneous nature of the isolates (Frerich *et al.,* 1988; 1989; Lilley & Frerichs, 1994), together with a low and inconsistent level of recovery from diseased specimens, suggested that they may only represent adventitious infections unrelated to outbreaks of EUS. Furthermore, experimental induction of the condition by direct exposure to isolated viruses was not achieved (Frerichs *et al*., 1993). Interest in viral agents has recently been revived by the finding that several more rhabdovirus isolates have been obtained from early stages of infection from ulcerated snakeheads in Thailand (Kanchanakhan *et al*., 1998). Snakeheads injected with a specific rhabdovirus culture, and bathed in spores of *A. invadans* have also been shown to develop typical EUS-lesions (Kanchanakhan, 1996). Therefore rhabdoviruses may be a component cause of EUS in Thailand, but in other areas where viruses have not been isolated, despite intensive efforts (Callinan, 1997; Callinan *et al.,* 1997; Roberts *et al.,* 1989), they are not considered to be involved in outbreaks.

1.2.3 Southeast Asian Aquatic Disease Control Project (SEAADCP)

It was partly as a result of the impact of EUS on aquaculture in the region that DFID initiated the Southeast Asia Aquatic Disease Control Project (SEAADCP) to promote research and training on health management of cultured aquatic animals. The project was based at AAHRI in Bangkok, and assisted AAHRI's development out of the Fish Health Section of the National Inland Fisheries Institute (NIFI) of the Thai Department of Fisheries. AAHRI has since expanded to become an international-class research institute and is currently the only OIE-approved laboratory for the diagnosis of EUS.

1.2.4 International Seminar

In 1994, DFID sponsored an EUS Regional Seminar in Bangkok that brought together most of the researchers on EUS. Research on EUS was comprehensively reviewed, participants agreed upon the first case definition of the disease, and future research priorities were identified (Roberts *et al.,* 1994).

1.2.5 Mycology

Following the Bangladesh survey (Roberts *et al.,* 1989), awareness of the involvement of mycotic granulomas in affected fish increased, and DFID research projects were arranged to assess the mycotic involvement, and identify the invasive fungus/fungi (R4723, R5680). As a result, a specific invasive fungus was identified and named *Aphanomyces invaderis*

(Willoughby *et al*., 1995). Subsequent projects were undertaken at IoA (R5997) and Glasgow university (R5902Cb) to characterise the pathogen in relation to other fungi. These studies showed that *A. invaderis* was the only fungus capable of reproducing EUS lesions (Lilley & Roberts, 1997), and that it was identical to *Aphanomyces piscicida* from mycotic granulomatosis (MG) outbreaks in Japan, and *Aphanomyces* isolates from red spot disease (RSD) outbreaks (Lilley & Thompson, 1997). These diseases are now all commonly referred to as EUS and the fungal pathogen is listed in the Index of Fungus as *Aphanomyces invadans* Willoughby *et al.* 1995 (David & Kirk, 1997).

1.3 The present project: R6979

1.3.1 Background

As research breakthroughs were made in previous DFID-EUS projects, this project was set up to make applied use of these findings. Knowledge about EUS causation had been established, and preliminary DNA and antibody work had been undertaken to characterise *A. invadans*. This project aimed to make this information more widely available to researchers; to test control measures against EUS that would benefit the farmers directly; and to use the molecular and immunological tools to study the spread of the fungus and provide improved diagnostic techniques to laboratories. At the time of the start of this project, the disease had been reported in Pakistan for the first time. Therefore, issues about the spread of the disease were considered of particular importance.

1.3.2 Identification of research strategies and partnerships

The long-standing relationship between Stirling and AAHRI, and the presence of the SEAADCP project at AAHRI, made this a logical partnership to pursue this research. Complimentary funding for work also became available from British Council-Bangladesh and Aquatic Vaccines Ltd, but these organisations had little influence on the identification of research priorities. Initial research strategies were decided primarily through discussions with AAHRI and other regional fish health researchers. A workshop was conducted at AAHRI at the beginning of the project, and it was envisaged that further strategies and collaborations would develop from this.

1.3.3 Collaborators

The individuals who worked on the project from IoA include J.H. Lilley, D.J.C. Miles and R.E. Campbell; from AAHRI include S. Chinabut, V. Panyawachira, S. Kanchanakhan, and K. Tonguthai, and from the Bangladesh Fisheries Research Institute (FRI) include M.H. Khan. Aside from these, other institutes and individuals that collaborated with project workers to obtain project outputs are listed in Table 1.1.

1.3.4 Research processes

This project combined laboratory studies; tank and pond trials; and farmers-participatory studies. The project purpose is given in Section 2, which emphasises that all activities were aimed at the control of EUS. The means by which project activities contributed to the project purpose are illustrated in Figure 4.1.

Table 1.1 Collaborators

Institution	Type	Key contact	Agreement	Functions
Bangladesh Agricultural University (BAU)	Univ[1]	MBR Chowdhury, GU Ahmed	Provision of lab facilities, supervision of Mr Khan	Epidemiology of EUS, pond trials
CARE-Bangladesh	NGO	MC Nandeesha, G Chowhan	Project-funded study	Farmer-participatory EUS trials
ICLARM & partners	Inter-Govn & NGOs	Paul Thompson	Provision of samples and questionnaires	Study of EUS in sanctuaries
Ayudthya Fish. Dev. Cent., Thailand	Govn[2]	Suparat Chatjariyawet	Provision of pond facilities	EUS pond trial
Office of Botanical Pesticides, Thailand	Govn	Attanon Paneeka	Provision of neem samples and information	Herbal treatments for EUS
Ministry of Public Health, Thailand	Govn	Ang-kana Herunsalee	Provision of Thai herbal treatments	Herbal treatments for EUS
Central Institute of Freshwater Aquaculture (CIFA), India	Govn	S Ayyappan, BK Mishra, RK Dey, SC Mukherjee	Project-funded study	EUS pond treatment trials
Fisheries Development Division, Nepal	Govn	Deep Swar, Shankar Dahal,	Proposed project-funded study	Prevalence & epidemiology of EUS
Aquatic Vaccines Ltd (AVL)	Indus[3]	Patrick Smith	Funding project PhD student, David Miles	Immunology of EUS
Bureau of Fisheries & Aquatic Resources (BFAR), Philippines	Govn	Juan Albaladejo, Susan Mayo	Provision of tank and sampling facilities	Joint seroprevalence and passive immunisation trials
Research Institute for Aquaculture 1 (RIA1), Vietnam	Govn	Phan Thi Van	Training provided by JHL (NORAD funded), joint studies proposed	Information dissemination
Fisheries Research & Training Institute (FRTI), Pakistan	Govn	Muhammad Ayub	Training provided by project, joint studies proposed	Dissemination, EUS prevalence
Sindh Fisheries Department, Pakistan	Govn	Qamar Baloach	Training provided by project, joint studies proposed	Dissemination, EUS prevalence
Ministry of Fisheries & Livestock, Pakistan	Govn	Mohammed Moazzam Khan, Rukshana Anjum	Training provided by project, joint studies proposed	Dissemination, EUS prevalence
NSW Fisheries, Australia	Govn	Richard Callinan	Participation on trip, joint publication	EUS manual, dissemination trips
Australian Centre for International Agriculture Research (ACIAR)	Govn	Barney Smith	Joint ACIAR-DFID funding of extension and dissemination materials	EUS manual, Pakistan pamphlet, Nepal-India-Sri Lanka trip
US Geological Survey (USGS)	Govn	Vicki Blazer	Exchange of techniques, materials, results, joint publication	Characterisation of US isolates
Liverpool University	Univ	Kenton Morgan, Flavio Corsin	Technical input, joint publication	Epidemiology
Uppsala University	Univ	Kenneth Söderhäll, Lage Cerenius	Joint studies, publication	Molecular characterisation, zoospore physiology
Glasgow University	Univ	Douglas Hart	Joint studies, publication	Molecular characterisation
Newcastle University	Univ	Gordon Beakes	Joint studies, publication	PyMS, Mabs
FAO	Inter-Govn	Rohana Subasinghe	Inputs by JHL into AAPQIS database	Internet information dissemination
NACA	Inter-Govn	Ian MacRae, Mike Phillips	Joint publication	EUS manual
Mangalore College	Univ	CV Mohan	Participation on trip	EUS dissemination

[1] Univ = University
[2] Govn = Government
[3] Indus = Industry

2. PROJECT PURPOSE

To generate the information needed for the formulation of strategies to contain EUS; and to develop and introduce improved prophylactic and therapeutic treatments to provide fish farmers with a means of reducing losses due to EUS.

3. RESEARCH ACTIVITIES, OUTPUTS AND CONTRIBUTION OF OUTPUTS

The research activities, outputs, and contribution of outputs are described in Sections 3.1 - 3.8 under the main subject headings. The contribution of outputs to the project purpose are summarised in Section 4 and illustrated in Figure 4.1.

3.1 DIAGNOSIS OF EUS

3.1.1 Introduction

One of the project objectives was to provide improved diagnostic procedures for EUS, based on the case definition for the disease given in Section 1.1.2. AAHRI is the OIE-approved diagnostic centre and reference laboratory for EUS cases, and requires the most specific and sensitive tests for the identification of *A. invadans* infections and the resulting granulomas. Other laboratories in the region would also benefit from the development of rapid, cheap diagnostic procedures. A further objective of the project was to help build a consensus on EUS causation among researchers in the region, and encourage the use of standard diagnostic techniques, so that reports of EUS occurrences would provide a more complete record of outbreaks and their impacts. Some of these latter activities are described in Section 3.8.

At the beginning of the project, EUS cases were generally diagnosed using histological samples stained with H&E to initially determine whether or not mycotic granulomas were present. Positive samples would be further processed with Grocott's (1955) methenamine silver (GMS) stain to identify whether or not the granulomas were caused by fungus. For confirmation that the invasive fungus was *A. invadans*, it was isolated and identified by morphology, growth and pathogenicity characteristics. The present study tested more rapid histological techniques, and attempted to develop *A. invadans*-specific molecular and immunological stains that could identify the fungus on histological slides. A PCR technique was also developed to identify cultures of *A. invadans*, based on information provided from projects R5902 and R5997.

An initial aim of the project was to make use of the DNA probe designed during project R5902 (Hart, 1997) to detect *A. invadans* DNA in water samples, as previous culture-based studies had failed to isolate *A. invadans* from water. It was envisaged that such a technique could be used to determine the geographical distribution of the fungus so that recommendations could be made to stop the spread of the disease to unaffected areas. An environmental probe could also be used in experimental pond trials to test intervention strategies to exclude or treat the fungus. Experiments

Box 3.1.1 FP1-FP2 PCR protocol

Primers:
FP1 5'-AAGGCTTGTGCTGAGCTCACACTC-3'
FP2 5'-GATGGCTAAGGTTTCAGTATGTAG-3'

PCR product:
98 base pairs

Reagents in 50µl reaction tube:
1x *Taq* buffer, 200µM NTPs, 25pmoles each of the primers FP1 and FP2, 2.5mM $MgCl_2$ and 1.25 units of *Taq* polymerase.

Amplifications cycle:
Initial denaturation of 3min at 95°C, followed by 35 cycles of 30sec at 95°C, 30sec at 68°C, and 30sec at 72°C, with a final extension of 5min at 72°C

undertaken to develop the technique are described in Section 3.1.2.1, however these proved unreliable when used on field samples, and other techniques were used to study the geographical distribution of the fungus (see Section 3.2) and pond interventions against EUS (see Sections 3.5 and 3.7).

3.1.2 Activities and outputs

3.1.2.1 DNA probe

A detailed description of the molecular characterisation of *A. invadans* was prepared and submitted for publication (Appendix 4). A pair of primers designed by Hart (1997) during project R5902, was used to develop a PCR-based technique for the specific amplification of *A. invadans* DNA (Box 3.1.1). Due to problems associated with the high annealing temperature of these primers, another set of primers were designed and PCR conditions optimised (Box 3.1.2). Use of these primers is described below.

Box 3.1.2 APH3-APH4 PCR protocol

Primers:
APH3 5'-ATAAGGCTTGTGCTGAGC-3'
APH4 5'-CATTTCTGATGGCTAAGG-3'

PCR product:
107 base pairs

Reagents in 50 µl reaction tube:
1x *Taq* buffer, 200 µM NTPs, 25 pmoles each of the primers APH3 and APH4, 2.5 mM $MgCl_2$ and 1.25 units of *Taq* polymerase

Amplifications cycle:
Initial denaturation of 3min at 95°C, followed by 35 cycles of 30sec at 95°C, 30sec at 50°C, and 30sec at 72°C, with a final extension of 5min at 72°C.

3.1.2.1.1 *Use of the probe to detect A. invadans in water samples*

Attempts to develop a procedure to detect *A. invadans* in the environment are described in Lilley & Chinabut (2000) (Appendix 2). A study framework was devised based on the one described by Hiney & Smith (1998). Quantitative, qualitative and reliability criteria were to be assessed at 4 levels of experimental complexity: (a) *in vitro*; (b) using a sterile seeded microcosm; (c) in a non-sterile incurred mesocosm; and (d) in non-sterile field samples.

(a) The probe succeeded in identifying DNA extracted from mycelium of 12 *A. invadans* isolates against a strain panel of 12 other fungal species. (b) A technique was developed whereby DNA could be extracted from fungal spores in a water suspension (Box 3.1.3). Using this technique, it was possible to detect down to 50 *A. invadans* spores in sterile water. (c) The technique was further tested on tank

Box 3.1.3 DNA extraction from water samples

1. Take one litre samples of pond water
2. Filter debris out using Whatman 541 filter paper
3. Filter samples onto Whatman 1.2µm disposable filters
4. Remove filter with a sterile needle and transfer to a labelled universal tube
5. Add 1 ml of DNA extraction buffer (50mM Tris-HCl (pH 8.0), 20mM EDTA (pH 8.0), 2% SDS)
6. Add Proteinase K to final concentration of 100 µg/ml
7. Incubate at 55°C for l hour in a heated shaker (or 37°C overnight in a heated shaker)
8. Store on ice for 10 min
9. Add 400µl cold saturated NaCl solution, mix
10. Store on ice for 5min
11. Spin down protein precipitate in benchtop centrifuge 3300 rpm 15 min at 4°C.
12. Remove supernatant to 1.5ml eppendorf
13. Spin at 21,000g for 10 min
14. Remove 500µl of supernatant
15. Add RNase A to final concentration of 20µg/ml
16. Incubate at 37°C for 15min in water bath
17. Add 1 ml ethanol (100% EtOH at room temp), mix/invert
18. Store -70°C for 30 min
19. Spin down DNA at 21,000g for 15 min
20. Remove supematant, wash pellet with ~500µl of 75% cold (-20°C) EtOH
21. Spin at 21,000g for 5min
22. Remove supematant. Vacuum dry pellet
23. Resuspend DNA in 20µl H_2O
24. PCR

water during bath challenge experiments (see Section 3.4). Either fungal spores were introduced to the tanks, or fungal mycelium was allowed to sporulate in the tanks. In both cases, abraded snakehead fish in the tanks became infected, indicating that infective *A. invadans* spores were present, but the probe failed to detect the *A. invadans* DNA. (d) The technique was also tested on field samples of pond water taken during an active EUS outbreak. The inability to use the DNA probe for detection of *A. invadans* in tank and pond water is probably due to the high level of contaminant DNA and low level of target DNA in the water. It is thought that *A. invadans* spores were lost during the initial process of filtering out the sediment and debris. There are also many other particles in tank and pond water of similar size to *A. invadans* spores, which would have been retained on the 1.2µm filters, and could have inhibited the PCR reaction. As the probe technique was not found to be sensitive or reliable enough for further experimental studies, other techniques were used to study the geographical distribution of the fungus (see Section 3.2) and pond interventions against EUS (see Sections 3.5 and 3.7).

3.1.2.1.2 *PCR of DNA extracted from fungal isolates*

Protocols for DNA extraction previously used in project R5997 were adopted at AAHRI (Nucleon II, Scotlab, UK). DNA preparations were obtained from some historical isolates and all new isolates of *A. invadans.* Both PCR protocols described in Boxes 3.1.1 and 3.1.2 were used to positively identify new *A. invadans* isolates from Bangladesh, Thailand and USA (Panyawachira *et al.,* 1999; Appendix 3; Appendix 4; Appendix 10). It is recommended that a PCR probe is used on all isolates of *A. invadans,* alongside demonstration of typical morphology, growth and pathogenicity characteristics, in order to confirm the identification.

3.1.2.1.3 *PCR of DNA extracted from paraffin embedded tissues (PET)*

The use of this technique to identify *A. inva*dans DNA in formalin-fixed fish tissues in histology blocks is described by Blazer *et al.* (2001). It proved possible to identify *A. invadans* in several histology samples taken coastal and inland eastern United States. However, the procedure did not work with Bouin's-fixed tissues or decalcified tissues. Two PET techniques were tested at AAHRI, that of Marchetti *et al.* (1998) and the procedure supplied with the QIAamp® kit (Qiagen, California). However, it was concluded that the technique was not reproducible enough for use in routine diagnostics (Appendix 6).

3.1.2.1.4 *In situ hybridisation*

Use of the DNA probe to identify *A. invadans* on histology slides would be of great value in EUS diagnosis and research. The involvement of *A. invadans* in infections that show invasive fungus and non-typical granulomas, such as those investigated by Panyawachira *et al.* (Appendix 6), could be positively determined. In studies at Stirling and AAHRI a variety of experimental conditions, primer labels (biotin, digoxigenin (DIG)) and chromagens (DAB, TrueBlue) were tested, but in no case was it possible to positively visualise a reaction with the target fungus. There are no reports of the successful development of an *in situ* hybridisation technique in the literature for any other fungal diseases of fish. It may be the case that the concentration of fungal DNA in the growing hyphal tips makes these organisms unsuitable for testing using *in situ* hybridisation.

3.1.2.2 Sequence data

The DNA sequences of the internal transcribed spacer region (ITS1) of the ribosomal DNA (rDNA) of four isolates sequenced by Hart (1997) during project R5902, and four isolates

sequenced during the present project, were submitted to the Internet database, GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) (Accession numbers AF349610-7). These sequences appear to be unique to *A. invadans*, and are the basis for the DNA probe described in Section 3.1.2.2. Therefore, where resources are available, PCR amplification of the ITS1 region using universal primers ITS1 and ITS2 as described by White *et al.* (1990), and sequencing of the resulting product, would provide a more reliable means of identifying *A. invadans* DNA than the PCR method described in Boxes 3.1.1 and 3.1.2. The partial sequences of the 18S region of 8 isolates provided by Hart (1997) were also lodged with GenBank (Accession numbers AF349602-9), but these do not provide a sequence unique to *A. invadans*.

Box 3.1.4 Uvitex - H&E staining protocol

1. Dewax
2. Rinse in flowing tap water for 30secs
3. Rinse in PBS (pH =6.47) for several seconds
4. 0.1% Uvitex 2B dissolved in PBS (pH =6.47) for 10mins
5. Rinse in PBS (pH =6.47) for 10mins
6. Stain with haematoxyline for 3mins
7. Rinse in flowing tap water for 15mins
8. Stain with eosin working solution for 30mins
9. Rinse in flowing tap water for 30secs
10. Dehydrate in ascending alcohol concentrations (70-100%) for 20mins
11. Clear in xylene for 30mins

3.1.2.3 Histological stains

A variety of fungal stains and background stains were compared. Appendix 6 illustrates the comparison between the fluorescent stain, Uvitex, Periodic-Acid-Schiff (PAS) stain and GMS stain for the visualisation of *A. invadans* hyphae in histology sections. Where fluorescent microscope facilities are available, Uvitex (Yorisada *et al*., 1999; Box 3.1.4) was considered the most practical and useful for the diagnosis of EUS. It is relatively cheap, very rapid, can be used in conjunction with H&E, provides very good visualisation of Oomycetes, and being a cellulose dye, does not bind well with non-Oomycete fungi. It is recommended that Uvitex-H&E is used in the routine histological diagnosis of EUS. Where fluorescent microscope facilities do not exist, GMS stain remains the best option.

3.1.2.4 Immunological procedures

Antibodies (Section 3.6) have been used in the identification of Oomycetes such as *Pythium* spp. and *Saprolegnia* spp. (e.g. Estrada-Garcia *et al.,* 1989; Beakes *et al.,* 1995). Cultures of hybridoma cells are prepared from mice that produce monoclonal antibodies (MAbs) that are specific to the species in question as they recognise a specific molecule, called the antigen. The antibodies bind to the antigen if it is present in a histological section, then stained. If applied to *A. invadans*, such techniques would allow pathologists with very little training to establish whether hyphae present in a histological section were *A. invadans* or not.

3.1.2.4.1 *Production of monoclonal antibodies (Mabs)*

Mice were immunised with formalin-fixed *A. invadans* cysts and germlings in order to provoke the production of antibodies to them. The antibody-producing cells were collected from the mice and fused with myeloma cells to make hybridomas, which are self-replicating in culture and continue to produce antibodies. The hybridomas that produced antibodies to *A. invadans* were then isolated into pure cultures producing only one type of antibody. The antibodies were characterised and their potential as diagnostic tools was

then examined. Unfortunately, there was insufficient time to complete the isolation procedure with all of the hybridomas raised from mice immunised with germlings or any of those from mice immunised with cysts, but these remain in liquid nitrogen at IoA. Of the hybridomas examined, five antibody-producing clones were identified and isolated. They are currently stored in liquid nitrogen at IoA.

Mammal antibodies are produced in several different arrangements, called the isotype. All five of the antibodies produced were identified as isotype IgM, which is a pentamer of five antibodies bound together. Unfortunately, IgM is the isotype with the poorest specificity and lowest affinity for its antigen, and makes poor quality MAbs (Harlow & Lane, 1988).

Characterisation of the antigens recognised by the MAbs has not been completed yet. Antigens will be separated by molecular weight by electrophoresis, and those recognised by the antibodies will be identified by Western blot. They will be compared to a second antigen preparation in which all proteins will have been removed with proteinase K, leaving only carbohydrates, in order to establish whether the antigens are proteins or carbohydrates.

3.1.2.4.2 *Immunohistochemistry (IHC)*

The most commonly used immunodiagnostic procedure is indirect immunohistochemistry (IHC), which involves using a conjugate to identify the MAb on a histological section. The conjugate is a commercially available MAb raised to mouse antibodies with an enzyme label attached to it. When the first MAb is bound to the antigen, the conjugate is applied to recognise the first MAb, then a dye is applied to react to the enzyme. The final result shows the antigen, and thus the pathogen, stained with the dye (Harlow & Lane, 1988). The main advantage of IHC over GMS is that if the MAb is species-specific, it only stains the appropriate pathogen. If carried out correctly, it would unambiguously indicate the presence or absence of *A. invadans* in a diseased fish.

A range of indirect IHC techniques were assessed, although there are so many possible tests (Mayer & Bendayan, 2001) that it was not possible to attempt all of them. At present, no reliable results have been obtained by the most commonly used methods, which use peroxidase-labelled conjugates to stain antigens brown or blue. Better results have been obtained using conjugates labelled with fluorescin isothiocyanate (FITC), which causes the antigen to fluoresce under ultra-violet light. However, these results have only been obtained using pure cultures and experiments with tissue sections from fish infected with *A. invadans* have not produced clear results.

Indirect IHC using FITC-labelled conjugates was used to compare the response of the MAbs to pure cultures of *A. invadans* with the response to other similar Oomycetes. Very little cross-reactivity with other species was recorded.

At present, the antibodies raised appear to be specific to *A. invadans*, but most are of such low affinity that their value in immunodiagnosis is limited. However, one of the MAbs shows promising results on *A. invadans* germlings when the technique of multiple reaction cycling (Linsenmayer *et al.* 1988) is employed. Studies are currently in progress to evaluate the protocol on tissue sections.

3.1.2.5 Pyrolysis mass spectrometry

Further analysis was undertaken on work started during project R5997, which successfully employed pyrolysis mass spectrometry to distinguish *A. invadans* from other fungal isolates. The work has been accepted for publication (Lilley *et al.,* 2001c; Appendix 5). The disadvantages of this technique are that the necessary facilities are generally not available outside of well-equipped hospitals, and that several fungal isolates, including a type strain, are required in order to make a positive diagnosis.

3.1.3 Contribution of outputs

The activities described in Section 3.1.4 have contributed to our knowledge about procedures for the diagnosis and characterisation of EUS and *A. invadans*. However, some activities did not yield results that were able to provide tangible contributions towards the project purpose. It is hoped that research laboratories will make use of these experiences, and be able to bring them to useable outputs in the future. Other activities described here did successfully improve diagnostic procedures. Use of the Uvitex stain will provide a means by which all diagnostic fish samples that are processed for H&E histology can be rapidly screened for involvement of Oomycete pathogens, particularly the EUS pathogen, *A. invadans*. The PCR diagnostic test was developed and optimised so that it also could be used routinely on fungal isolations. Both methods are being publicised and used in other laboratories both within the region (e.g. FRI-Bangladesh, FDD-Nepal, CIFA-India) and internationally (e.g. USGS-USA, Louisiana State University-USA). These EUS diagnostic procedures were also used for studies undertaken during this project (Sections 3.2, 3.4, 3.5 and 3.7).

Early diagnosis of EUS will help reduction in losses in more intensive systems, by enabling control measures to be advised and adopted before the disease affects the whole susceptible population in a pond. However, small-scale farmers are rarely in contact with fish disease diagnosticians, and in these cases, research findings using these diagnostic methods to determine which clinical signs represent EUS would be useful to allow farmers to make decisions based on simple clinical observations. Such studies are described in Section 3.2, where it was found that 87% of EUS-susceptible fish showing lesions sampled from Bangladesh farms could be diagnosed as EUS.

Rapid EUS diagnosis would also be useful for laboratories, such as AAHRI, which are involved in the health certification of live fish for export. Certification against EUS is not yet required by any country, but industry-led initiatives to ensure that shipments were EUS-free would help to prevent the spread of the disease.

3.2 ECOLOGY & GEOGRAPHICAL DISTRIBUTION OF *A. INVADANS*

3.2.1 Introduction

The project aimed to provide a better understanding of the geographical distribution of *A. invadans* so that areas at risk could be identified, and measures proposed to prevent the spread to unaffected areas (Figure 4.1).

It did not prove possible to develop a reliable environmental probe for *A. invadans* using the DNA sequences (Section 3.1.2.1.1). Another proxy technique of detecting *A. invadans* by measuring anti-*A. invadans* antibodies was tested (Section 3.2.2.8). However, most of the information was gathered by obtaining samples of fish with lesions and sampling them for histology, or isolation and characterisation of fungus. Samples of affected fish were obtained using a combination of active surveillance (Sections 3.2.2.1 & 3.2.2.5), passive surveillance (Section 3.2.2.7), use of samples from observational studies (Sections 3.2.2.2-4) and through contact with international scientists (Sections 3.2.2.9-10). An attempt was made to obtain detailed reports of outbreaks from farmers and fisheries officers by distribution of questionnaires, however an inadequate response was achieved and some direct interviews were undertaken instead (Section 3.2.2.6).

The project also aimed to generate information about the numbers of fish affected in outbreaks, so that a better assessment could be made of the impacts of EUS. Most of the activities described below provide some information of the numbers and proportions of fish affected.

3.2.2 Activities

3.2.2.1 Bangladesh cross-sectional surveys

A cross-sectional survey of randomly-selected farmed and wild fishery sites was conducted in all 64 districts from December 1998 to April 1999 (Section 3.7.2.1). Fish with lesions were recorded from fish farms in 32 (50%) districts and 30 (47%) were confirmed EUS positive, and from wild fisheries 52 (81%) districts demonstrated lesions and 49 (77%) were confirmed as EUS-positive. A map showing affected districts is given in Appendix 7. In total, 6434 wild fish and 6401 farmed fish were examined, and average prevalence of lesions was calculated as 16.0% and 15.5% respectively. Although disease was more widespread in the wild fisheries, the percentage fish with lesions was generally higher at farm sites (0-45%) than wild fisheries (0-32%). A total of 471 fish with lesions was sampled for histology from the 84 affected sites and 80% of these were diagnosed as EUS-positive. Thirty-one species of fish were confirmed as being EUS-positive out of 47 recorded with lesions. Combining data on affected species from both farmed and wild fisheries sites, the highest prevalences of EUS were recorded in *Channa marulius* (30%); *Glossogobius* sp. (25%); *Cirrhinus cirrhosus* (24%); *Channa striata* (22%); *Channa punctata* (21%); and *Anabas testudineus* (20%); and the lowest prevalence was in *Lepidocephalichthyes guntea* (3%). Detailed lists indicating species susceptibility are given in Appendix 7, Tables 2-3.

Unpublished data from a fish disease survey undertaken by the Bangladesh Flood Action Plan 17 (FAP17) Project, from October 1992 to March 1994, was also obtained and analysed. This showed that 26% of 34,451 freshwater fish examined had lesions. No attempt was made to diagnose the lesions, but the species most affected are those that

are considered most susceptible to EUS. Further details are provided in Appendix 1 and 7, Table 1.

3.2.2.2 Bangladesh case-control study

A study comparing EUS-affected (case) ponds with unaffected (control) ponds was conducted in Mymensingh from November 1998 to February 2000 (Section 3.7.2.2). In this study, 33% of the 5206 fish sampled from case ponds had lesions. Seventy-four of these were sampled for histology and 74% of these were confirmed EUS-positive (Appendix 7, Table 4).

3.2.2.3 Bangladesh cohort study

Several projects are underway in Bangladesh that aim to develop beels (floodplain lakes) into over-wintering fish sanctuaries. However, concerns have been raised due to the possible increased risk of EUS. This is because a greater than normal density of EUS-susceptible fish will inhabit these areas during the period that EUS outbreaks are most likely to occur. In order to study the relative prevalence of EUS in over-wintering sites, and determine whether they offer a significant risk of EUS, a cohort study was proposed. Field collaborators were asked to sample fish and conduct interviews with key informants at a number of over-wintering sites and nearby non-sanctuary sites.

Unfortunately only eight sites were sampled, which was insufficient for risk analysis. In addition, all 8 sites sampled (sanctuary and non-sanctuary) contained diseased fish. Out of 6 sites sampled by ICLARM staff at Dikshi Beel, Ashura Beel and Goakhola Hartiara Beel, there was a slightly lower proportion of fish affected at sanctuary sites (11.7% with lesions) than non-sanctuary sites (19.7%). Overall, the numbers of affected fish were: 67% of 6 *Mastacembalus puncalus;* 33% of 3 *Channa striata;* 29% of 7 *Nandus nandus;* 25% of 49 *Anabas testudineus;* 20% of 46 *Channa punctatus;* 15% of 48 *Colisa fasciatus;* 15% of 165 *Mystus vittatus*; 14% of 234 *Puntius sophore*; 11% of 9 *Glossogobius giurus;* 0% of 8 *Lepidocephalus guntea;* 0% of 7 *Heteropneutes fossilis;* 0% of 5 *Chanda baculis;* 0% of 4 *Tetraodon cutcutia;* 0% of 4 *Puntius ticto;* 0% of 4 *Gudusia chapra;* and 0% of 1 *Ompok pabda.* Of 44 fish sampled for Uvitex-H&E histology, 75% were confirmed EUS-positive at AAHRI. These were: 9 *Puntius sophore; 7 Mystus vittatus; 3 Macrognathus pancalus; 3 Anabas testudineus; 2 Nandus nandus; 2 Channa punctata; 1 Glossogobius giurus; 1 Colisa fasciata* and 1 *Channa striata.*

3.2.2.4 Bangladesh CARE study

A farmer-based treatment trial facilitated by CARE-LIFE staff is described in Section 3.5.2.6. Several samples were collected from farms to confirm the presence of EUS. Samples were processed for Uvitex-H&E histology at BAU. 70% of 30 fish were confirmed as EUS-positive. These were: 10 *Cirrhina cirrhosa;* 3 *Anabas testudineus;* 2 *Mystus tengara;* 1 *Catla catla;* 1 *Channa punctata;* 1 *Esomus danrica;* 1 *Labeo rohita;* 1 *Barbodes gonionotus;* and 1 *Puntius sp.*

3.2.2.5 Nepal cross-sectional survey

Over 6000 fish have been examined for lesions during a cross-sectional study conducted from November 2000-January 2001 (Section 3.7.2.3). The data is not yet available, but correspondence has indicated that the occurrence of lesions at sample sites was low initially, but increased later in the season.

3.2.2.6 Thailand questionnaire survey

Seventeen snakehead farmers from Suphanburi, Nakhon Pathom, Singburi and Chanchoengsao districts in Thailand were interviewed using a structured questionnaire. Their responses with regards to EUS outbreaks are given in Box 3.2.1. Further information about control strategies employed by Thai farmers is provided in Section 3.7.2.5.

Box 3.2.1 Interview survey of snakehead farmers

General statistics (averages):

- 8 workers per farm
- 12.5 years experience
- 13 ponds (area=1300m^2, depth=2.8m)
- Production 14,400 kg/pond

EUS:

- 82% had heard of "rok rabat" (EUS)
- 65% said it had occurred on their farm
- Information was obtained mainly from friends or neighbours
- 91% said the lesions appear in winter
- Categorisation of the problem:
  - 33% minor
  - 42% medium
  - 25% major
- Mortality:
  - 73% less than 10%
  - 9% 10-30%
  - 9% 30-60%

3.2.2.7 Samples submitted to AAHRI

AAHRI provides a diagnostic service to Thai farmers, and as the OIE registered centre for EUS, accepts samples from abroad for diagnosis. Box 3.2.2 lists the cases confirmed as EUS by histology in 1998-2000. It shows that in 1999 cases were not confined to the winter season, as is normally the case for EUS. Also a number of giant gourami farms had EUS outbreaks, sometimes, but not in all cases, causing high losses. It is possible that *A. invadans* is maintained in an area throughout the non-EUS season by sublethal, if not subclinical, infections of a small proportion of fish in the population.

A number of new isolations of *A. invadans* were made (Table 3.2.1). These were characterised in terms of pathogenicity (Section 3.4.2.2), growth (Panyawachira *et al.,* 1999; Appendix 3) and using PCR probes (Section 3.1.2.1.2).

In order to ensure that type cultures are available to all researchers, *A. invadans* isolates RF6, PA7 and T99G2 have been deposited at the International Mycological Institute (IMI) culture collection.

Table 3.2.1 Fungal isolates obtained during current project and tested for pathogenicity in snakeheads

Isolate	Origin	Date	Location	Worker
		Aphanomyces invadans isolates		
T99G	*Osphronemus goramy*	Sept 99	Bangkok noi, Thailand	Lilley
T99G2	*Osphronemus goramy*	Nov 99	Thailand	Panyawachira
T99S	*Channa striata*	Dec 99	Rayong, Thailand	Panyawachira & Lilley
B99C	*Cirrhinus ariza*	Mar 99	Mymensingh, Bangladesh	Lilley & Khan
NJM9701	Ayu (*Plecoglossus altivelis altivelis*)	Aug 97	Shiga Prefecture, Japan	Hatai
UM3	Menhaden (*Brevoortia tyrannus*)	Aug 98	Wicomico River, USA	Blazer
		Saprophytic isolates		
99ExtAc	External *Achlya* infection of *Channa striata*	Mar 99	AAHRI wet lab	Lilley
Achlya99	External *Achlya* infection of *Anabas testudineus*	Dec 99	AAHRI wet lab	Lilley
99ExtAp	External *Aphanomyces* infection of *Channa striata*	Mar 99	AAHRI wet lab	Lilley
EN1	*Aphanomyces* causing leaf blight disease of *Eichhornia crassipes*	?	Bangkok, Thailand	Kiewong

3.2.2.8 Philippines immunoprevalence study

Monitoring by immunoprevalence utilises the fact that fish develop antibodies specific to pathogens that they have been exposed to (Kaattari & Piganelli, 1996). Fish from populations exposed to certain pathogens express antibodies to those pathogens far more than fish from unexposed populations (Yoshimizu *et al.,* 1992; Aaltonen *et al.,* 1997), even if they are not actually infected with the pathogen at the time. Consequently, it is possible to establish whether the pathogen exists in the area by the presence or absence of antibodies to it in the blood.

Box 3.2.2 Confirmed EUS cases at AAHRI, Thailand 1998-2000

98 Jun - Bangladesh - *C. catla, M. vittatus, M. pancalus, P. sophore* - D98039
98 Dec - Suphanburi, Thailand - snakehead - D98099
99 Jan - Chainat, Thailand - giant gourami - D99006
99 Feb - Chainat, Thailand - Anabas - D9909
99 Mar - Chainat, Thailand - Anabas - D99010
99 May - Nepal – snakeheads from 96 - D99019
99 June - Nonthaburi, Thailand - giant gourami - D99020
99 June - Nonthaburi, Thailand - giant gourami - D99021
99 Jul - Nakhon Pathom, Thailand - giant gourami - D99047
99 Aug - Bangkok noi, Thailand - juvenile giant gourami - D99066
99 Nov - Thailand - adult giant gouramies
99 Dec - Rayong, Thailand - snakeheads - D99097
99 Dec - Chainat, Thailand - Anabas - D99098
00 Jan - Bungchawag reservoir, Suphanburi, Thailand - *Labeo rohita*, Anabas - D0003
00 Jan - Singburi, Thailand - mud carp - D0005

Striped snakeheads were selected, as other studies have shown that they do express anti-*A. invadans* antibodies (Lilley, 1997; Thompson *et al.,* 1997; Section 3.6.2.1). Also, they are common throughout much of the range of EUS and regularly caught by capture fisheries through much of the Asia-Pacific region. The sera of snakeheads of different populations were collected and their response to *A. invadans* compared in the context of the history of EUS in the geographical region inhabited by the populations. Most sampling was carried out in the Philippines, as the EUS status of different islands differs considerably, and has been monitored by the Bureau of Fisheries and Aquatic Resources (BFAR) for some years. Particular attention was given to the island of Mindanao, as EUS only appeared there two years before the study, and its spread across the island was well documented. Concentrating on Mindanao allowed comparison of populations from EUS-endemic regions that were adjacent to non-endemic regions, but were very similar in every other way.

The ability of the sera to inhibit the germination of *A. invadans* was assessed as described in Appendix 21. The concentration of anti-*A. invadans* antibodies was assessed by ELISA. The specificity of the anti-*A. invadans* antibodies was assessed by comparing the response of sera to *A. invadans* with the response to two *Aphanomyces* saprophytes and one *Saprolegnia* saprophyte previously isolated from EUS lesions.

Sera collected from Mindanao showed that fish from two of the three EUS-endemic populations had a higher antibody response than EUS-naïve fish, although statistical significance was equivocal in some cases. The remaining case was undergoing its first recorded EUS outbreak at the time of sampling, so it is possible that the fish had not had time to develop a recognisably higher response. There was one other case undergoing its first outbreak, which had the highest antibody response recorded in the study, indicating that snakeheads can develop antibodies very quickly when exposed to *A. invadans* in the environment.

However, the most inhibitory sera from the Philippines were those collected from Luzon, although their antibody level was only slightly higher than that of EUS-naïve fish. Some of the fish collected in Thailand also showed high inhibitory activity in spite of a relatively low antibody level. As both Thailand and Luzon have been EUS-endemic for over ten years while the first outbreaks in Mindanao only occurred two years prior to the study, it is likely that fish in those regions have a far more effective antibody response, which probably targets antigens more important to the pathogen.

The response to *A. invadans* was only significantly different to that to other Oomycetes in a few cases, indicating that there is very little specificity in the responses to any of them.

These results show that antibodies that recognise *A. invadans* are present even in fish that have never been exposed to it, as antibodies produced in response to other Oomycetes cross-react with it. Overall concentration of antibodies gives some indication of the ability of serum to inhibit *A. invadans* in recently exposed populations, and so immunoprevalence may not be useful in predicting EUS outbreaks in regions that have been EUS-endemic for any length of time. Nevertheless, sera from previously naïve fish in the presence of an outbreak was more inhibitory than that of completely naïve fish, indicating that an adaptive response does take place and probably confers some protection (Section 3.6.3.3). Such a response can evidently be produced very rapidly, as it was identified in populations during the first outbreak that they had been exposed to. The response becomes more efficient in populations that have been exposed to *A. invadans* for some time, where higher levels of inhibition were induced by relatively low antibody levels. It was not clear whether the change in the nature of the response was indicative of adaptation of individuals or selection within populations for those individuals able to produce protective antibodies. If selection is involved, it is possible that the introduction of EUS into a region causes a genetic bottleneck among snakeheads and other highly susceptible species, and the impact on biodiversity within populations of such species is a matter of some concern.

These results provide evidence for the presence of an adaptive immune response to *A. invadans* mediated through serum, which almost certainly takes the form of antibody production. It is likely that antibodies are produced to certain antigens of *A. invadans* that it is particularly vulnerable to, although such antibodies were not positively identified in the present study. The study of fish from Mindanao suggests that immunoprevalence monitoring may show the spread of EUS through previously naïve populations. It is not clear from the present study whether the antibody levels within populations remain indicative of the EUS status in regions that have been exposed to EUS in the past but where no EUS outbreak has been recorded for some time.

3.2.2.9 <u>USA samples</u>

In order to define the boundaries of EUS outbreaks, it is a priority to compare EUS with other known diseases that show similar signs. Project R5997 succeeded in showing that the MG in Japan and RSD in Australia were identical to EUS by demonstrating that the invasive fungus involved in all cases was identical (Lilley & Thompson, 1997). In contrast, the *Aphanomyces* isolates from ulcerative mycosis (UM) outbreaks in USA (Dykstra *et al.,* 1986; 1989) were shown to be different from *A. invadans*. However, it was suspected that these isolates were not the true invasive pathogens of UM as they were incapable of invasive growth in UM- and EUS-susceptible fish (Noga, 1993; Lilley & Roberts, 1997).

Recently, Dr V. Blazer, USGS, used techniques given in the project manual (Lilley *et al.,* 1998) to isolate invasive cultures of *Aphanomyces*. These were sent to AAHRI and characterised as *A. invadans* by morphology, growth, pathogenicity, PCR and sequencing of the ITS1 region (Blazer *et al*., 1999; Appendix 8; Blazer *et al*., 2000; Appendix 9; Appendix 10). The only difference observed was that the American isolate had a smaller hyphal width in snakehead fish (6-15µm) compared with Asian isolates (13-30µm). This observation is consistent with other reports, which indicate that the American fungus has a width of 7-15µm in infected fish (Noga & Dykstra, 1986; Noga, *et al*., 1988; 1991), whereas the Asian and Australian *A. invadans* has a width of 12-27µm in infected fish (Egusa & Masuda, 1971; Callinan *et al*., 1989; Roberts *et al*., 1993). McKenzie & Hall (1976) recorded the largest hyphal widths of 30-45 µm in the original RSD outbreaks in Australia.

It is probable, therefore, that *A. invadans* was involved in the original UM outbreaks in the mid-1980s. These findings have implications for our understanding of the boundaries of EUS outbreaks, and for measures to contain the spread of the disease, as discussed in Section 3.2.4.

3.2.2.10 Other reports

Contact was made with a number of fisheries officers and fish disease scientists from South America and Africa, but none reported observing EUS-like signs in histological sections of diseased fish. One report from Egypt (Shaheen *et al*. 1999) describes the isolation of a thermo-labile *Aphanomyces* from ulcerated mullet, but no histology was done, and the culture is no longer available (Faisal, pers. comm.).

Regional scientists working with AAHRI staff have succeeded in histologically demonstrating EUS in areas that were previously unconfirmed, e.g. Vietnam (Phan Thi Van, pers. comm.), Nepal (S. Dahal, pers. comm.) and Pakistan (R. Anjum, pers. comm.). Contact with other scientists has uncovered unpublished work that revealed EUS in histological sections from Sri Lanka (P. Vinobaba, pers. comm.) and India (R.K. Dey, pers. comm.).

Histological studies of a large number of ornamental fish have not revealed signs of EUS (R. Del-Rio-Rodriguez, pers. comm., S. Kueh, pers. comm.). Although this indicates the risk of transmission via ornamental fish is small, ornamentals have in the past been found to be infected (Table 3.2.2), and therefore this remains a real risk. Hatai (1994) and Hanjavanit *et al.* (1997) have shown that particular ornamental species imported to Japan from Singapore have had EUS, and It has been hypothesised that EUS was transmitted to Sri Lanka through shipments of ornamental fish (Balasuriya, 1994).

Some histological sections of fish samples have been examined, which show lesions not typical of EUS (Appendix 6). Samples from Murray cod in South Australia provided by R. Reuter show invasive fungal infection, but no inflammatory response. No fungus was isolated from these fish, so full characterisation was not possible. Use of the PCR probe on DNA extracted from the paraffin-embedded tissue was negative, which indicates that the fungus was not *A. invadans*, but this technique is liable to produce false negatives due to histological processing (Section 3.1.1.1.3). EUS has never been shown from South Australia, and the bulk of evidence from these samples indicated that this was probably *Saprolegnia* sp. that had managed to penetrate muscle tissues due to the small size of the

fish. A similar infection has previously been recorded from South Australia by Puckeridge *et al*. (1989).

An ulcerative disease of *Toxotes* sp. from Bangsai, Thailand also showed invasive fungus (Appendix 6). In this case there was a significant granulomatous response, but very unlike typical EUS. It is possible that this was the result of a combination with a parasitic infection. However as no fungus was isolated, therefore there was insufficient evidence to categorise this as EUS.

The histology of Vietnamese redspot disease of grass carp remains unclear. A series of samples were examined, which were labelled as redspot disease from Vietnam, however the histology of the samples was very different from each other. One sample showed typical EUS, but it is thought that it was not from grass carp at all. Grass carp is considered to be resistant to EUS, therefore it is most likely that redspot of grass carp is distinct from EUS, but that this sample was EUS of a different fish species.

3.2.3 Outputs

3.2.3.1 Affected species

An updated list of species confirmed with EUS is given in Table 3.2.2. Most of the new entries are the result of activities described in Sections 3.2.2.1 and 3.2.2.10. The table shows that 72 different species have been confirmed as affected by EUS.

3.2.3.2 Affected areas

To date, 13 countries have been confirmed affected by EUS, and EUS has been reported from 18 countries (Figure 3.2.1). The latest outbreaks are listed in Box 3.2.3. This indicates that the disease continues to cause problems, particularly where flooding (Vietnam), or other changes to water systems (Cambodia), have occurred, or in areas where it has been not been previously reported (Mindanao, Philippines).

3.2.3.3 Unaffected areas

As it has not been possible to develop a test for the presence of *A. invadans* in the environment, it cannot be definitively stated that the fungus is not present in areas where EUS has not been reported. The widespread presence of susceptible fish (e.g. snakeheads and catfish) in areas where the disease is thought not to occur (e.g. Africa) provide support to the belief that *A. invadans* is not present in these areas. However, a statistically-valid sampling protocol would be required in order to demonstrate freedom from disease.

Table 3.2.2 Species confirmed with EUS by presence of typical mycotic granulomas in histological section or isolation of confirmed *Aphanomyces invadans* culture

***Latin name* (common name)**	Jap[†]	Aus[‡]	Ino	Tha	Lao	Vie	Mya	Phi	SL	Ban	Ind	Nep	Pak	Sco	US
Acanthopagrus australis (yellowfin bream)		I													
Anabas testudineus (climbing perch)								S		M					
Barbodes gonionotus (silver barb)				G						M					
Bidyanus bidyanus (silver perch)		D													
Brevoortia tyrannus (Atlantic menhaden)															B
Carassius auratus auratus (goldfish)	O														
Carassius carassius (crucian carp)	O														
Catla catla (catla)										A					
Channa gachua (snakehead)												H			
Channa maculata (Formosan snakehead)	O														
Channa marulius (giant snakehead)										M					
Channa micropeltes (red snakehead)				G											
Channa orientalis (walking snakehead)										M					
Channa pleurophthalmus (snakehead)	J[ψ]														
Channa punctata (spotted snakehead)										N		H	L		
Channa striata (striped snakehead)				F*,N	G		G	C		M	P				
Channa sp. (snakehead)						R									
Cirrhinus ariza (reba)										T					
Cirrhinus cirrhosus (mrigal)										A			G		
Clarias batrachus (walking catfish)								C		M					
Clarias gariepinus (African catfish)								D*		M					
Colisa fasciatus (banded gourami)										M					
Colisa lalia (dwarf gourami)	K[ψ]														
Epinephelus sp. (grouper)											Y				
Esomus sp. (flying barb)											W				
Etroplus sp. (chromide)											W				
Fluta alba (swamp eel)				G											
Glossogobius giurus (bar-eyed goby)								C		A					
Glossogobius sp. (goby)										M	W				
Heteropneustes fossilis (stinging catfish)										M	W				
Ictalurus punctatus (channel catfish)															B
Johnius sp. (croaker fish)								S							
Labeo rohita (rohu)										T					
Lepidocephalichthys guntea (guntea loach)										M					
Lepomis macrochirus (bluegill)	O														B
Liza diadema (mullet)		Q													
Liza sp. (mullet)									V						
Macquaria ambigua (golden perch)		D													
Macrognathus aculeatus (lesser spiny eel)										M					
Macrognathus pancalus (barred spiny eel)										M					
Mastacembalus armatus (armed spiny eel)										G					
Morulius calbasu (orange-fin labeo)										M					
Mugil cephalus (grey mullet)	O	I						C			P				
Mugil sp. (mullet)									V		W				
Mystus cavasius (Gangetic mystus)										M					
Mystus sp. (catfish)											X				
Mystus tengara (tengara)										M					
Mystus vittatus (striped dwarf catfish)										M					
Nandus nandus (Gangetic leaf fish)										M					
Nematalosa sp. (gizzard shad)									V						
Notopterus notopterus (grey featherback)				G						M					
Oncorhynchus mykiss (rainbow trout) [‡]	K*													U*	

***Latin name* (common name)**	Jap[†]	Aus[‡]	Ino	Tha	Lao	Vie	Mya	Phi	SL	Ban	Ind	Nep	Pak	Sco	US
Osphronemus goramy (giant gourami)				G											
Osteobrama cotio cotio (keti)							G			M					
Oxyeleotris marmoratus (sand goby)			N												
Oxyeleotris sp (gudgeon)															
Parambassis ranga (Indian glassy fish)										M					
Platycephalus fuscus (dusky flathead)		D													
Platycephalus sp. (flathead)											X				
Plecoglossus altivelis altivelis (ayu)	K,O														
Psettodes sp. (spiny turbot)								S							
Punitus chola (swamp barb)												H			
Punitus ticto (ticto barb)										M					
Puntius sophore (pool barb)										A			G		
Puntius sp (puntius)										T	P				
Rhodeus ocellatus ocellatus (rosy bitterling)	K*														
Scardinius erythrophthalmus (rudd) [‡]	K*														
Scatophagus argus (spotted scat)								S							
Scatophagus sp. (scat)											W				
Siganus sp. (spinefoot)									V						
Sillago ciliata (sand whiting)		I,E*													
Sillago sp. (sillago)											W				
Terapon sp. (therapon)											W				
Trichogaster chuna (honey gourami)										M					
Trichogaster pectoralis (snakeskin gourami)				G											
Trichogaster trichopterus (3-spot gourami)	J[Ψ]	E*		N											
Tridentiger obscurus (Japanese trident goby)	O														
Tylosurus sp. (needlefish)									V						
Upeneus bensai (goatfish)								S							
Valamugil sp. (mullet)											W				
Wallago attu (wallago)										M	X				
Xenentodon cancila (round-tailed garfish)										N					

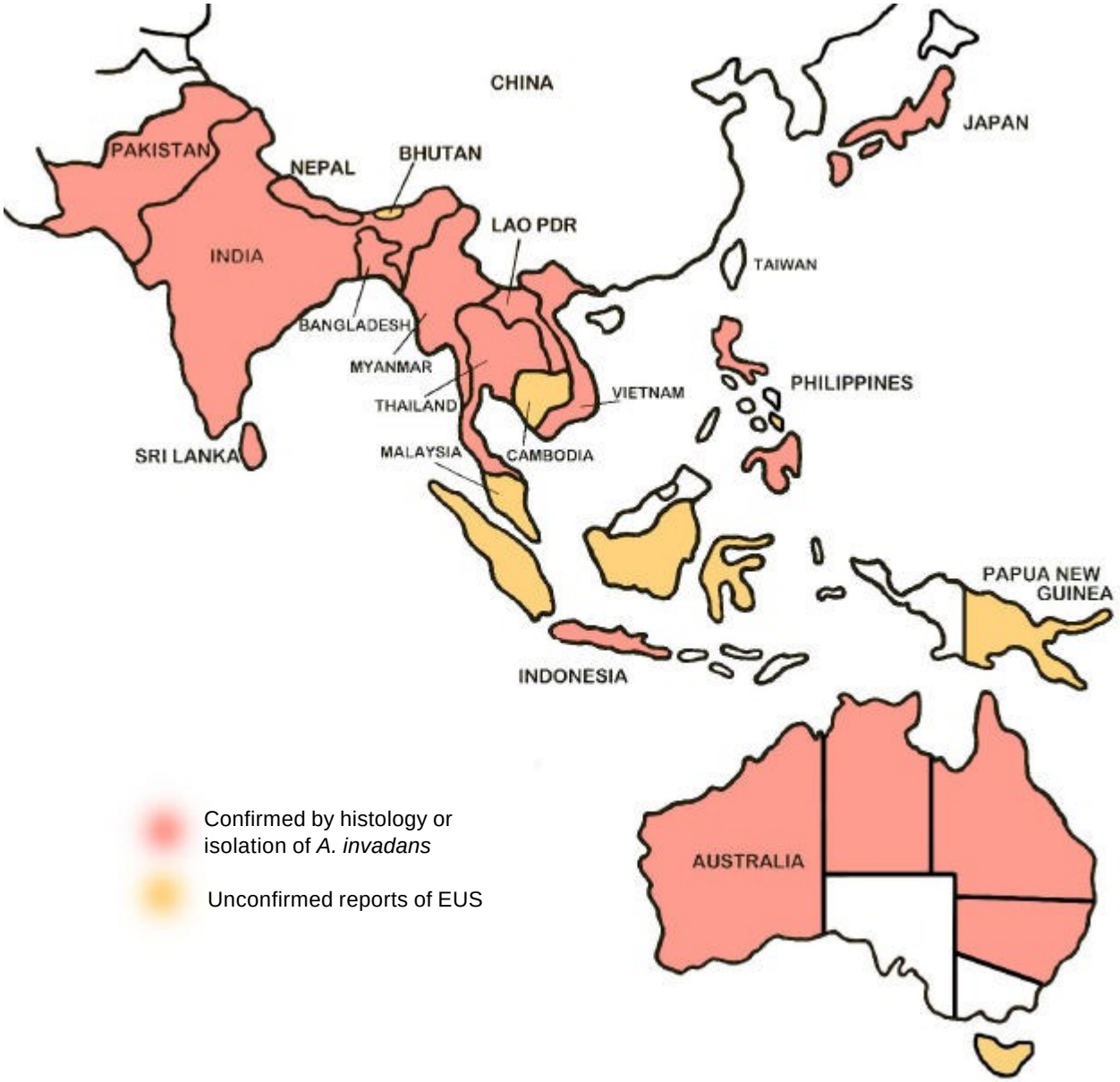
[*] Artificial challenge
[Ψ] Ornamental fish imported from Singapore
[‡] Marginally susceptible

COUNTRY KEY:
Jap = Japan; Aus = Australia; Ino = Indonesia; Tha = Thailand; Lao = Lao PDR; Vie = Vietnam; Mya = Myanmar; Phi = Philippines; SL = Sri Lanka; Ban = Bangladesh; Ind = India; Nep = Nepal; Pak = Pakistan; Sco = Scotland; US = USA

REFERENCE KEY:

A Ahmed & Hoque (1999)
B Blazer *et al*. (2001)
C Callinan *et al.* (1995b)
D Callinan (unpublished)
E Catap (2000)
F Chinabut *et al.* (1995)
G Chinabut (unpublished)
H Dahal (unpublished)
I Fraser *et al.* (1992)
J Hanjavanit *et al.* (1997)
K Hatai (1994)
L Kanchanakhan (1996a)
M Khan *et al*. (2001a)
N Lilley & Roberts (1997)
O Miyazaki (1994)
P Mohan & Shankar (1995)
Q Pearce (1990)
R Phan Thi Van (unpublished)
S Reantaso (1991); Chinabut (unpublished)
T Roberts *et al.* (1989)
U Thompson *et al*. (1999)
V Vinobaba (unpublished)
W Vishwanath *et al.* (1997)
X Vishwanath *et al.* (1998)
Y Viswanath *et al.* (1997)

Figure 3.2.1 Areas affected by EUS



Box 3.2.3 Recent outbreaks of EUS

(Information from project activities (Sections 3.2.2.1-11), OIE quarterly reports and personal communications)

Australia
- Queensland - confirmed by histology (Sept 1998; Feb-Aug 1999; May 2000)
- Northern Territory - reported (1998), confirmed by histology (April-Sept 1999)
- Western Australia - confirmed by histology (Aug 1998)
- NSW - confirmed by histology (1997; Jun 2000)
- South Australia - passive surveillance - never reported
- Victoria - passive surveillance - never reported
- Tasmania - reported (1981) but never confirmed
- ACT - never reported

Bangladesh
- Confirmed by histology in 51 out of 64 districts (Dec 1998 - April 1999)
- 16% of 12,835 had lesions
- 80% of 471 fish with lesions were EUS positive
- 31 species confirmed
- Confirmed by histology in Mymensingh (Dec 1999; Jan-Feb 2000)
- *A. invadans* isolated in Mymensingh (Mar 1999)

Bhutan
- No information

Cambodia
- Reported in Suay Rieng Province and other areas (Jan-Jun 1999), not confirmed
- Reported annually in wild and cage cultured snakeheads in Prey Veng, Kompong Chhnang, Condar and Siem Reap.
- Reported in seasonal streams and wetland ponds of Se San River Basin for over a decade (Anon, 2000)
- Reported to have recently spread to the mainstream Se San River due to hydrological changes, causing high mortalities (Anon, 2000)

China
- Not reported

Hong Kong
- No information

India
- Reported in various areas (1998; 1999)
- Very high losses reported from community ponds in Tripura State (2000)
- Confirmed in sample from Tamil Nadu (2000)

Indonesia
- Not reported, but thought to occur (1999)

Iran
- Never reported

Japan
- Reported (Aug-Sept 1998; Aug 99), but not confirmed
- Confirmed in Shiga Prefecture (Aug 1997) by isolation of *A. invadans*

Korea (DPR)
- No information

Korea (RO)
- Never reported

Lao PDR
- Reported (1998), not confirmed
- Reported 9000kg mortality of *Labeo chrysophekadion* in Nam On tributary of Mekhong (Mar 1999), not confirmed as EUS

Malaysia
- One suspected case in cultured *Channa micropeltes* in Pahang (Mar 1997) 60% morbidity and 10% mortality
- Reported in 1998, not confirmed

Box 3.2.3 Recent outbreaks of EUS (contd)

(Information from project activities (Sections 3.2.2.1-11), OIE quarterly reports and personal communications)

Myanmar
- No information

Nepal
- Reported from several districts in the Terai and mid hills (Sept-Dec 1998; Jan-May, July, Sept, Dec 1999)
- Confirmed in samples from Kathmandu valley (Feb-July; Dec 1998)
- Confirmed in samples from Terai (Jan 2000)

New Zealand
- Never reported

Pakistan
- Confirmed by histology from Punjab (April 1996)
- Confirmed by histology from Sindh (Jan 1998)
- Reported from areas of Sindh and Punjab (Aug-Dec 1998; Oct-Dec 1999)

Philippines
- Up to 30% prevalence in susceptible fish from Laguna Lake and Mangabol swamp, Luzon (Jan 1996)
- Reported from Lakes Lanao, Dapao and Maiinit, Mindanao (Jan 1998)
- Clinical signs in cage cultured grouper in Davao, Mindanao (Dec 1998)
- Confirmed in snakehead in Compostela Valley, Region XI, Mindanao probably due to floods, no significant mortality (Jan-Mar 99)
- Reported from Caraga, Region XII, Mindanao, (Jan-Mar 1999), confirmed in *Scatophagus argus* and *Mugil* sp (Mar 1999)
- Confirmed in snakheads in Lake Siloton, Lake Sebu & South Cotabato, Mindanao (July 1999)
- Confirmed in 30% of 100 snakeheads transported from Oton, Guimaras, & Dumangas, Iloilo (Nov 1999)

Singapore
- Never reported

Sri Lanka
- Reported annually in Batticaloa Lagoon, last confirmed in 1996
- Reported from Colombo and Kaluthara (Jan 1996)

Taiwan
- Never reported

Thailand
- Confirmed histologically (1998, 1999, 2000) - see Box 3.2.1
- Giant gourami from Nonthaburi (June 1999), death in 75% of 20 fish
- Giant gourami from Bangkok (Aug 1999), death in 20% of 10,000 fish
- Confirmed by isolation of *A. invadans* from Bangkok (Sep, Nov 1999) and Rayong (Dec 1999)
- Histologically confirmed in trapped wild fish from Ban Sa Tein Yai, Amphur Ban Mee, Lopburi (Feb 2000), *Anabas testudineus* (24% of 344 with lesions) *Puntius* spp. (24% of 100), *Channa* spp. (21% of 105), stocked *Labeo rohita* (0% of 35) stocked *Pangasius sutchi* (0% of 14)

USA
- 121 menhaden from Chesapeake Bay and 31 from Pocomoke and Wicomico rivers all showed EUS-like histology (July-Aug 1997) (Blazer *et al*. 1999)
- Incidence of lesions on commercial fish species dropped from 3.2% in 1997 to 0.19% in 1998 (Jordan *et al*., 1999)
- *A. invadans* isolated from menhaden in Wicomico river, Maryland (Aug 1998) (Blazer *et al*. 2001)
- *A. invadans* probe positive for samples from Maryland, South Carolina, Virginia, New Jersey, Georgia and Louisiana
- EUS-like histology observed in channel catfish (*Ictalurus punctatus*) in southeast Louisiana since about 1992. Chronic in nature with low daily mortality but over time losses can be high. Consistently associated with low temperature, low hardness, low alkalinity and low pH.
- *Aphanomyces* isolate from channel catfish shows identical ITS1 sequence to *A. invadans*

Vietnam
- Suspected in cage-cultured grouper (1998) but not confirmed
- Reported from Tien Giang Province (1999) but not confirmed
- Reported following floods in the Mekhong Delta (winter 2000), but not confirmed
- Confirmed histologically in wild fishes from northern Vietnam (2000)

3.2.4 Contribution of Outputs

The 72 species listed in Table 3.2.2 should be considered the ones most at risk of contracting EUS. However, there is a clear difference in susceptibility of the fish listed, as shown in Appendix 7. It is interesting to note any differences in susceptibility within genera for fish that are being considered as candidate species for aquaculture development. For example, in Appendix 7 Table 1, *Puntius terio* is listed as one of the worst affected species, whereas no lesions were recorded on any of 361 *Puntius phutunio* examined.

It should be pointed out that the species that have been shown to be affected by EUS may not be the only species that can transmit EUS. There remains a possibility that resistant species may also act as carriers.

EUS outbreaks appear to be restricted between latitudes 40^{o} North and 30^{o} South (including reports of UM from USA), probably because fish adapted to low temperatures can mount a sufficient immune response to the fungus. This is supported by the observation that in experimental trials, rainbow trout can be infected with *A. invadans* only at the upper levels of their temperature tolerance range, and then only by injection of high numbers of spores (Thompson, pers. comm.).

The most severe outbreaks occur in areas where fish are adapted to high temperatures, but are exposed to periods of low temperature. EUS is present in areas near the equator that show less marked seasonality (e.g. Indonesia, Malaysia), but it is considered to be a minor problem in these areas.

It has been postulated that *A. invadans* originated from an isolated area (e.g. the Pacific islands) and was brought to Japan with live fish imports (Lilley, 1997). From the discussion above, it is possible that the origin was from an equatorial area where the fungus infected local fish, but due to high temperatures the disease generally did not progress and result in mortalities.

In the areas where EUS occurs (Fig. 3.2.1), it is probably (as has been shown in Bangladesh) endemic in the wild fish population. It is apparent that the disease in these areas shows a typical disease cycle, with initial outbreaks subsiding, but with subsequent flare-ups when a particular set of conditions occurs. Such conditions include unusually low temperatures, flooding earlier in the year, or development in the culture of a susceptible species (as in the occurrence of EUS in giant gourami in Thailand). In EUS endemic areas, control in farms would be possible by treatment and exclusion of the fungal pathogen (Sections 3.5 & 3.7), immunostimulation of susceptible fish (Section 3.6) and maintaining a suitable pond conditions (Section 3.7).

Areas that remain unaffected by EUS, but have a suitable climate and susceptible fish, should be considered at most risk from EUS. Initiatives like the FAO/NACA/OIE/AAHRI Regional Programme for the Development of Technical Guidelines on Quarantine and Health Certification, and Establishment of Information Systems for the Responsible Movement of Live Aquatic Animals may help to prevent introduction of EUS to these areas. At the national level, countries may define unaffected areas and develop guidelines to prevent spread to these areas. In the Philippines, BFAR developed the following action plan to prevent the spread of EUS within Region XI in Mindanao:

1) Restrict introduction of fish from EUS endemic areas to Region XI
2) If introduction is unavoidable, disinfect incoming fish
3) Limit transfer of EUS-susceptible fish within Region XI
4) Assess the spread of EUS through fish dispersal and water systems
(The study described in Section 3.2.2.8 was intended to contribute to activity 4)

Of the various techniques tested during the project to establish the geographical distribution of *A. invadans*, it is concluded that sampling for diseased fish, targeting known susceptible species, during the season when EUS is likely to occur, is the most reliable method. Diagnosis of EUS should follow the recommendations given in Section 3.1. It has not proved possible to determine the presence of *A. invadans* in the absence of an active outbreak, either by culture based methods (Willoughby, 1999) or proxy methods using DNA probes and by testing for antibodies in fish serum (This Section).

3.3 ZOOSPORE PHYSIOLOGY

3.3.1 Introduction

Motile zoospores provide Oomycete fungi with a capacity for dispersal (Lange and Olson, 1983) and host/substrate location (Deacon and Donaldson, 1993). Therefore, the study of adaptations in the structure, physiology and behaviour of zoospores are likely to provide information on the processes by which pathogenic fungi locate and infect hosts (Unestam, 1969), and also, methods for the strategic control of disease (Willoughby & Roberts, 1992).

The project aimed to determine the optimal and lethal conditions for zoospore production and motility. It was felt this would improve understanding of pond conditions that are at risk of EUS, and management procedures that would prevent EUS outbreaks by inhibiting *A. invadans* sporulation or zoospore motility (Figure 4.1).

3.3.2 Activities and outputs

3.3.2.1 Polyplanetism

Polyplanetism is a phenomenon of some Oomycete fungi whereby several successive tertiary generations of zoospores may be produced from the secondary cyst. This repeated emergence of zoospores was considered to be an adaptation to parasitism among particular species of *Aphanomyces* (Cerenius and Söderhäll, 1985). Experiments were carried out to show that *A. invadans* is capable of polyplanetism in autoclaved lake water, but this ability is limited compared to other *Aphanomyces* strains studied (Lilley *et al*., 1999; Appendix 11). In the presence of a general nutrient background, some *A. invadans* cysts germinated (as did the saprophytic *Aphanomyces* spp.), but some produced a further zoospore generation. It was thought that this latter behaviour is an adaptation to aid the location of a specific host. Identification of compounds that can trigger encystment of *A. invadans* at very low concentrations may be useful in helping to prevent the zoospores locating a host.

3.3.2.2 "Mini-sporangium"

During zoospore studies, it was found that production of a "mini-sporangium" is a distinctive feature of *A. invadans* (Lilley *et al*., 1999; Appendix 11). Such structures also produce infective spores, and inhibition of the production of these structures should be investigated.

3.3.2.3 Geotaxis

Motile secondary zoospores of particular Oomycete fungi show negative geotaxis so that they accumulate towards the surface of a water body (Cameron and Carlile, 1977). Previous microscope observations of suspensions of motile secondary zoospores of *A. invadans* had indicated that they are less inclined to accumulate at the surface, and could actually be positively geotactic (Callinan, pers. comm.; Lilley, 1997). As this would have implications for treatment of the fungus in ponds, this was investigated further. Lilley & Panyawachira (2000; Appendix 12) showed that *A. invadans* secondary zoospores are negatively geotactic, and therefore treatments should be effective primarily in surface waters.

3.3.2.4 Sporulation

Optimal procedures for production of *A. invadans* zoospores were investigated, and described by Lilley *et al*. (2001e; Appendix 13). Sporulation and production of good yields of motile zoospores are required for diagnosis of *A. invadans,* and a variety of experimental studies. The studies were initiated primarily for the work described in Section 3.1.2.1.1, to

find the best sporulating medium that was not derived from pond water, to ensure that no external sources of *A. invadans* DNA would be added to sporulating cultures. Autoclaved pond water (APW) gave the best results, but MSM (Kurata *et* al., 2000) proved a suitable alternative.

3.3.2.5 Cyst viability

To date, *A. invadans* has only been isolated from internal tissues of fish affected with epizootic ulcerative syndrome (EUS), and there is no experimental evidence to show that it can survive for long in water, or on non-fish substrates. Attempts to isolate the fungus from pond water and soil have not been successful (Willoughby *et al*., 1999). Observations of the fungus have revealed no resistant spore stages that would indicate an ability to survive long periods away from a host fish.

Lilley *et al*. (1998) therefore proposed that eradication of *A. invadans* from a pond may be achieved by excluding susceptible and carrier hosts. This has also been recommended as a means of controlling crayfish plague (Unestam, 1969; Söderhäll *et al.,* 1977). Matthews and Reynolds (1990) showed that crayfish did not get infected after spores of the pathogen *Aphanomyces astaci* were left for nine days at 10^{o}C.

Experiments described by Lilley *et al.* (2001f; Appendix 14) showed that encysted zoospores of *A. invadans* can survive for at least 19 days *in vitro* at 20^{o}C. It also demonstrated that cultures isolated from cysts 17 days post-sporulation are still pathogenic to snakeheads. It was therefore suggested that if high-risk ponds can not be dried and limed before stocking, it is recommended that they are fallowed for at least 20 days. Similarly, inlet water taken from high-risk areas that can not otherwise be disinfected, could be retained for at least 20 days in a fish-free reservoir prior to use.

3.3.2.6 Chemotaxis

Zoospores of Oomycete fish pathogens have shown chemotactic behaviour towards host material, including particular sugars and amino acids (Cerenius & Söderhäll, 1984; Smith *et al.,* 1984). Rand & Munden (1993) suggested that manipulation of particular chemical messengers could provide a biologically rational means of controlling fungal fish infections. Sihalath (1999) undertook some experiments at IoA, and showed that *A. invadans* zoospores responded to extracts of a variety of EUS-susceptible and non-susceptible fish. He also showed chemotactic response to lower concentrations of particular amino acids than shown by other fish-parasitic fungi, but concluded that more detailed experimentation was needed to make any practical use of these findings.

3.3.2.7 Zoospore treatment

Experiments to test the effect of various compounds on zoospore motility and viability are described in Campbell *et al*. (2001; Appendix 17), and discussed further in Section 3.5.

3.3.4 Contribution of outputs

The most significant contribution of the zoospores physiology studies is probably the finding that *A. invadans* cysts can remain viable for at least 20 days. On the basis of this, it is recommended that ponds, which cannot be drained and dried prior to stocking, should be fallowed (kept free of fish and other possible *A. invadans* substrates) for at least 20 days prior to stocking.

A number of staff and students from various institutions undertook zoospore studies to gain experience in research on *A. invadans*, and Oomycete fish pathogens in general. It is hoped that the series of articles published in the AAHRI newsletter on the topics described above will also provide researchers with a basis for work in this field.

3.4 TANK STUDIES

3.4.1 Introduction

Previous studies have shown that EUS lesions can be reproduced in susceptible fish by injection with *A. invadans* spores, or subcutaneous insertion of *A. invadans* hyphae (Chinabut *et al.,* 1995; Lilley & Thompson, 1997). However, in order to test preventative treatments against *A. invadans* spores in water, a bath challenge system was required in which fish would be infected if kept in tanks or ponds with free-swimming *A. invadans* propagules. Callinan (1997) used a bath challenge system to test the hypothesis that exposing sand whiting (*Sillago ciliata*) to water of pH 5, followed by immersion in *A. invadans* spores would reproduce the process of EUS infection that occurs in Australian estuaries. The present project aimed to test this challenge system, and a system of abrading fish followed by immersion in *A. invadans* spores, using EUS-susceptible fish found in Thailand. The project also planned to undertake further investigations of other agents, to test whether, in combination with *A. invadans* spores, they could reproduce EUS.

During the project, other tank trials were also carried out to test susceptibility of European fish species to *A. invadans* in order to investigate the possibility that EUS could spread in Europe. In other studies, new isolates of *A. invadans* were also tested for pathogenicity in snakehead fish. Fish were also exposed to treatments identified in Section 3.5 to determine whether they could be harmed by the compounds at the concentrations required to kill fungal spores.

3.4.2 Activities and outputs

3.4.2.1 Infection of different species

A list of confirmed EUS-affected species is given in Table 3.2.1. However this does not provide an indication whether fish species in other regions would be susceptible to the disease, so that the risk of it spreading to other areas can be assessed. Khan *et al*. (1998; Appendix 15) describe attempts to experimentally infect rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*) and stickleback (*Gasterosteus aculeatus)* by injection of over 1000 *A. invadans* spores. The latter two showed very little signs of infection given the high challenge dose, and gave results that were comparable to injected tilapia (*Oreochromis niloticus*), which is generally considered to be an EUS-resistant fish. Rainbow trout, however, could be infected, but progression of the disease and mortality were low compared to the susceptible rosy barbs (*Puntius schwanenfeldii*). Thompson *et al*. (1999; Appendix 22) undertook further challenges of rainbow trout, and showed that at doses of less than 100 spores the fish did not become infected. Therefore, the studies demonstrated a potential for infection to trout, but under conditions that were unlikely to exist in natural circumstances.

3.4.2.2 Pathogenicity testing of new isolates

During Project 5997 26 *A. invadans* isolates were injected into snakeheads and shown to be pathogenic and 32 other fungal species were shown to be non-pathogenic (Lilley & Roberts, 1997). This technique is therefore an important means of characterising *A. invadans.* All new isolates obtained during the present study (Table 3.2.1) were therefore injected into snakehead fish. Six *A. invadans* isolates were shown to be pathogenic and four other fungi were non-pathogenic in snakeheads. For a discussion of experiments on

American isolate UM3, see Section 3.1.2.1.2; Section 3.2.2.9; Blazer *et al.,* 2001; and Appendix 10.

3.4.2.3 Fish toxicity tests

Selected treatments were tested for toxic effects against silver barb, (*Barbodes gonionotus*) at AAHRI and rainbow trout, (*Oncorhynchus mykiss*) at IoA (Campbell *et al.,* 2001; Appendix 17). The results for barbs are shown in Table 3.4.2. These results helped to determine concentrations of treatments used in pond trials described in Section 3.5.

Table 3.4.2 Toxicity of fungicides on *Barbodes gonionotus*

Treatment	Highest concentration at which no adverse effects were recorded	Lowest concentration at which adverse effects were recorded
Crude neem seed extract	250ppm	500ppm
Commercial neem (0.27%	20ppm	50ppm
Commercial neem & *OP10	20ppm + 0.5ppm	50ppm + 0.5ppm
D-limonene + *E-Z-Mulse™	5ppm + 0.5ppm	20ppm + 0.5ppm
*OP10	2.5ppm	5ppm
*E-Z-Mulse™	0.5ppm	2.5ppm
*Coconut diethanolamide	2.5ppm	5ppm

*surfactants

3.4.2.4 Development of bath challenge model

Initial attempts to develop a bath challenge model at AAHRI are described by Fairweather (1999). Sixteen 20-litre tanks, each containing ten snakeheads, were used to compare challenges using sporulating *A. invadans* cultures and (i) tap water, (ii) distilled water (low alkalinity, low hardness) and (iii) pH5 distilled water (low pH, low alkalinity, low hardness). After 30 days, the average percentages of EUS-affected fish in each of 4 replicate tanks were: (i) 42% in tap water; (ii) 50% distilled water, and (iii) 73% in the acidified water. However, the treatments were not significantly different, probably because the fish showed aggression to each other in the small tanks, which may have lead to biting and the development of lesions which in groups (i) and (ii). A subsequent treatment trial was carried out using 150-litre tanks, which helped to prevent the fish showing aggression (Fairweather, 1999).

In a second trial, the larger 150-litre tanks were used, and both striped snakeheads (*Channa striata*) and silver barbs (*Barbodes gonionotus*) were tested. Fish with external lesions were confirmed as EUS-positive using Uvitex-H&E histology. The results of the bath challenge are illustrated in Appendix 16. All the snakeheads injected with *A. invadans* spores (300 spores in 0.1ml) (Appendix 16 Fig. B), scraped and bathed in spores (Appendix 16 Fig. D), or exposed to pH5 water and bathed in spores (Appendix 16 Fig. E), became EUS-infected, and controls (no *A. invadans* spores) remained unaffected. However, in the silver barb trial, only the injected fish became infected (Appendix 16 Fig. A). Abraded, acid-exposed, and control fish remained unaffected. In subsequent pond trials (Section 3.5), *Channa striata* and mrigal (*Cirrhinus cirrhosus*) proved suitable models, whereas juvenile giant gouramis (*Osphronemus goramy*) failed to become infected in sufficient numbers. A summary of the tank bath challenge procedure is given in Box 3.4.1.

3.4.2.5 Combined challenges with other biological agents

Sarkar *et al*. (1998; 1999) undertook challenge experiments at BAU, Mymensingh, and showed that a variety of *Aeromonas* spp. caused lesions and mortality in *Barbodes gonionotus* when bath challenged at very high doses. On the advise of project staff, challenge experiments were conducted with natural levels of bacteria in combination *with A. invadans* spores, and EUS-type lesions and mortality were reported (M.G.A. Sarkar, pers. comm.). This would indicate that bacteria could be an initiating factor for EUS in some circumstances.

Box 3.4.1 Bath challenge procedure using *A. invadans*

- Acclimatise 10 snakeheads for 14 days in each 150-litre tank filled with tap water at 22-24^{o}C
- For each challenge tank, produce 7 wads of *A. invadans* using the hemp seed-V8-APW technique described in Lilley *et al.* (2001e; Appendix 13)
- Put the wads in a 15ml plastic tube with holes burnt along the length, and suspend the tube in the middle of the tank (Appendix 16 Fig. C)
- About 12 hours later, remove the fish and either:

(i) Put in a bucket containing pH 5 water (prepared by adding 1.5 g/litre NaH_2PO_4 to distilled water) for 30 min; or

(ii) Abrade a small (0.5cm^2) area on the left flank of the fish

- Return the fish to the challenge tank
- Feed at 2% body weight daily
- Change water every 5 days
- Fish will start to show lesions about 14 days post-challenge
- Confirm as EUS-positive using Uvitex-H&E histology (Box 3.1.4.
- In tank and pond trials, *Channa striata* and *Cirrhinus*

Previous experiments undertaken by Kanchanakhan (1996) have shown that intramuscular injection with rhabdovirus isolate T9412, followed by immersion in *A. invadans* spores, results in typical EUS in snakeheads. Arrangements were made for studies to bath challenge with both the rhabdovirus and *A. invadans,* in order to demonstrate probable component causes of an EUS outbreak in snakeheads in Thailand (Kanchanakhan, pers. comm.). However these were abandoned due to the same problems of aggression in the fish as encountered by Fairweather (1999).

3.4.4 Contribution of outputs

The development of the bath challenge model was essential to enable work to commence on pond trials to test preventative treatments for EUS (Section 3.5).

The trials also provided some information on the possible development of EUS in natural outbreaks. However, it is unknown why some EUS-susceptible species (striped snakeheads and mrigal) were readily infected with this challenge, and others (silver barbs and giant gouramy) were not. It may be possible that due to a more rapid rate of skin healing in the latter two species, the dermal insult provided by abrasion or acid water did not coincide with the release *of A. invadans* spores. Alternatively, these forms of dermal insult do not provide the correct route of entry for *A. invadans* spores in those species of fish.

Sufficient work has now been carried out using injection trials for this to be a standard procedure for testing pathogenicity of fungal isolates, and susceptibility of different fish species to invasive fungal growth (although the immersion trial would be a more suitable test for susceptibility of fish to EUS infection).

The toxicity tests described here are not sufficient to provide recommendations for use of novel chemicals in the culture ponds. However, they do provide a rapid method of determining whether the compound under investigation is worth pursuing further.

3.5 TREATMENT STUDIES

3.5.1 Introduction

As the fungus *A. invadans* is generally considered to be the necessary cause of EUS (Section 1.1.3), a major component of the project was aimed at identifying and testing treatments against *A. invadans* (Figure 4.1).

Compounds were initially screened *in vitro* for activity against *A. invadans* hyphae, zoospores and sporulation (Section 3.5.2.1). Candidate treatments were checked for toxicity to *Barbodes gonionotus* (Section 3.4.2.3). Some treatments were tested in tank trials against naturally infected fish (Section 3.5.2.21). Treatments were then assessed as preventative control measures in pond trials in Thailand (Section 3.5.2.3) Bangladesh (Section 3.5.2.4) and India (Section 3.5.2.5). In collaboration with the CARE-LIFE project, treatment interventions were tested by farmers in Rajshahi and Kishoreganj, Bangladesh in 1999-2000 and 2000-2001 seasons.

3.5.2 Activities and outputs

3.5.2.1 *In vitro* studies

A large number of chemicals have been tested for activity against *A. invadans* hyphae and zoospores (Campbell *et al*., 2001; Appendix 17; Taukhid, 1999; Fairweather, 1999). These include: (a) chemicals with previous reported activity against Oomycete fungi; (b) chemicals in use in Asia to treat ulcerative disease outbreaks; (c) commercial biocides and fungicides; (d) natural products with potential anti-microbial activity; and (e) surfactants tested separately, and in combination, with some of the above treatments.

No compounds tested proved as effective as malachite green, but some low toxicity natural compounds and particular surfactants showed potential for further studies. Some compounds that are currently in use in Asian aquaculture were shown to have no effect on *A. invadans* hyphae at recommended treatment rates. A range of compounds which demonstrated activity against the mycelium were selected for further testing in a zoospore motility assay. Lower treatment concentrations were required to inhibit zoospore motility than were required to inhibit hyphal growth. Zoospore activity ceased within 1 hour exposure to 2.5ppm coconut diethanolamide; 1.25ppm propolis + 0.5ppm 13/6.5; 5ppm neem (*Azadirachta siamensis*) seed extract + 0.01ppm OP10; 20ppm tea tree (*Melaleuca alternifloria*) oil; and 25ppm D-limonene + 0.05ppm E-Z-Mulse™. The treated spores were shown to be non-viable in culture medium. Selected compounds were further tested for ability to inhibit zoospore production by *A. invadans* mycelium over a 72-hour period. In toxicity trials (Section 3.4.2.3.) silver barbs suffered no mortalities and no obvious behavioural changes from candidate treatments.

3.5.2.2 Therapeutic treatments

Various treatments were tested on naturally infected fish during the winter season of 1999-2000. In one experiment, 100 EUS-infected climbing perch, *Anabas testudineus,* from a farm in Chainat province, Thailand were transported to AAHRI. Five fish were placed in each of twelve 120-litre tanks. these were randomly allocated to one of the following treatments: (i) 37.5ppm lime $Ca(OH)_2$; (ii) 37.5ppm lime, 2.5ppt salt, 0.1ppt $CaCl_2$ & 0.1ppt $MgCl_2$; (iii) 2.5ppt salt, 0.1ppm malachite green & 25ppm formalin; (iv) Salar bec at 0.5% of feed; (v) 50ppm neem seeds & 0.01ppm 13/6.5 surfactant (vi) Control (no treatment).

None of the treatments were able to prevent the development of lesions and mortality in the fish. The fish did not feed well, and therefore the Salar bec (immunostimulant) was probably not taken up by the fish. External fungus was apparent on most fish at the start of the trial, and only in the salt, malachite green and formalin treatment (iii) was this successfully controlled. Indeed, one of the treatment (iii) tanks was the only tank in which fish survived until day 10. Fish died rapidly in all other tanks, with invasive fungus and external *Achlya* infections.

3.5.2.3 Pond trial - Thailand

The scrape bath challenge model described in Section 3.4 was adapted for use in pond trials to test preventative treatments for EUS. The trials are described in more detail and illustrated in Appendix 18. Briefly, five 400m^2 earthen ponds were used for the trial. Two 1m^2 hapas with bamboo walkways were fixed in each treatment pond. On the morning of the challenge (day 0), one container with 10 *A. invadans* mats was placed in each hapa and treatments were added to the ponds. The following treatment regimes were tested: 0.15ppm malachite green; 2.5ppt salt and 38ppm $Ca(OH)_2$; 125ppm dried, crushed neem (*Azadirachta siamensis*) seed; and control (no treatment). On the afternoon of day 0, 20 striped snakeheads or 20 juvenile gouramies were weighed, artificially abraded and put in each of the 8 small hapas. On day 10 post-challenge, all fish were examined for clinical signs, weighed, and sampled for Uvitex-H&E histology. New fungal wads were added on the morning of day 10, and 20 new pre-acclimatised fish were abraded and added to the hapas in the afternoon of day 10. These were examined, weighed and sampled on day 20.

The results of the first part of the snakehead trial are given in Table 3.5.1. Malachite green was the most effective of the treatments, with only four fish from this group showing low levels of fungus in skin tissue, and none showing clinical lesions. The snakeheads in the salt and lime and neem groups also showed lower percentage infection than untreated controls.

Giant gourami was not a suitable model for EUS treatment trials, as low numbers of fish were affected in all groups, including untreated controls. Low numbers of both snakeheads and giant gouramis were infected in the second phase of this study, probably due to a rise in water temperatures (>26^oC).

Table 3.5.1 Percentage of snakeheads confirmed with EUS after 10 days

Treatment	Number fish with EUS	Number sampled	Percentage of fish with EUS
Control	18	20	90%
Malachite green	4	19	21%
Salt and $Ca(OH)_2$	4	20	20%
Neem seeds	6	20	30%

3.5.2.4 Pond trial - Bangladesh

The trial is described in Appendix 18. Briefly, ten earthen ponds (46-113m^2) were used for the trial. On the morning of the challenge (day 0), five containers with four *A. invadans* mats were placed in each pond and treatments were added to the ponds. The following treatment regimes were tested: green water (250ppm pre-stock cowdung + 5ppm urea + 5ppm TSP and 3-4 day post-stock treatments of 25ppm cowdung + 2.5ppm urea + 2.5ppm TSP); 25ppm pre-stock liming + 6ppm weekly post-stock liming ($CaCO_3$); 25ppm sieved ash; 50ppm crushed neem seed; and control (no treatment). On the afternoon of day 0, 20 *Cirrhinus mrigala* fingerlings (12-15 cm) were weighed, artificially abraded and put in each of the 10 ponds. On day 14 post-challenge, all fish were examined for clinical signs, weighed, and sampled for Uvitex-H&E histology.

The results of the sampling of the trial fish is given in Table 3.5.2. None of the treatments were totally effective in preventing infection by *A. invadans*. The fertilised ("green water") ponds had the lowest levels of infection followed by neem, lime and ash ponds. The untreated control groups showed high levels of infection

Table 3.5.2 Percentage of mrigal confirmed with EUS after 14 days (number of fish sampled is given in brackets)

Treatment	Pond 1	Pond 2
Control	88% (8)	100% (10)
Green water	14% (7)	40% (10)
Neem seeds	33% (9)	75% (8)
Lime	44% (9)	89% (9)
Ash	78% (9)	89% (9)

3.5.2.5 Pond trial - India

The Central Institute for Freshwater Aquaculture (CIFA), Bhubaneswar has extensive experimental pond facilities, and the staff have been engaged in EUS research since its first occurrence in India in 1988. A drug that is being marketed region-wide as a preventative and therapeutic treatment for EUS (CIFAX) was developed at CIFA. Therefore, project staff were keen to collaborate with CIFA staff in order to undertake replicated, controlled experiments to test a variety of treatments for EUS, including CIFAX. Following the DFID-ACIAR sponsored visit to CIFA by project staff in 1999, plans were made for pond trials that winter (Callinan *et al.,* 1999; Appendix 30). However, these trials were postponed until winter 2000-2001 due to the devastation caused by the cyclone that affected Orissa in 1999.

Given the success of the immunostimulant tank trials (Section 3.6.3.2), project staff were keen to use the opportunity to test these compounds at CIFA. In addition, CIFA staff have undertaken work on *Aeromonas hydrophila,* and are convinced that it has a strong involvement with EUS outbreaks in India. Therefore, four separate studies were designed in order to test immunostimulants and preventative treatments against *A. invadans* and *A. hydrophila:*

Trial 1. Immunostimulant trial - Aphanomyces invadans (8 Dec 2000 - 21 Jan 2001)
Nine 1000-litre earthen-bottomed ponds were used for the trial. Three ponds were randomly allocated to one of two immunostimulants (Salar bec or Ergosan) or one control. Ten clinically-normal *Cirrhina mrigala* fingerlings (3-4 inches) were acclimatised in each pond. Ten days before challenge immunostimulant was incorporated in the feed (at 0.25% of feed, and fish were fed at 2% body weight per day). On the morning of the challenge, five *A. invadans* mats were placed in a plastic container with holes and suspended in the middle of the pond. In the fish were artificially abraded on the left flank and returned to the tanks. Mortality was recorded over the experimental period and any moribund/recently dead fish sampled for histology. On day 30, all the remaining fish were examined for clinical signs and sampled for histology. Uvitex-H&E histology of formalin-fixed samples is being undertaken at CIFA.

Trial 2. Preventative treatment trial - Aphanomyces invadans (7 Dec 2000 - 10 Jan 2001)
Sixteen 1000-litre ponds were used for this trial. Two tanks were randomly allocated to one of 7 treatments or 1 control: (a) 0.15ppm malachite green (b) 10ppm CaO and 1ppm turmeric (c) 5ppm calcium carbide (CaC2) (d) 0.1ppm CIFAX (e) 200ppm ground neem seeds (f) 2.5ppm coconut diethanolamide (g) green water (fertiliser) (h) control. Treatments were added to the ponds the day before challenge. Water quality was monitored periodically. Fish were challenged and sampled as described for Trial 1. However in this case, the number of fish infected with A. invadans, not mortality, was the main outcome variable, therefore all fish could be sampled by day 14 post-challenge.

Trial 3. Immunostimulant trial - Aeromonas hydrophila (7 Dec 2000 - 21 Jan 2001)
The trial was conducted as for Trial 1, except that the challenge comprised of an intramuscular injection with 0.1ml *A. hydrophila* suspension (10^7 cells/ml).

Trial 4. Preventative treatment trial - Aeromonas hydrophila (11 Dec 2000 - 10 Jan 2001)
The trial was conducted as for Trial 2, except that only four treatments and one control were used: (b) 10ppm CaO and 1ppm turmeric (d) 0.1ppm CIFAX (e) 200ppm ground neem seeds (g) green water (fertiliser) and (h) control. The challenge comprised of an intramuscular injection with 0.1ml *A. hydrophila* suspension (10^7 cells/ml). CIFA staff felt that it was not necessary to bath challenge the fish, as the treatments were capable of preventing disease even though fish had been injected with the pathogen.

All studies were scheduled to be completed by the end of the project. However, histological processing of samples have taken longer than envisaged. At present, no mortality or histology data is available. When results are provided, a report will be provided, and it is hoped that an article can be prepared to publicise the results.

3.5.2.6 Farmer based trial - Bangladesh

The Locally Intensified Farming Enterprises (LIFE) project run by CARE – Bangladesh is working at Rajshahi and Kishoregonj districts located in the northern part of Bangladesh to enhance food security through improving farmer's knowledge and skills with regard to major agricultural activities. Farmers working with the LIFE project identified EUS as the main problem they encounter in fish cultivation.

During the 1998-9 winter season, LIFE assisted farmers to investigate four possible treatment that the farmers had identified (Nandeesha *et al.,* 2001). LIFE staff contacted project R6979 staff and proposed further joint investigation of the treatments. The study aimed to determine whether the treatments reduced the incidence of EUS as reported by farmers, and to assess whether the farmers were satisfied with the treatments and were prepared to continue using the treatment in subsequent years. A manuscript describing these studies has been prepared and is reproduced in Appendix 19. A report to CARE and DFID was also produced (Islam *et al.,* 2001). In addition to the CARE-LIFE supervised farmers, this reports on the results provided by farmers supervised by Partner NGOs, and the reported effect of the treatments on the recovery of affected fish.

Six candidate prophylactic treatments were identified through consultation with farmers. These comprised of: ash, lime, salt, salt&lime, neem branches (*Azadirachta indica*), and fertiliser. Two hundred and seventy-eight farmers (94 in Rajshahi district and 184 in Kishoregonj district) participated in the trial. Each farmer selected one of the treatments for their experimental pond. Lime and ash were the most popular selections in both districts. For each treatment except neem, fortnightly applications were advised over the study period from October 1999 to February 2000. However, at the end of the study, the number of applications of these treatments averaged only 3.3 times. The occurrence of ulcerative disease was reported by farmers in a structured questionnaire at the end of the study. A proportion of affected sites were visited by CARE staff during the study and 30 fish samples were processed at BAU to determine whether the disease was EUS. Seventy percent of the samples could be confirmed as EUS-positive.

Farmers from all treatment groups reported a lower incidence of ulcerative disease than farmers with control ponds (Table 3.5.3). In total, 9.4% of farmers adopting a treatment reported ulcerative disease, compared to 61.9% of farmers with control ponds. No farmers applying fertiliser reported disease, but only three farmers adopted this treatment. Only two of the 60 farmers adopting lime (3.3%) reported disease, and 9.8% of ash farmers, 10% of salt farmers and 15.4% of salt&lime farmers reported disease. Neem was the least effective of the treatments, with 19% of farmers reporting disease. Fish production levels, as reported by farmers, were higher in all treatment groups than controls in Kishoregonj, but production from lime, neem and ash treatment ponds was lower than controls in Rajshahi. The percentage increase in production on the previous year, as reported by farmers, was higher in all treatment groups than controls in both districts. The cost of production compared to the previous year, as reported by farmers, was higher than controls for all treatment farmers, except for those using ash. However, in all cases, the reported increase in production was higher, or equal to, the increase in costs. Farmers adopting the salt treatment reported the highest increase in input costs.

Of the farmers that adopted one of the treatments, 95% indicated they were satisfied with the results. The majority indicated that this was due to no occurrence of disease. The most popular treatments were lime and ash, with 38% and 32% of farmers expressing a desire to adopt these respective treatments in the next season. Other pond variables were recorded to check whether they were associated with the occurrence of ulcerative disease in the experimental ponds. The entry of wild fish in ponds, and the occurrence of ulcerative disease in nearby ponds, were significant risk factors for ulcerative disease in Kishoregonj and Rajshahi respectively.

Table 3.5.3 Reporting of disease by farmers adopting different treatments

Treatment	Rajshahi				Kishoregonj			
	No. of ponds	Farms affected		% farms affected in 1999-00	No. of ponds	Farms affected		% farms affected in 1999-00
		1998-99	1999-00			1998-99	1999-00	
Salt	4	2 (50%)	0 (0%)[a]	8.6%	16	13 (81%)	2 (13%)[a]	9.9%
Lime	30	11 (37%)	1 (3%)[a]		30	22 (73%)	1 (3%)[a]	
Salt&lime	11	4 (36%)	1 (9%)[a]		15	13 (87%)	3 (20%)[a]	
Neem	6	4 (67%)	3 (50%)		15	14 (93%)	1 (7%)[a]	
Ash	19	14 (70%)	1 (5%)[a]		42	37 (88%)	5 (12%)[a]	
Fertiliser	0	-	-		3	3 (100%)	0 (0%)[a]	
Control	11	8 (73%)	7 (64%)	63.6%	31	22 (71%)	19 (61%)	61.3%
Total	**81**	**43**	**13**		**152**	**124**	**31**	

Figures in the parentheses show data as a proportion of the total
Figures with superscript show significant differences with Control at 5% level of significance

The trial was repeated, with a number of adaptations, with new farmers during the 2000-2001 season. Farmers were selected according to a number of criteria to minimise variables, and to ensure that the trial ponds were at high risk of EUS. The ponds were better monitored other measurable indicators were used to independently verify data provided by farmers. The results of these trials are not yet available. Correspondence with CARE-LIFE staff indicates that uptake of treatments by farmers continues to be good.

3.5.3 Contribution of outputs

Although none of the treatments that were tested completely prevented EUS, a number of the treatments resulted in significantly less fish affected in experimental ponds, or significantly less ponds affected as reported by farmers. The experiments on some of these treatments (fertilisation and lime) are in agreement with the study of risk factors (Section 3.7), which showed that Bangladesh polyculture farmers that fertilise and lime ponds are at lower risk of getting EUS. These are probably the two most cost-effective preventative treatments, and they have the added benefit of increasing fish production in the ponds.

Ash was a popular treatment among farmers in areas where it is readily available (Section 3.5.2.6), but in controlled pond trials it had little effect in reducing EUS occurrences (Section 3.5.2.4).

Neem appeared to have some effect in controlled trials, but in the farmer-based trial (Section 3.5.2.6), none of the farmers were prepared to use seeds, and the leaves were used at levels which are very unlikely to have any effect.

Salt can also be effective, but very high doses are required to raise the salinity in ponds to a level that *A. invadans* will not sporulate. The expense of this would be prohibitively high for most small-scale farmers. Salt may have some has an effect at lower doses, probably in improving the condition of the fish, rather than any effect on the fungus.

A study of the use immunostimulants is described in Section 3.6.3.2, and they were found to be effective in reducing mortality due to EUS. The results of the pond trials (Section 3.5.2.5) are awaited.

Tank trials testing therapeutic treatments on naturally infected snakeheads and climbing perch showed that the chemicals were not effective against the fungus once it had already infected the fish.

3.6 IMMUNOLOGY

3.6.1 Introduction

Most studies on the control of EUS have concentrated on the exclusion of *A. invadans* from culture systems (Lilley *et al.,* 1998). However, health management in European, North American and Japanese aquaculture usually involves consideration of the immunity of the cultured fish to disease. Although less is known about the immunology of species cultured in the EUS-endemic regions of Asia, it is possible that technologies may be transferred to the region in order to reduce the impact of diseases such as EUS on aquaculture.

The fact that EUS outbreaks are usually restricted to the temperatures below 30°C (Tonguthai, 1985), even though *A. invadans* grows in culture at much higher temperatures (Willoughby *et al.,* 1995), implies that even species that are typically regarded as susceptible must have some inherent immunity to EUS. More direct proof that apparently susceptible species mount an immune response to *A. invadans* was demonstrated when Chinabut *et al.* (1995) showed that striped snakeheads, *Channa striata*, can eliminate injected *A. invadans* at temperatures above 30°C.

Although there is a considerable body of literature on the immune response of other organisms such as mammals, plants and crustaceans to fungi and similar hyphal pathogens, the immune response of fish has not been widely studied in this context. Further, most of the studies of the immune system of fish in any context have focused on species of economic importance in developed countries such as salmonids and channel catfish *Ictalurus punctatus*.

One of the most widely reported aspects of the immune response to *A. invadans* is the infusion of granulocytes, cells of the non-specific immune system, into the infection site (e.g. McKenzie & Hall, 1976; Wada *et al.,* 1994; Vishwanath *et al.,* 1997). The granulocytes surround the invasive hyphae with granulomata, so-called because of their granular appearance. Granuloma formation is such a characteristic feature of *A. invadans* infection that it is incorporated in the case definition of EUS (Section 1.1.2). Rapid granuloma formation appears to be a feature of fish resistant to EUS. Wada *et al.* (1996) showed that resistant common carp *Cyprinus carpio* form granulomata far more rapidly than susceptible ayu *Plecoglossus altivelis*. Similarly, Chinabut *et al.* (1995) showed that striped snakehead at high temperatures form granulomata rapidly after injection with *A. invadans* and recover, while snakeheads at low temperatures form granulomata more slowly and are far more likely to die. One of the main granulocytes is the macrophage, which commonly ingests bacteria or surrounds larger pathogens, and destroys them with reactive oxygen compounds such as superoxide or nitric oxide (Secombes & Fletcher, 1992). The activity of macrophages against *A. invadans* was assessed in studies described here (Section 3.6.2.1).

Several components of the immune system are carried in the blood, but their importance in immunity to *A. invadans* has received little attention. Complement proteins are used by mammals to destroy hyphal pathogens (Lehman, 1985). Fish use complement proteins in defence against several microbial pathogens (Alexander & Ingram, 1992), and complement deficiency has been correlated with susceptibility of channel catfish to winter saprolegniasis (Hayman *et al.,* 1992), an Oomycete disease which shares several of the characteristics of EUS. The only adaptive immune mechanism that has been shown in fish

is the production of antibodies (Yano, 1996). Antibodies are produced in response to foreign substances, such as toxins or pathogens. It has been shown that striped snakeheads (Lilley, 1997; Thompson *et al.,* 1997) produce antibodies in response to infection with *A. invadans*, though it has not been established whether the antibodies actually confer protection (see Section 3.6.2.3).

There are two main methods by which it is possible to enhance the immune response of fish. Vaccination involves exposing the fish to the pathogen involved to stimulate the production of antibodies to it. Immunostimulation involves supplementing feed with substances that enhance several factors of the immune system.

Vaccination presents logistical problems, as vaccines are usually applied by injection, which may not be appropriate for many Asian aquaculture systems where fish are not handled until harvest. Handling fish purely for the purpose of vaccination may not be advisable, as there is evidence that the stress and damage caused by such procedures renders fish more vulnerable to opportunistic pathogens (Strasdine & McBride, 1979). Also there is some doubt as to whether a vaccine would be affordable to low income, low input systems. The study described in Section 3.6.2.3 examined the possibility that the anti-*A. invadans* antibodies previously shown may confer protection, to establish whether vaccination may be worth considering in more detail.

There has been considerable interest in immunostimulation since it was found that much of the benefit of vaccinating salmonids was conferred through the stimulation of the innate immune system intended to ensure the uptake of the vaccine, rather than through the vaccination itself (Olivier *et al.,* 1985; Cipriano & Pyle, 1985). Since then, a wide range of substances have been found to enhance the resistance of fish to many different pathogens (Sakai, 1999). Most studies have concentrated on bacterial pathogens, although Catap & Munday (1998) reported that β-glucans, commonly used as immunostimulants for salmonids, improved the immune response of sand whiting *Sillago ciliata* to *A. invadans*. Vadstein (1997) suggested that immunostimulation may be most appropriate in situations where vaccination is not possible. At present, there is no available vaccine for any of the diseases most commonly encountered in freshwater aquaculture in Asia, including EUS, so the potential for immunostimulation for reducing the impact of such diseases, is worth investigating. Even if a vaccine to EUS were available, the logistics of applying it and the likely cost of the vaccine would make it inappropriate for many EUS-affected systems, while the same limitations may not apply to immunostimulation (see Section 3.6.2.2).

Both immunostimulation and vaccination are best regarded as prophylactic rather than therapeutic measures, and so should be applied to farms that are at risk from EUS rather than those that are already undergoing an outbreak. In order to establish what farms are at risk, it is necessary to know the distribution of *A. invadans*. At present, monitoring is carried out by diagnosis of fish already infected with *A. invadans* (Section 3.2.1). Consequently, it is not possible to establish the presence of EUS in a region until an outbreak is already in progress, by which time farms may already be incurring losses. Testing of a technique to monitor presence of *A. invadans* in the environment by the presence or absence of antibodies in the blood is described in Section 3.2.2.8.

The immune studies described here initially investigated which mechanisms are important in the immune response to *A. invadans* (Section 3.6.2.1). A range of potential

immunostimulants were then used to try to enhance those mechanisms and the most successful used in trials that replicated EUS outbreaks on farms as far as possible (Section 3.6.2.2). Further studies investigated the antibody response of susceptible fish in more detail, in order to establish how quickly antibodies are produced in response to natural outbreaks, whether they confer protection (Section 3.6.2.3), and whether immunoprevalence has potential for monitoring the occurrence of *A. invadans* (Section 3.2.2.8).

A further study, initiated during Project 5997, describes the immune response of rainbow trout (*Oncorhynchus mykiss*) against *A. invadans* with a view to using trout as a model for *A. invadans* experiments (Thompson *et al*., 1999; Appendix 22). This work provided the basis for some of the studies described here in Section 3.6.2.1. It showed that rainbow trout produce macrophages that are capable of killing *A. invadans*, and antibodies in response to infection with *A. invadans*.

3.6.2 Activities

3.6.2.1 Comparison of immune response of different species

This study is described in detail in Miles *et al.* (2001a; Appendix 21). Practical aspects of macrophage culture were discussed in a separate article (Miles, 1999; Appendix 20).

The immune responses of four EUS-susceptible species were compared to that of non-susceptible Nile tilapia. All the species used in the study (Table 3.6.1) are of some economic importance in Thailand and all other than the swamp eel are cultured. The resistance of Nile tilapia has been established conclusively by injection with high concentrations of *A. invadans* zoospores (Khan *et al.,* 1998; Appendix 15). All fish were acquired from ponds or natural water bodies in central Thailand, a region known to be EUS-endemic (Tonguthai, 1985; Chinabut, 1998).

Table 3.6.1. Species, source and EUS-susceptibility of fish used for comparison of the immune response of different species

Species	Source	EUS-Susceptibility*
Striped snakehead *Channa striata*	Pond farms, Suphanburi	Yes
Silver barb *Barbodes* (= *Puntius*) *gonionotus*	National Aquaculture & Genetic Research Institute, Patum Thani	Yes
Giant gourami *Osphronemus gouramy*	Pond farms, Uthai Thani	Yes
Swamp eel *Monopterus* (=*Fluta*) *albus*	Wild caught fish from Saphanmai market, Bangkok	Yes
Nile tilapia *Oreochromis niloticus*	Singburi Province Fisheries Station.	No

*Lilley *et al.* (1998)

The fish were acclimatised to low temperature, at which EUS occurs, for at least three weeks, in order to induce the condition that the fish are likely to be in at the time that they would normally be exposed to a natural epidemic. Other snakeheads, silver barbs and tilapia were acclimatised in aquaria at warmer temperatures at which EUS does not occur, in order to compare the immune response of the fish at EUS-permissive and non-permissive temperatures.

It was not feasible to examine every factor involved in the complex immune system of fish, so the study concentrated on two areas, macrophages and blood serum. Some of the serum was heated to inactivated complement proteins.

The effect of serum, heated serum and macrophages on *A. invadans* was assessed by incubating them with freshly prepared *A. invadans* cysts overnight in a microplate. The following day, the number and length of the germlings that had grown was assessed and compared to the number and length of germlings from a culture without serum or macrophages. The number of macrophages that adhered to the microplate was also assessed, as there is evidence that only activated cells adhere (Kwak *et al.,* 1998), so the proportion of adherents may indicate the proportion of macrophages activated.

Other macrophages were incubated in the presence or absence of *A. invadans* overnight, and superoxide production, a major mechanism of killing microbes, assessed the following day.

Other serum from snakeheads, gourami and tilapia was used for quantification of the concentration of antibodies that reacted to *A. invadans* by enzyme linked immunosorbent assay (ELISA). ELISAs could not be carried out in the case of swamp eels or silver barbs as species-specific reagents are required, and are not available for those species.

3.6.2.2 Immunostimulation

This study is described in detail in Miles *et al*. (2001b; Appendix 23). It assesses the ability of five feed supplements supplied by Aquaculture Vaccines Ltd. (AVL) in enhancing resistance to *A. invadans* (Table 3.6.2).

Table 3.6.2. Active ingredients of putative immunostimulants used for *in vitro* trial

Product	Active ingredient*
Salar-bec	300g kg^{-1} vitamin C, 150g kg^{-1} vitamin E, trace quantities of vitamins B_1, B_2, B_6, B_{12}
Ergosan	0.002% unspecified plant extract, 1% alginic acid from *Laminaria digitata*, 98.998% algal based carrier
Betamak C85	Brewers' yeast containing 32% β 1,3 & 1,6 Glucans, 30% unspecified mannan
Lysoforte	Lysophospholipids
Oro glo layer dry	Yellow xanthophylls derived from marigold *Tagetes erecta*, principally lutein with significant amounts of zeaxanthin

*Information courtesy of AVL and Elorisan GmbH-Biostimulatoren, Deggendorf, Germany.

A preliminary study was carried out by injecting all the potential immunostimulants into striped snakehead. After 14d, the ability of the macrophages and serum of those fish to inhibit the germination and growth of *A. invadans* cysts was assessed, as described in Section 3.6.2.1. Inhibition was compared to the macrophages and serum of control fish. Salar-bec and Ergosan were the only substances that were identified as potential immunostimulants in this preliminary experiment, and were used in a larger scale trial.

Striped snakeheads and silver barbs were selected as the study species as they are highly susceptible to EUS (Tonguthai, 1985; Roberts *et al.,* 1988; Chinabut, 1998), and some information on the immune response of both species to *A. invadans* was available from previous studies (Thompson *et al.,* 1997; Khan *et al.,* 1998; Section 3.6.2.1).

Immunostimulants were mixed with feed pellets at 2g kg^{-1}, and attached to the pellets by spraying 10ml kg^{-1} vegetable oil into the mixture. The feed pellets were of a brand widely used in Asia, and probably similar in nutritional content to other common feeds. Vegetable oil was used rather than the specialised adhesives recommended by AVL, as it is widely and cheaply available. As far as possible, the supplemented feed preparations were as similar as possible to those which may be expected if the immunostimulants were used on farms. The fish were fed with either the supplemented feed or control diets of pellets and oil only for 14d. After 14d, the feed was changed to pellets only and *A. invadans* zoospores were injected into the dorsal muscle of the fish, a technique frequently used to replicate EUS infection (Chinabut *et al.,* 1995; Lilley & Roberts, 1997).

Two tanks of 40 fish were used to assess the effect of each diet on each species. Fish were sampled at regular intervals and the presence or absence of *A. invadans* infection was established histologically. Where infection was present, the cellular response of the fish was assessed by comparing the area of granulomatous tissue with the area of acute inflammation. The anti-*A. invadans* antibody concentration of the snakeheads was assessed. Mortalities in the tanks were also recorded over the course of the trial, which was terminated after 40d.

3.6.2.3 Passive immunisation

The efficacy of snakehead antibodies was assessed by injecting three types of sera into snakeheads from a population that had never been exposed to EUS (Table 3.6.3).

Table 3.6.3. Source of sera used for passive immunisation

Treatment	Source
1	Wild fish, River Polangi, Bukidnon Province, Mindanao, Philippines
2	Fish injected with *A. invadans* isolate B99C, collected from Suphanburi, Thailand
3	Wild fish from first recorded natural EUS outbreak, Iloilo, Panay, Philippines

Duplicate tanks were used per treatment, each containing eight snakeheads. They were fed to satiation on live Nile tilapia, guppy or goldfish fingerlings in order to provide as natural a diet as possible (Chinabut, 1989). Immediately after injection, the fish were immersion challenged (Section 3.4) by placing them in low pH distilled water containing 5g l^{-1} sodium dihydrogen orthophosphate and abrading an area of the dorsal skin. The fish were then replaced in the tanks and 14 mycelial mats that had been grown on GPY-agar and broth (Lilley *et al.,* 1998; Appendix 4) were placed in each tank to sporulate. During the trial all moribund fish were collected and sampled for histological examination for the presence of invasive hyphae and the development of the granulomatous response. The trial was run for 30d, after which all surviving fish were sampled.

3.6.3 Outputs & Contribution of outputs

3.6.3.1 Comparison of immune response of different species

Macrophages were found to inhibit *A. invadans* in all species except for the highly susceptible snakehead. Although this finding suggests a reason why snakehead are one of the most susceptible species, tilapia macrophages were not more effective than those of gourami or silver barbs, indicating that the EUS-resistance of tilapia is not mediated through granulocytes alone.

Superoxide production of snakeheads appeared to be one of the most important mechanisms by which snakehead macrophages interacted with *A. invadans*. However, *A. invadans* was able to suppress superoxide production by snakeheads, which may explain the lack of effectiveness of snakehead macrophages. Such suppression is not unprecedented, as it has been shown in Oomycete plant pathogens (Doke, 1983) and bacterial fish pathogens (Stave *et al.,* 1987), but it has not previously been shown in Oomycete pathogens of animals.

Tilapia and snakehead macrophages produced more superoxide at low than high temperatures. Similar results have previously been reported from other warm water fish such as striped sea bass *Morone saxatilis* (Carlson *et al.,* 1995), implying that superoxide is most important at low temperatures. If so, the findings of the present study shows that a major immune mechanism of snakeheads is ineffective at low temperature, explaining their high susceptibility at low temperature compared to high temperature (Chinabut *et al.,* 1995).

Macrophage adherence was higher at high temperature in both silver barbs and snakeheads, but not in tilapia. Although the evidence that adherence indicates activation state (Kwak *et al.,* 1998) is not conclusive, it is possible that the resistance of tilapia may be partly due to the fact their immune systems are not suppressed at low temperatures, while those of susceptible species are. If so, the temperature at which macrophages are inhibited may indicate what geographical regions and what time of year a species is prone to infection by EUS, which may be used as a guide to species selection for aquaculture.

Studies on the inhibitory activity of serum indicated that complement was important in preventing the germination of *A. invadans*, although it had no effect on growth in any of the species examined. Kurata *et al.* (2000) described a serum protein from common carp that

inhibited *A. invadans* and suggested that it was partly responsible for the EUS-resistance of that species. However, tilapia serum was not more inhibitory than that of any other species, so they plainly do not possess a similar mechanism, indicating that there is more than one way by which the resistance of resistant species is conferred.

Anti-*A. invadans* antibodies were found in tilapia, snakeheads and gouramis. More were present in tilapia and snakehead at high temperature. Serum from high temperature snakeheads was the only serum able to inhibit germination after heating, implying that the antibodies are inhibitory. An effective antibody response by susceptible species may explain the decrease in prevalence in successive EUS seasons (Chinabut, 1998).

Any protection afforded by antibodies is evidently incomplete, as EUS outbreaks continue to occur, although they become more confined to the colder months (Tonguthai, 1985). The reduced production of antibodies at low temperatures may explain why protection does not extend to the colder seasons, though further studies are needed to confirm that the antibody response is actually protective before this conclusion could be supported.

All factors of the immune response of fish are likely to be inhibited if the fish are in poor condition. For example, the speed at which superficial wounds of snakeheads are repaired is significantly reduced by poor diet (Chinabut, 1989). Similarly, severe EUS outbreaks are often induced by environmental stress (Section 3.7). The present study shows that even susceptible fish can mount an immune response to *A. invadans*, so the condition of fish in aquaculture systems is likely to be a significant factor in their vulnerability to EUS.

3.6.3.2 Immunostimulation

Five substances were injected into striped snakehead to establish whether they enhanced the ability of serum or macrophages to inhibit *A. invadans*. Fish injected with the xanthophyll preparation, Oro Glo Layer Dry, suffered high mortality and experiments on this product were abandoned. The fish injected with the brewer's yeast, Betamak C85, and the lysophospholipid preparation, Lysoforte, showed a response to *A. invadans* that was little or no better than that of control fish. The biggest improvements were conferred by the vitamin supplement, Salar-bec, which significantly improved the inhibition of germination by serum, and the inhibition of growth by macrophages and serum. The alginate, Ergosan, also conferred benefits in the inhibition of growth by serum and macrophages, though not as great as those conferred by Salar-bec.

Ergosan and Salar-bec were mixed with feed, and fed to fish that were subsequently injected with *A. invadans.* Salar-bec improved survival of silver barbs by 23% and of snakeheads by 59%, calculated by relative percent survival (Ellis, 1988). It also increased the antibody concentration and rate of granuloma formation in snakeheads. Both are characteristic of snakeheads at high temperatures at which they are resistant to EUS (Chinabut *et al.,* 1995; Section 3.6.2.1). Granulomatous response was observed in very few silver barbs, but inflammation developed much more quickly in those fed with Salar-bec.

Difficulties with temperature control led to a very low prevalence of infection in the Ergosan trial, among both control and treated fish. Survival of snakeheads was improved by 60%, though it is uncertain whether the mortality was caused by EUS. Mortality among silver barbs was extremely low among both treatments and controls.

Many previous studies have discussed the possible value of vitamin supplements (such as Salar-bec) as immunostimulants (Sakai, 1999), especially in the light of the rapid deterioration of the vitamin supplement of feeds during processing and storage (Soliman *et al.,* 1987). The present study indicates that they may be valuable in enhancing the protection of farmed fish to EUS.

The immunostimulatory potential of alginates (such as Ergosan) in cold water aquaculture has also been previously investigated (Dalmo *et al.,* 1998; Gabrielson & Austreng, 1998). The failure of the challenge in the present study makes it impossible to conclude that Ergosan confers protection to EUS, though the considerable reduction in mortality among snakeheads indicates that it does have some immunostimulatory properties.

While recommendations for dose rates of both substances exist for application in salmonid culture, their relevance to tropical aquaculture is doubtful. Before either can be used on farms, it will be necessary to conduct further studies to establish the dose rate and the length of time for which they should be applied to feed. Further, controlled farm trials will be needed before either substance can be recommended for large scale distribution in the Asia-Pacific, as the response of fish to immunostimulation may be different on farms to that observed in a closely controlled aquarium trial (Galeotti, 1998). A pond trial of Salar-bec and Ergosan has been carried out in collaboration with CIFA, India (Section 3.5.2.5) and the samples are currently being processed.

3.6.3.3 Passive immunisation

The sera used in the passive immunisation trial were investigated, and serum from recovered fish was found to have the highest levels of anti-*A. invadans* antibodies. However, the only serum that inhibited the germination of *A. invadans* was that derived from Thai fish that had been injected with *A. invadans.* The serum of EUS-naïve fish actually stimulated germination. No specificity in the antibody response was found in any of the sera.

During the passive immunisation trial itself, there was no significant difference in the incidence of invasive fungal hyphae between the three treatments. Several fish died without evidence of EUS at the site of scraped skin and one fish was found to have invasive fungal hyphae at another site.

When treatment groups were compared in terms of survival, fish injected with the serum of Thai fish challenged with *A. invadans* had a significantly lower mortality than fish injected with the serum of naïve fish. The serum of fish that had recovered from their first exposure to EUS also reduced mortality, though statistical significance was equivocal ($p = 0.08$). The most important single factor in defining survival was the weight of the fish, as larger fish tended to survive for longer than smaller fish.

The lower mortality in fish injected with serum from fish previously exposed to *A. invadans* indicates that snakeheads do have a protective acquired immune response. The lack of a relationship between the anti-*A. invadans* antibody concentration and mortality supports the suggestion, discussed in Section 3.2.2.8, that the quantity of antibodies present does not reflect immunity to EUS.

The inhibitory activity of the serum provided a much better indication of its protective activity. The fish with the highest mortality had received the (naïve) serum that stimulated germination, while the fish with the significantly reduced mortality had received the serum that inhibited germination. The suggestion that fish from areas that had been endemic for some time have a more effective antibody response (Section 3.2.2.8) is further supported by the fact that the most inhibitory, and also most protective, sera came from a population that had been exposed for many years.

3.7 EPIDEMIOLOGY

3.7.1 Introduction

Although some specific causes of EUS have been conclusively identified (Section 1.1.3), there are a large number of other factors that influence the probability of an outbreak occurring. These factors may influence fish susceptibility, fungal availability, or progression of the disease after infection (Figure 4.1). It is not necessary to positively determine how a factor influences EUS occurrence in order to show that there is an association, which enables interventions to be proposed that reduce the risk of EUS outbreaks. Epidemiological observational studies are used to determine such statistical associations between putative risk factors and disease.

Very few epidemiological studies have been carried out that have been able to positively demonstrate determinants for any fish disease. Ahmed and Rab (1995) conducted a cohort study with retrospective and prospective data to investigate a number of risk factors for EUS. They showed that stocking of silver barb and use of piscicides increased the risk of EUS and post-stocking use of lime and culture in newly excavated ponds reduced the risk of EUS. These workers called for more study on the effects of flooding, disease history, pond conditions and species selection on the occurrence of EUS. These factors, among others, were addressed in a cross-sectional study of Bangladesh in the 1999-2000 winter season (Section 3.7.2.1) and in a case-control study in the Mymensingh district of Bangladesh in both 1998-9 and 1999-2000 winter seasons (Section 3.7.2.2). Collaborators on these studies were from Bangladesh Fisheries Research Institute (FRI) and Bangladesh Agricultural University (BAU). The prevalence data from the cross-sectional study is discussed in more detail in Section 3.2.2.1 and Appendix 7. A further cross-sectional study was conducted in collaboration with the Fisheries Development Division (FDD), Nepal in the 2000-2001 winter season (Section 3.7.2.3).

Box 3.7.1 Factors that increase the likelihood of EUS occurring in ponds (identified using univariate analysis)

POND CONNECTION
- Wild fish observed in ponds
- Pond embankment not high enough to prevent entry of outside water
- Holes observed in the pond bank
- Pond connected to other water body (ricefield, ditch or beel)
- Pond water is not exclusively rainfed or underground
- Pond is close to other water body
- Floodwater entered the pond during the previous rainy season

PRE-STOCKING POND PREPARATION
- Pond is not dried
- Bottom mud is not removed
- Pond is not limed
- Pond is not fertilized

POST-STOCKING MANAGEMENT
- Pond is not limed
- Pond is not fertilized
- Compost is prepared in another water body before applying to pond

WATER COLOUR
- Water is black (high organic debris) or transparent (nutrient-poor)
- Water is not green (high phytoplankton content) or red (high zooplankton content)

HYGIENE/HEALTH
- Fry source water released in pond
- Cattle wash or drink at the pond
- Farm nets are not dried/disinfected

3.7.2 Activities and outputs

3.7.2.1 Bangladesh cross-sectional study

An interview-based questionnaire survey of one fish farmer and one fisherman randomly selected in each of the 64 districts of Bangladesh was carried out to study risk factors associated with EUS outbreaks. The survey was carried out during the EUS season, December 1998 to April 1999. At each site, 100 fish were examined for lesions, and one fish of each species with lesions was sampled for histological diagnosis. A fish farm or

wild fishery was classified as affected with EUS if one or more fish of any species had a positive diagnosis based on the presence of characteristic mycotic granulomas in histological sections. Univariate analyses were used to examine the association between EUS occurrence and putative risk factors using crude relative risk (RR) as the measure.

Box 3.7.2 Factors that increase the likelihood of EUS occurring in wild fisheries sites (identified using univariate analysis)

TYPE OF HABITAT
- Site is a beel or haor
- Site is not a river or floodplain
- The site floods every year

HEALTH
- EUS occurred in the previous season

Data showed that there is a significantly higher relative risk of EUS occurring in farmed fish when wild fish are present in the pond; EUS occurred in the previous season; pond embankments are not high enough to prevent in-coming flood water; ponds are connected to natural waters; ponds are not dried or limed prior to stocking; ponds are not limed post-stocking; nets are not dried or disinfected and pond water colour is black indicating high levels of organic waste (Box 3.7.1).

Of the wild-caught fish, those sampled from haors (flood plain depressions) had a significantly higher relative risk of getting EUS. Fish from rivers and flood plains were at a lower risk of EUS infection (Box 3.7.2). Artificial stocking of the wild fishery was found to have no association with the occurrence of EUS.

The results of the surveys are described in two papers, one including socio-economic data (Khan & Lilley, 2001; Appendix 24) and the other providing more details of the prevalence of EUS in different species (Khan *et al.,* 2001a; Appendix 25). A third paper is in preparation, which will include the multivariate information described below. Some images from the study are given in Appendix 26.

Multivariate analyses

Multivariate analyses of the data were undertaken by F. Corsin and P.J. Cripps, University of Liverpool. Variables that were significant at the 25% level in univariate analysis, together with all variables that were thought to be biologically significant, were added to the logistic regression models.

A total of 36 variables were offered into the fish farm model. From these, a model was developed fitting 4 fish farm variables: (a) - (d) (Box 3.7.3). Each of these caused a decrease in the deviance significant at the 0.01 level. Post regression diagnostics aimed at assessing the robustness of the model showed that the effect of (a), (b) and (d) was consistent after omission of the observations with more leverage on these variables. However, the removal of 4 observations with largest delta-betas for (c) led to a lack of

Box 3.7.3 Risk factors associated with EUS identified using logistic regression models

Farm sites
(a) Pond banks are high enough to prevent the entry of run-off water from the surrounding area
(b) Cattle bath \ drink in the pond
(c) Wild fish observed in the pond
(d) Shrimp observed in the pond

Fishing sites
(e) Fishing site is a river
(f) Fishing site is a floodplain
(g) The water body floods every year
(h) Ulcerative disease occurred at the site in the previous year

convergence, even when an attempt to fit this variable by itself was made. The final model using EUS in fish farms as outcome is reported in Table 3.7.1. (b), (d) and (c) significantly increased the risk of a farm being infected with EUS by 40.24, 19.04 and 15.75 respectively. On the contrary, (a) was protectively associated with the outcome variable, reducing the risk of EUS by 0.03.

Table 3.7.1 Results of the logistic regression model using EUS in fish farms as the outcome

Factor	Coefficient	Std.Error	p-value	Odds Ratio	Lower(C.I.)	Upper(C.I.)
(a)	-3.38	1.24	0.006	0.034	0.003	0.387
(b)	3.695	1.056	< 0.001	40.24	5.076	319
(c)	2.757	1.281	0.031	15.75	1.28	194
(d)	2.946	1.267	0.020	19.04	1.59	227.9

Deviance with 56 df = 34.05
Likelihood Ratio Statistic at 4 df = 50.37
p-value < 0.0010

Nine variables were offered for inclusion into the wild fisheries model. Owing to some missing values, only 62 observations were used. Four variables (e-h) (Box 3.7.3) fitted into the model and all reduced the deviance with a p-value < 0.02. When post-regression diagnostics was carried out, the effect of (e) and (h) was consistent. On the contrary, the removal of observations with a higher leverage on (g) led the model to a failure in reaching convergence also when this was the only variable fitted, therefore preventing the assessment of the effect of this variable on the outcome. In addition, the removal of the 3 observations with the largest delta-betas for (f) led to the expulsion of this variable from the model however, owing to the large reduction in the deviance, (f) was included in the final model. Table 3.7.2 shows the final model developed with (g) and (h) significantly increasing the risk of a wild fishery site being infected with EUS by 33.39 and 8.64 times respectively. On the contrary, (e) and (f) were protectively associated with EUS infection, reducing the risk by 0.08 and 0.05 respectively.

Table 3.7.2 Results of the logistic regression model using EUS in wild fisheries as the outcome

Factor	Coefficient	Std.Error	p-value	Odds Ratio	Lower(C.I.)	Upper(C.I.)
(e)	-2.547	1.026	0.013	0.078	0.010	0.585
(f)	-2.895	1.205	0.016	0.055	0.005	0.586
(g)	3.508	1.469	0.017	33.39	1.876	594.2
(h)	2.156	0.9742	0.027	8.638	1.28	58.3

Deviance with 57 df = 38.39
Likelihood Ratio Statistic at 4 df = 27.84
p-value < 0.0010

3.7.2.2 Bangladesh case-control study

Case-control studies were carried out in Mymensingh district from November 1998 to March 1999, and December 1999 to February 2000.

Pond level study

Fifty EUS-affected (case) and 50 unaffected (control) ponds were compared with respect to pond structure, pond management, disease history, and water quality parameters. A pond was categorised as a case when at least 5 out of 100 fish examined had clinical EUS-like lesions, and at least one of the fish was confirmed as EUS positive by histology. After sampling of a case pond, a nearby corresponding control pond was selected to ensure the best possible matching. Associations were measured by univariate odds ratio (OR).

Box 3.7.4 Risk factors significantly associated with EUS affected ponds in Mymensingh district

- Pond water sourced from ricefields, and not from underground or rain
- Shallow ponds
- High stocking density
- Previous occurrence of EUS in the pond
- No pre-stock liming
- Flood water entering the pond
- Connection to wild water
- Low alkalinity levels
- High ammonia levels
- Presence of *Puntius sophore* or *Channa punctata* in pond

A table showing the results is given in Appendix 27. There was a significant difference between the water source of case and control ponds ($\chi 2$ = 38.92, P = 0.000). Case ponds were more likely to receive water from ricefields (73%) than controls (10%); and less likely to be exclusively rainfed (23%) or use underground water (5%) than controls (66% and 24% respectively). Other statistically significant variables are listed in Box 3.7.4 and the level of association is shown in Table 3.7.3.

Table 3.7.3 Association between pond variables and EUS

Variable	Case ponds	Control ponds	Statistic	P value
Depth (metres)	Mean – 0.98	Mean – 1.27	t-test = 4.746	0.000
Stocking density (per ha)	Mean – 97000	Mean – 62000	KW = 3.799	0.051
History of EUS	70%	43%	OR = 3.03	0.016
Pre-stock liming	20%	60%	OR =0.17	0.000
Pond floods	62%	6%	OR = 25	0.000
Connection to wild water	66%	4%	OR = 50	0.000
Temperature (^{o}C)	Mean – 21.7	Mean – 21.8	KW = 0.261	0.610
Transparency (cm)	Mean – 20.8	Mean – 21.4	KW = 0.362	0.547
Salinity (ppt)	Mean – 0.8	Mean – 0.8	KW = 1.107	0.293
PH	Mean – 7.3	Mean – 7.4	KW = 2.042	0.153
DO (ppm)	Mean – 3.7	Mean – 3.8	t-test = 0.459	0.647
Alkalinity (ppm)	Mean – 86.6	Mean – 105.6	KW = 3.019	0.082
Hardness (ppm)	Mean – 76.4	Mean – 92.9	KW = 2.167	0.141
Ammonia (ppm)	Mean – 1.3	Mean – 0.7	KW = 6.841	0.009
Puntius sophore	70%	30%	OR = 5.56	0.000
Channa punctata	30%	2%	OR = 20	0.000

OR = Odds Ratio (OR=1 no association, OR>1 variable is a putative risk factor for EUS, OR<1 variable is a sparing factor against EUS)
KW = Kruskal-Wallis

Fish level study

A total of 500 fish, 250 with lesions (cases) and 250 healthy fish (controls) were compared with respect to clinical signs, species, length, parasite loading and presence of external fungus. 150 case fish and 150 control fish were tested for presence of aeromonad bacteria. 75 of the case fish were sampled for histology to determine whether or not they

were EUS positive. Case fish were EUS-susceptible fish with clinical lesions, which were sampled from confirmed EUS case ponds. Control fish were EUS-susceptible fish with no clinical lesions from control ponds.

A table showing the results is given in Appendix 27. Of the 74 fish sampled for histology, 74% were confirmed as EUS-affected by presence of mycotic granulomas. Most of the lesions on the case fish were categorised as medium (85%), 12% were severe and 3% were mild. With regards to site of the lesions, 44% of cases had caudal lesions, 31% had lateral lesions, 24% dorsal lesions, 24% ventral lesions, 8% fin rot, 6% head lesions, 3% eye protrusion, and 2% were thin or suffering from malnutrition. The statistically significant variables associated with case fish are listed in Box 3.7.5 and the level of association is shown in Table 3.7.4.

Table 3.7.4 Association between fish variables and EUS

Variable	Case fish (n)	Control fish (n)	Statistic	P value
Fish length (cm)	mean = 10.4 (250)	mean = 8.4 (250)	KW = 8.75	0.003
Fungus (external)	83% (250)	0% (250)	OR = 160	0.000
Parasites (any type)	34% (250)	13% (250)	OR = 3.45	0.000
Trichodina spp	9.6% (250)	4% (250)	OR = 2.55	0.021
Chilodonella spp.	6% (250)	1% (250)	OR = 4.88	0.014
Myxosporidean	2% (250)	1% (250)	OR = 2.02	0.681
Apiosoma spp.	13% (250)	0.4% (250)	OR = 33.95	0.000
Epistylis spp.	0.4% (250)	0% (250)	-	1.000
Schyphidia spp.	1% (250)	0% (250)	-	0.499
Dactylogyrus spp.	1% (250)	1% (250)	OR = 0.66	1.000
Gyrodactylus spp.	4% (250)	3% (250)	OR = 1.60	0.471
Monogeneans	1% (250)	1% (250)	OR = 0.66	1.000
Piscicola spp.	1% (250)	0% (250)	-	0.499
Lernaea spp.	2% (250)	1% (250)	OR = 3.05	0.285
Argulus spp.	1% (250)	1% (250)	OR = 0.33	0.623
Ergasilus spp.	0% (250)	0.4% (250)	-	1.000
Aeromonas spp.	82% (150)	21% (150)	OR = 17.49	0.000
A. hydrophila	27% (49)	41% (26)	OR = 0.52	0.350
A. veronii biovar *veronii*	18% (49)	27% (26)	OR = 0.60	0.592
A. sobria biovar *sobria*	37% (49)	18% (26)	OR =2.61	0.200
A. schuberti	10% (49)	14% (26)	OR = 0.72	0.700
A. jandaei	8% (49)	0% (26)	-	0.300

OR = Odds Ratio (OR=1 no association, OR>1 variable is a putative risk factor for EUS, OR<1 variable is a sparing factor against EUS)
KW = Kruskal-Wallis

3.7.2.3 Nepal cross-sectional study

During an EUS extension trip to Nepal in 1999 (Section 3.8.2.3; Callinan *et al.,* 1999; Appendix 30) fisheries officers and farmers commented that EUS occurs annually in Nepal, considered it to be the main disease affecting capture and culture fisheries. Although mortality rates appear to have declined, there remains a fear of EUS outbreaks among fish farmers, which may limit further development of the aquaculture sector in Nepal. A request was made by the Fisheries Development Division (FDD) to the project for assistance to research the problem. As the Bangladesh cross-sectional study was considered to be very successful in determining prevalence and risk factors for EUS, a similar study was arranged

in Nepal. The objectives of the study were to: (i) measure risk factors for EUS in village carp polyculture grow-out ponds in the Terai area of Nepal, with the aim of providing recommendations for control; (ii) provide quantitative data on the prevalence and geographical distribution of EUS in the Terai area of Nepal; and (iii) assess the current livelihood conditions of participating farmers and identify potential impacts of any fish health problems on the farmers. An overview of the study is provided in Appendix 28.

In 1999, staff from FDD were separately funded to undertake training at AAHRI in histological techniques for the diagnosis of EUS. This expertise was integral to the study, and efforts were made to ensure that histological facilities at FDD were functional and adequate for EUS diagnosis and research. By August 2000 a questionnaire had been translated into Nepali, pre-tested and finalised. Particular attention was paid to assessing the ability and willingness of farmers to make control-based interventions. Study sites were randomly selected from sampling frames provided by Assistant Fisheries Development Officers (AFDOs). Between October 2000 and January 2001, 6 sites from each of 10 districts were sampled (Jhapa, Sunsari, Siraha, Mahottari, Rautahat, Parsa, Nawalparasi, Kapilbastu, Banke and Kailali). At the present time, data input and histological processing of samples is nearly complete. It is envisaged that analysis will be undertaken over the next two months and extension material and an article will be prepared, based on results.

3.7.2.4 Epidemiological studies in Pakistan

Pakistan was first reported as being affected by EUS in 1996. Being an EUS "frontier" state, it provided a unique opportunity to study factors involved in the spread of the disease. A visit was made in April 1998, and in addition to the dissemination activities described in Section 3.8.2.2, two study designs were prepared: (i) A study of the prevalence and geographical distribution of EUS in the Indus, Chenab, Jhelum, Ravi and Sutlej rivers; and (ii) A case-control study of EUS in the Punjab. Some pre-testing of survey procedures was undertaken with farmers in Punjab and Sindh. However, the Federal Ministry of Food, Agriculture and Cooperatives did not approve the study proposals on the grounds that EUS research studies should be integrated within a country-wide fish disease umbrella project.

3.7.2.5 Thailand questionnaire survey

Seventeen snakehead farmers from Suphanburi, Nakhon Pathom, Singburi and Chanchoengsao districts in Thailand were interviewed using a structured questionnaire. Their responses with regards to EUS control are given in Box 3.7.5. Due to the low number of farmers interviewed, none of the variables showed significance. However, similar trends to those found in Bangladesh can be seen. The presence of wild fish and flooding of the pond pose a risk for EUS, and the practice of pond drying reduces the risk of EUS.

Box 3.7.5 Interview survey of snakehead farmers, Suphanburi

Pond management variables associated with EUS having occurred at the farm

- Presence of wild fish (RR = 0.51<3.50<23.81)*
- Pond floods (RR = 0.44<1.78<7.25)
- Pond drying (RR = 0.10<0.38<1.40)
- Pond liming (RR=0.28<1.09<4.32)

Popular preventive measures

- Decrease water exchange and reduce feed over winter
- During pond preparation, remove mud
- Use well water
- Don't overstock
- Make the water green
- Add salt and lime

Popular treatment procedures

- Remove diseased fish
- Stop water exchange and reduce feed
- Add salt and lime
- Treat with antibiotics

3.7.3 Contribution of outputs

The epidemiological studies that have been completed have successfully identified risk factors for EUS that enable rational control measures to be proposed. Suggestions for use of these measures as part of an EUS control strategy are given in Section 4.

Experimental studies on the risk factors identified here could provide further details about how EUS spreads and affects a water body. In addition to the pond and farm trials described in Section 3.6, Khan (2001) undertook some preliminary intervention studies to determine the effect of pond variables such as pond drying, water depth and stocking density on EUS occurrence, and confirmed that all had a significant effect on outbreaks.

As EUS is shown to be endemic in the natural waterways of Bangladesh, it is not surprising that ponds that have been flooded, are connected with natural water bodies, and contain wild species, are at high risk of EUS. Some farmers questioned have indicated that they would be willing take measures such as increasing the height of pond embankments and excluding wild fish, if these measures significantly reduced the risk of disease. However, there is often less willingness to change pond management practices in the case of community ponds or multiple ownership ponds. This is particularly true where the change would impact on other important uses of the pond (e.g. for cattle bathing / drinking). The harvest of wild species from fish ponds are often provided cheaply, or free of charge, to labourers or poorer members of the community. Therefore, care must be taken in advising the exclusion of wild fish, to ensure that the most vulnerable groups are not negatively impacted.

Environmental conditions always affect disease outbreaks, and in the case of EUS, low alkalinity and high ammonia are both risk factors for outbreaks. These can be mitigated by the addition of lime and better water quality management. The most commonly used type of lime in Bangladesh is calcium carbonate ($CaCO_3$), which does not have any sterilising effect against pathogens (Lilley *et al.,* 1992). As pre-stock and post-stock liming is shown here to reduce the risk of EUS, the effect is probably in terms of reducing stress in fish by increasing alkalinity and stabilising pH. Lime, however, is still a purchased input and may not be available to the poorest farmers. Ash (Section 3.5) was investigated as a cost-free alternative, and appears to have some positive effect. The advantages of adding fertiliser to ponds that are too low in nutrients are also clear from the present studies. Fertiliser is a low-cost input, and has the added benefit of increasing fish production. The effect against disease may be due to the improved nutritional status of the fish, or may be due to lower levels of fungal propagules in the water. Lilley (1992) showed that spore counts of all Oomycete fungi were lower in water of high algal content. There are plans by regional scientists to further investigate this aspect of disease control.

The studies also showed that parasites and aeromonad bacteria were associated with the lesions on fish. They probably contribute to the deterioration of the lesions, and monitoring and treatment of these agents could reduce the severity of EUS outbreaks. However, no particular species of parasite or bacteria was present on more than 30% of case fish (the highest was *A. sobria* biovar *sobria,* which was isolated from 37% of fish with *Aeromonas*,

and 82% of the case fish had *Aeromonas*). Therefore, as previous studies have indicated, no particular ecto-parasite or aeromonad can be considered a necessary cause of EUS.

3.8 DISSEMINATION

3.8.1 Introduction

As previous projects had succeeded in delivering new information on EUS causation, the present project aimed to present this information to regional scientists, fisheries officers and farmers, in the hope that a consensus could be built on the frequently asked question, "what causes EUS?". Many research groups in Asia have had limited access to the results of work by other groups, resulting in the duplication of studies. It was hoped that more productive research to develop control methods in local systems would result once there was some agreement on disease determinants. The results of project studies on control measures would also be presented to regional scientists, fisheries officers and farmers, to gauge obtain comments on the feasibility of the strategies proposed.

3.8.2 Activities

Planned pathways for the uptake of project outputs are listed in Table 3.8.1. These are resented at three different levels. Primary uptake is limited to the individuals who have been involved directly with project activities. Secondary uptake includes groups that collaborators will interact with. Tertiary uptake is through distribution of publications and extension material. Dissemination strategies to impact specified numbers of individuals in target groups are listed in Table 2.8.2.

3.8.2.1 AAHRI Workshop

A workshop on EUS was conducted at AAHRI early in the project (26th - 30th January 1998) for 11 participants from 9 regional countries (Appendix 29). The workshop aimed to: disseminate up-to-date information on EUS causation; provide a forum for discussion of experiences regarding EUS in each of the countries; invite comments on the EUS manual that was in preparation; and to invite the participants to collaborate in project R6979 by monitoring EUS outbreaks and conducting small research studies supported by the project. However, of the participants, only Masud Khan was in a position to undertake related research, and he joined the project to study epidemiology of EUS in Bangladesh. Collaboration with other regional scientists was instead achieved through correspondence after the workshop.

3.8.2.2 Training in Pakistan

As a follow-up to a joint DFID-ACIAR funded mission to Pakistan in March 1997, a trip was organised in April 1998. Professor K. Morgan, a veterinary epidemiologist from Liverpool University, joined project staff (S. Chinabut and J. Lilley) to hold 2 one-day workshops at the Fisheries Research and Training Institute (FRTI), Lahore and the Sindh Fisheries Department Hatchery and Training Centre. FRTI (1998) produced a report on the team's activities in the Punjab. In addition to the dissemination activities, two detailed epidemiological study designs were prepared (Section 3.7.2.4).

3.8.2.3 Extension tour

A joint ACIAR-DFID funded EUS extension tour of five centres in Nepal, India and Sri Lanka was undertaken from 7-20 June 1999. The objectives were: (i) Through structured workshops, to ensure the combined research benefits resulting from the ACIAR, AAHRI and DFID programmes on EUS are embedded in key institutes in India, Nepal and Sri Lanka. (ii)

Table 3.8.1 Uptake Pathways

Who	How
PRIMARY	
(i) AAHRI staff and participating Thai farmers	AAHRI staff are integrally involved in the generation and dissemination of project results. 40 farmers were involved in detailed interviews.
(ii) FRI (Bangladesh) staff and participating Bangladesh farmers and fishermen	Masud Khan, a researcher on the project, is a permanent staff member at FRI. 114 farmers and 64 fishermen were involved in detailed interviews.
(iii) BAU (Bangladesh) staff and participating students	Project collaborators are permanent staff at BAU. 4 BAU students have worked on various project studies. 80 staff and students attended an EUS seminar.
(iv) CARE-LIFE (Bangladesh) participating farmers and staff	In total, 567 farmers from Kisheroganj and Rajshahi are participating in the EUS treatment trials. Study results have been discussed in detail at Farmer Science Congresses (FSC) and field days.
(v) CIFA (India) staff	Study collaborators are permanent staff at CIFA. 15 attended a one-day seminar.
(vi) BFAR (Philippines) staff	Study collaborators are permanent staff at BFAR.
(vii) RIA1 (Vietnam) staff	Trainees and study collaborators are permanent staff at RIA1.
(viii) FDD (Nepal) staff and seminar attendees	Study collaborators are permanent staff at FDD. 60 fisheries officers and farmers attended a one-day seminar
(ix) FRTI (Pakistan) staff and workshop attendees	Collaborators on a proposed study are permanent staff at FRTI. 100 Punjab and Sindh fisheries officers and farmers attended 2 half-day workshops.
(xi) Other attendees at AAHRI workshop, CICFRI seminar, Kerala University seminar, NARA seminar, Stirling University lectures and practicals.	Participation in workshops and seminars.
SECONDARY	
(i) Thai DoF extension officers, farmers, Kasetsart and AIT fisheries students	AAHRI holds approx. 18 seminars/year training 460 extension officers, farmers and Kasetsart students in fish disease. In addition, approx. 430 farmers/year come with disease samples for diagnosis, and are provided with specific disease control advice.
(ii) Bangladesh fish farmers	The Health Section of FRI gives seminars to farmers and extension officers. Masud Khan has also produced FRI extension pamphlets on EUS in Bengali. FRI staff (primarily Masud Khan) does receive samples for diagnosis and makes field visits within Mymensingh district. In the past, limited expertise and manpower have restricted these activities, but the Health Section is presently being upgraded.
(iii) Bangladesh fisheries students	A high proportion of fisheries students in Bangladesh graduate from BAU, and project collaborators provide the disease component of this course.
(iv) Bangladesh fish farmers	CARE is producing audio-visual material for use in future activities with carp farmers.
(v) Indian fish farmers	CIFA is the Indian Government aquaculture institute responsible for disseminating research outputs via leaflets and training seminars to farmers throughout India.
(vi) Philippine fish farmers	BFAR has field stations in main aquaculture areas in the Philippines and undertakes dissemination activities.
(viii) Nepalese fish farmers	There is a network of fisheries extension officers under FDD.
(ix) Pakistan fish farmers	FRTI undertakes some training and EUS leaflets are being distributed by the Office of the Fisheries Development Commissioner.
TERTIARY	
Other regional fisheries officers and scientists and farmers	EUS manual distribution, conferences and seminars
Wider scientific community	Peer-reviewed publications
Further interested parties	Internet postings

Through detailed consultation with scientists and extension officers at these institutes, to develop response strategies to control and prevent EUS outbreaks appropriate to the circumstances in each country. Project staff (S. Chinabut and J. Lilley) were joined by Dr R.B. Callinan (NSW-Fisheries) and Dr C.V. Mohan (College of Fisheries, Mangalore). About 160 participants attended the workshops. The report of the tour, including conclusions and recommendations, is reproduced in Appendix 30 (Callinan *et al.,* 1999). An application has been made to ACIAR for funds for an extension tour of Laos, Cambodia, Vietnam and southern China.

3.8.2.4 Other workshops / seminars / conferences

Seminars on EUS have also been given to fisheries officers and researchers in Vietnam, Bangladesh, Thailand and Philippines. Project staff also presented study results at international conferences at Chiang Mai, Bangkok, Baltimore, Athens, Hong Kong, Cebu and Dhaka. Details of these presentations are given in Section 6 of the Project Completion Summary Sheet.

3.8.2.5 Extension publications

A handbook describing EUS causation and control methods was produced early in the project (Lilley *et al.,* 1998; Appendix 31). The production and dissemination costs of the leaflet were covered by ACIAR. To date, over 800 copies have been distributed, mainly on request.

During the 1998 trip to Pakistan, assistance was provided in the production of an EUS extension pamphlet in Urdu (Appendix 32). The pamphlet is published by the Office of the Fisheries Development Commissioner with funds provided by ACIAR.

A leaflet describing EUS control strategies using information gained during the course of this project, is being produced. It is hoped that this will form the basis of extension leaflets in Thai, Bengali and Nepali.

Table 3.8.2 Dissemination strategy

Target group	Numbers	Messages to disseminate	Means of dissemination	Evidence of uptake
CARE-LIFE farmers	567	EUS treatment options	farmer-based research	Continued use of treatment
Organisations responsible for extension	10+	EUS causation and control	Seminars and distribution of pamphlets, manuals	Incorporation of pamphlet information in extension materials
Regional fisheries officers	800	EUS causation and control	Distribution of manuals	Demand for manual, use of information
Regional aquaculture research institutes	10+	EUS causation and control	Conferences, seminars and publications	Subsequent research outputs from institutes
Publishing fisheries scientists	10+	EUS research achievements	Peer-reviewed publications	Publication citations

3.8.3 Impact analysis

Table 3.8.3 lists the main targeted impacts of the project and measurable indicators. CARE-LIFE is one of the few collaborating organisations that are committed to directly measuring uptake and impact on farmers. It should be noted that most of the collaborating organisations do not have the resources or institutional linkages to carry out continued fish health monitoring surveys. A further limitation to impact assessment is that standardised fish sampling techniques have not been developed for effective disease surveillance in small-scale aquaculture systems.

Table 3.8.3 Impact analysis

Impact expected	How created	How measured
(i) Reduction in losses due to EUS	Knowledge of control strategies provided to organisations responsible for extension/dissemination Efforts made to halt the spread of the disease	• Secondary statistical data • Questionnaire studies • Uptake of treatments by CARE farmers • EUS considered at regional level for trans-boundary movement of fish. • Latest diagnostic techniques in use in regional laboratories
(ii) Access to basic EUS diagnosis and control information for extension officers and farmers	Distribution of leaflets, manuals	• Incorporation of leaflet information in extension materials
(iii) Up-to-date knowledge of EUS causation and control embedded in key regional aquaculture research institutes	Seminars and publications	• Presentations / publications from regional research institutes demonstrate a building of a consensus on EUS causation / control
(iv) Wider understanding of EUS research achievements	Publications	• Publication citations

3.8.3.1 Statistical data

Insufficient statistical data is available to make accurate projections of the impact of the project in reducing losses. ACIAR (1998) used fishery statistics to calculate the Net Present Value (NPV) of the ACIAR EUS Project 9130. The most conservative estimate showed that Project 9130 would provide potential benefits of Aus$56m (£20m) to fisheries in Australia, Indonesia and Philippines. R6979 has focused more on Thailand, Bangladesh, Nepal, India, Sri Lanka, Pakistan, Philippines and Vietnam, which have a higher susceptible fish harvest (4.2m tonnes compared with 0.5m tonnes in 1997, FAO Fishstat Plus V. 2.21), and therefore has a greater potential for impact. In the event, ACIAR provided support for further dissemination activities beyond the countries listed in ACIAR (1998) and worked jointly with R6979 with regards to several activities described in this Section. ACIAR (1998) also calculated that Project 9130 would provide value if it succeeded in reducing EUS losses in Australia, Indonesia and Philippines by 0.1%. In comparison, R6979 was less than half the cost of the ACIAR project, and is therefore expected to have a positive NPV.

3.8.3.2 Incorporation of leaflet information in extension materials

On completion of R6979, some programme development funds will be spent on maximising outputs from the project in terms of assisting in the production of extension materials and other outputs.

3.8.3.3 Regional publications/presentations demonstrate a building of a consensus on EUS causation/control

There is an increasing consensus on EUS causation. At the 1996 World Aquaculture Society (WAS) meeting in Bangkok, 5 out of 6 presentations showed results in agreement with the findings of 1994 Regional Seminar (Roberts *et al*., 1994). At the 1998 Fifth Asian Fisheries Forum in Chiang Mai, 4 out of 6 presentations were in line with the 1994 case definition. At the 1999 WAS meeting in Sydney, 2 out of 3 presentations used this case definition. At the 1999 Asian Fisheries Society Fish Health Section meeting in Cebu, all 7 presenters were aware of current research, and 6 of these were in agreement with the 1994 case definition.

3.8.3.4 Publication citations

By 28 February 2001 the Web of Science citations lists indicate that project publications have already received 7 citations (Table 3.8.4). As most of the project publications are still in press, and as several other articles are not yet listed on Web of Science, this number is expected to rise significantly. Publications from the previous project R5779 have received 35 citations in peer-reviewed articles, and the previous EUS manual has 12 citations. Citations have been made by researchers from: Australia, Bangladesh, China, Egypt, Finland, India, Japan, Malaysia, Philippines, Spain, Sri Lanka, Taiwan, Thailand, UK and USA.

Table 3.8.4 Citations of project publications

Reference	Number of citations
Project R6979	
Lilley *et al*. (1998) Handbook of EUS	3
Blazer *et al.* (1999) J. Aquatic Animal Health	2
Thompson *et al.* (1999) Fish & Shellfish Immunology	2
Project R5779	
Lilley & Roberts (1997) J. Fish Diseases	12
Lilley *et al.* (1997) Veterinary Record	8
Lilley *et al.* (1997) Diseases of Aquatic Organisms	5
Lilley & Inglis (1997) Aquaculture Research	4
Thompson *et al.* (1997) Fish & Shellfish Immunology	3
Lilley *et al.* (1997) Aquaculture	3

4. SUMMARY OF OUTPUT CONTRIBUTIONS

A summary of diagnostic and control techniques is given here, drawing on a variety of studies to date. The contribution of the various R6979 studies is illustrated in Figure 4.1.

4.1 Diagnosis

4.1.1 Clinical signs

EUS is associated with ulcerative lesions on particular species of fish (listed in Table 3.2.2). The gross appearance of lesions varies between species, habitat and stage of lesion development. The most distinctive EUS lesion is the open dermal ulcer. This is often most conspicuous in snakeheads, which are often used as an "indicator species" for EUS in an area. However, other diseases may also result in similar clinical lesions and it is important not to rely on clinical signs as a means of diagnosis. EUS has been associated with mass mortality, but evidence from EUS-endemic areas shows that it can also occur at low intensity, with fish often recovering towards the end of the cool season.

4.1.2 Rapid muscle squash preparation

A presumptive diagnosis of EUS in susceptible fish showing dermal lesions can be made by demonstrating aseptate hyphae (7-30 µm in diameter) in squash preparations of the muscle underlying the visible lesion.

4.1.3 Histology

Confirmatory diagnosis requires histological demonstration of typical granulomas and invasive hyphae using Uvitex-H&E (Box 3.4) or GMS (Grocott 1955). Callinan *et al.* (1989); Viswanath *et al.,* (1997) and Chinabut & Roberts (1999) have described the sequential histopathology of EUS. Early EUS lesions are erythematous dermatitis with no obvious fungal involvement. *A. invadans* hyphae are observed growing in skeletal muscle as the lesion progresses from a mild chronic active dermatitis to a severe, locally extensive, necrotising, granulomatous dermatitis with severe floccular degeneration of the muscle. The fungus elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae, a typical characteristic of EUS. The most typical lesions are large, open, haemorrhagic dermal ulcers about 1-4 cm in diameter. These are commonly infected with bacteria, particularly *Aeromonas* spp.

4.1.4 Fungus culture and characterisation

A. invadans can be isolated using the methods described in Lilley *et al.* (1998). The fungus should be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces* (Lilley *et al.,* 1998). *A. invadans* is characteristically slow growing in culture and fails to grow at 37^{o}C on GPY agar. Details of the temperature-growth profile of *A. invadans* is given in Lilley & Roberts (1997). Confirmation that the isolate is *A. invadans* can be made by injecting a 0.1 ml suspension of 100+ motile zoospores intramuscularly in EUS-susceptible fish (preferably *Channa striata*) at 20^{o}C, and demonstrating growth of aseptate 7-30µm wide hyphae in muscle of fish sampled after 7 days, and typical mycotic granulomas in muscle of fish sampled after 14 days. Confirmation can also be made by PCR of *A. invadans* DNA using FP1-FP2 primers (Box 3.1) or APH-APH4 primers (Box 3.2), or sequencing of the ITS1 region (Appendix 4).

4.2 Control

4.2.1 Control of the spread of EUS

Figure 3.2.1 and Box 3.2.3 provide details of areas affected by EUS. Mechanisms of spread have not been conclusively identified, but the movement of fish is considered to be the most likely method. Therefore, susceptible fish (Table 3.2.2) with any form of clinical lesion should not be moved from an affected area to an unaffected area, particularly an unaffected area with known susceptible fish species located between 40°N and 30°S (Section 3.2.4). All fish (including resistant fish) should be bathed in 30ppm formalin or 0.1ppm malachite green before transport. Movement of fishing equipment across national boundaries are thought to have spread crayfish plague (Alderman, 1996) and therefore drying or disinfecting transported nets and equipment should also be advised.

Flooding (Section 3.7) and marine migration of diadromous fish (K. Morgan, pers. comm.) also considered possible mechanisms of spread, but little can be done to control these, and they are less likely to result in a spread of the disease beyond current affected areas.

4.2.2 Control of EUS in pond aquaculture

The proposed intervention strategies to control EUS in ponds are listed in Figure 4.2, and discussed below.

Treat cause of skin damage

Where there are clear causes of skin damage that lead to EUS (such as low pH water), this information can be used to manage ponds so that skin damage does not occur. Several of the treatments that reduced the occurrence of EUS in pond trials (Section 3.5) were thought to act to improve the condition of the fish, rather than to kill the pathogen. Where the source of the skin damage is unknown, epidemiological studies, like those described in Section 3.7, can be used to clearly identify causes and risk factors for skin damage.

Eliminate A. invadans zoospores

Where possible, *A. invadans* should be eradicated from ponds prior to stocking (e.g. by drying, mud removal and/or disinfecting with quicklime or bleaching powder). Entry of *A. invadans* during pond filling should also be prevented (e.g. by rain or tube-well water, and preventing the entry of wild fish). Alternatively, application of piscicides and fallowing the pond for at least 20 days prior to stocking should help to eliminate *A. invadans*. Hatchery-reared seed should be used, preferably prophylactically treated with 30ppm formalin. Nets and other equipment should be dried or disinfected before use in the pond.

Where *A. invadans* has potentially entered the pond, prophylactic treatments before the onset of winter are advised. Use of commercial compounds to kill *A. invadans* in ponds is possible in more intensive systems. Although *A. invadans* propagules are relatively easy to kill, compared to other fungi (Lilley & Inglis, 1997), treatments at levels that do not harm fish remain problematic. Malachite green (0.1ppm) and chelated copper (5ppm) are the most effective chemicals, but they present potential health or environmental risks. Salt (3ppt), quicklime (40ppm), or formalin (30ppm) should have a positive effect, but doses may vary depending on the species treated and the water quality. Other treatments have showed potential (Coconut diethanolamide, D-Limonene and hydrogen peroxide), but further trials are required before these can be advised. The results of pond trials at CIFA (Section 2.5.2.5) are expected soon.

In rural, small-scale aquaculture, the options are more limited. Of the herbal treatments that were tested here, neem was one of the most promising, but even this would require substantial effort to gather and process the seeds. Results of the LIFE trial (Section 3.5.2.6) showed that none of the participating farmers were prepared to use the advised dose. Farmers were prepared to use lime and ash, but these are not thought to act against the fungus at the levels used.

Box 4.1 Control of EUS in polyculture ponds in Bangladesh

Preventative measures:
- If EUS occurred in the previous season, dry ponds and remove mud prior to stocking.
- Lime ponds prior to stocking.
- Keep pond banks in good repair to prevent entry of flood water during the rainy season.
- Where possible, stock with bighead carp instead of catla; and common carp instead of mrigal.
- Use only hatchery-reared seed.
- Reduce stocking density over winter. Where possible harvest puntius and mrigal.
- Where possible, use only rain/well water over winter, especially if EUS is reported in the area. If not possible, supply from a fast-flowing river or canal is preferable.
- Try and maintain water depth above 1.5m throughout the winter.
- Maintain alkalinity above 90ppm and hardness above 80ppm by adding lime and gypsum.
- Fertilise ponds to maintain green water.
- Try and maintain total ammonia levels below 1ppm (by low stocking/ low organic input/ mechanical aeration)
- Dry (or disinfect) nets or other equipment before use in the pond

Where this does not conflict with other water uses:
- Do not let cattle into the pond
- Exclude wild fish and other aquatic animals

If EUS occurs:
- Remove diseased fish
- Add lime and salt
- Consider emergency harvest

Grow resistant species/strains

The relative resistance of different fish species has been demonstrated by sampling natural populations (Appendix 7) and by challenge experiments (Appendix 15). Selection of resistant species for aquaculture would be a sure means of preventing EUS. Similarly, harvesting of susceptible fish before the winter season would also prevent EUS occurring in farmed fish.

The reduction in EUS severity over time, and reported recovery of stocks of certain susceptible wild fish (Appendix 1), provides indications that there may be hereditary traits for resistance. Nilsson (1992) has reported that there is a genetic basis for resistance to fungal disease in Arctic Char. Selective breeding programmes of EUS-susceptible species should therefore consider disease resistance characters.

Affected fish

Removal of affected fish is an important measure to prevent the spread of any infectious disease. Where outbreaks have been severe, emergency harvest should be considered. No compound has proved effective as a treatment for EUS outbreaks, although use of antibiotics and fungicides are can be effective in preventing death due to opportunistic bacteria and fungi.

Prophylactic immunostimulation has been shown to reduce mortality in challenged snakeheads (Section 3.6.2.2.), but further work is required to optimise dosage for different species and culture systems. Although this may be a useful measure for fish in ponds where affected fish have been observed, it would not be capable of treating severely affected fish themselves, as they are unlikely to take the feed and immunostimulant, or be able to mount an effective immune response at that stage.

Reduce risk

Data provided by epidemiological studies in Section 3.7 have clearly demonstrated risk factors for EUS in carp polyculture systems in Bangladesh. Some measures (e.g. don't overstock ponds, careful pond preparation) are advisable for most aquaculture systems against a variety of diseases, whereas others are more specific against EUS (e.g. exclude susceptible species, limit connection with natural water bodies). Epidemiological studies can be used to identify specific risk factors for other species and culture systems.

Box 4.2 Control of EUS in intensive monoculture snakehead ponds

Preventative measures:

- Site farm to prevent entry of flood water during the rainy season.
- Dry and lime ponds prior to stocking.
- If EUS occurred in the previous season, also remove the bottom mud.
- Reduce stocking density and feeding over winter.
- Reduce water exchange over winter. If possible, use water from a fast-flowing river or canal.
- If EUS is reported in the area, stop inlet water and use only rain/well water.
- Try and maintain water depth above 1.5m throughout the winter.
- Maintain alkalinity above 90ppm and hardness above 80ppm by adding lime and gypsum.
- Fertilise ponds to maintain green water.
- Dry (or disinfect) nets or other equipment before use in the pond

If EUS occurs:

- Remove diseased fish
- Add lime and salt
- Consider treating with antibiotics, fungicide
- Consider emergency harvest

Boxes 4.1 and 4.2 provide specific recommendations for low-input carp polyculture ponds and high-input snakehead farms respectively. Recommendations are based on studies described in this report, and aim to be feasible for most farmers. Many farmers already employ some of the methods advised. Recommendations to snakehead farmers provided by the Thai DoF include some of the measures listed in Box 4.2, and the responses of the snakehead farmers in Section 3.7.2.5 indicate that they are employing many of these measures.

4.2.3 Control of EUS in cages

As cages are open to the natural water, it is impossible to exclude *A. invadans* from the culture systems and there are therefore fewer options for control. However, some strategies are listed in Figure 4.2.

Parasite infections are sometimes a cause of skin damage in cage aquaculture and attempts should be made to treat these. In areas where cage aquaculture is still in early stages of development, careful species selection avoiding EUS-susceptible fish can be advised (e.g. use of tilapia and pangasius). As with pond culture, careful monitoring and removal of affected fish should be recommended. Stocking densities can be very high in cage systems, and it is advised that these are reduced during the winter season.

4.2.4 Control of EUS in wild fishes

There are even fewer options for control of EUS in wild fishes. In this case, initial prevention of spread to the area is of critical importance (Section 4.2.1). However, once an area is affected, there still may be options for control. (Figure 4.2). In areas where human activity are likely to impact on water systems, the likelihood of causing EUS should be considered. Examples of this include the creation of drainage and irrigation systems which result in acid sulphate runoff and EUS (Sammut *et al.* 1996), or the reduction in water levels and

increased EUS occurrence due to dam construction (Anon, 2000). The establishment of over-wintering sanctuaries in Bangladesh is a cause for concern as these beels have been shown to be at high risk of EUS (Box 3.7.2). Sites should be carefully monitored during the establishment of the sanctuaries to determine whether any particular management interventions increase the risk of EUS. Data from Section 3.7.2.1 indicated that artificial stocking of natural waters in Bangladesh is not a risk factor for EUS. In fact, stocking with resistant species could be an effective strategy against EUS.

Figure 4.1 Project activities aimed at controlling EUS

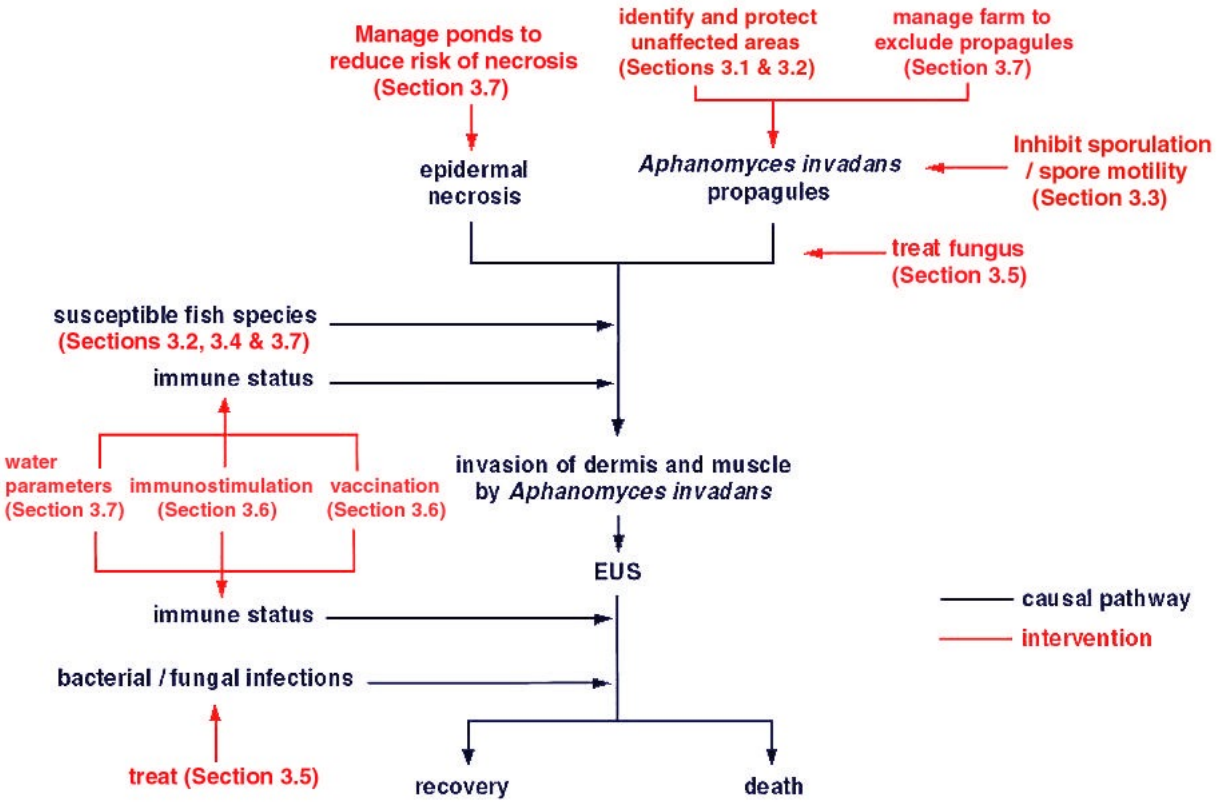
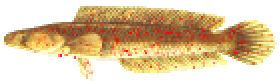
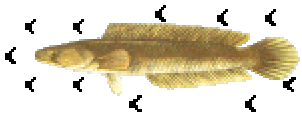


Figure 4.2 Strategies to control EUS

Control of EUS in ponds



Identify cause of dermatitis/skin damage; treat/control



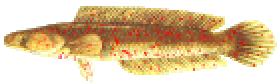
Add chemical to pond to kill zoospores

Consider growing resistant species or strains

Feed immunostimulant to increase rate of destruction of fungus in tissues

Optimise husbandry; reduce risk

Control of EUS in cages



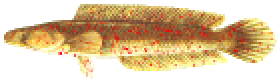
Identify cause of dermatitis/skin damage; treat/control

Consider growing resistant species or strains

Feed immunostimulant to increase rate of destruction of fungus in tissues

Optimise husbandry; reduce risk

Control of EUS in wild fishes



Identify environmental causes of dermatitis/skin damage; manage to reduce impacts

Stock water with resistant species or strains

Protect habitat to minimise stress

5. RECOMMENDED FUTURE STUDIES

DFID-funded research into EUS has successfully identified causes and control methods for EUS. However, disease processes change, and research should be an ongoing process in affected areas. In addition to studies specifically aimed at controlling EUS, there is a pressing need to evaluate fish disease research as a whole, and how greater impacts can be made in reducing losses to aquaculture in Asia.

5.1 Molecular studies

5.1.1 An assessment of strain differences between Asian and American isolates would provide information on the spread of the fungus.

5.1.2 DNA Sequencing has become a cheaper and more rapid process. It is envisaged that workers in mycological laboratories will soon provide more detailed sequences of *A. invadans*. This would help in developing more specific tests for EUS, and for stain comparisons of *A. invadans.*

5.2 Impacts of EUS on wild fish populations

An assessment of inland capture statistics of important affected fisheries would be useful to identify impacts of EUS on biodiversity of susceptible fish populations. This was a recommendation in the report of the dissemination trip to Nepal, India and Sri Lanka (Callinan *et al.,* 1999). CICFRI hold detailed catch statistics for water bodies in India and an agreement was made for R6979 project staff to study these data, however, the data was not forthcoming during the period of the project. Most of the other recommendations arising from the report were addressed by this project.

5.3 Surveillance

5.3.1. There is a need to develop standardised fish sampling techniques to monitor fish health and production in small-scale systems, for use by farmers and researchers.

5.3.2. Studies to show freedom from EUS in areas that are thought to be unaffected would enable specific guidelines to be prepared to reduce the risk of introduction of the disease (see Section 3.2.3.3).

5.4 Investigation of the relationship between plankton and fungal levels in ponds

Reduced levels of EUS (Sections 3.5 & 3.7) and fungal counts (Lilley, 1992) have been found in fertilised green water. More detailed studies to show optimal plankton levels and species are required. Some work on this aspect has been proposed at RIA1, Vietnam.

5.5 Detailed evaluation of optimal doses of Salar bec for protection of susceptible fish

Salar bec proved to be a useful measure for reducing EUS mortalities, but further optimisation of levels would be required before it can be recommended to fish farmers (Section 3.6).

5.6 Selective breeding for resistant strains

With the development of aquaculture of susceptible fish (e.g. snakeheads in South India), selective breeding of EUS-resistant strains is being considered.

5.7 Evaluate guidelines

Further work with defined farmer groups would be useful to evaluate the wider impacts of adopting measures advised in Section 4. For example, assessing which farmers would be

able to adopt the method of excluding wild fish, and in what circumstances this measure would negatively impact fishermen or other groups.

5.8 Institutional analyses

Institutional analyses to investigate how the results of fish disease research can be best utilised for the benefit of rural farmers.

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APPENDIX ONE

Paper one - Lilley, J.H., Callinan, R.B., and Khan, M.H. (2001) Social, Economic and Biodiversity Impacts of EUS. In: Proceedings of DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999. (In press)

SOCIAL, ECONOMIC AND BIODIVERSITY IMPACTS OF EPIZOOTIC ULCERATIVE SYNDROME (EUS)

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ABSTRACT

Few surveys have been conducted that accurately assess impacts of EUS on fish populations and associated fishing communities. A review of past information on the disease is hampered by the fact that a pathological diagnosis of EUS was not used in most studies prior to 1994, and many accounts of ulcerative fish diseases in Asia may be unrelated to EUS. Data from the Bangladesh Flood Action Plan 17 (FAP17) Project, from October 1992 to March 1994, showed that as much as 26% of 34,451 freshwater fish examined had lesions of some sort. A recent cross-sectional survey in Bangladesh revealed that 80% of 471 fish with lesions sampled from 84 sites were diagnosed as EUS-positive. This indicates that EUS studies in Bangladesh that just examined fish with lesions were not grossly overestimating the impact of the disease.

EUS outbreaks have subsided in many areas, but new occurrences are being reported in previously unaffected areas, and in newly developed farming systems and fisheries management systems. There is recent evidence that EUS does not always occur in seasonal outbreaks, or always cause high mortalities, but may be prevalent at a low level throughout the year. It may therefore have an effect on productivity, which cannot be measured in terms of mortalities.

Particular communities that are heavily dependent on local fish catches have been affected by outbreaks of EUS. Social impacts may extend beyond persons directly affected by fish losses. For example, one of the main constraints to aquaculture development identified in Nepal is fear of ulcerative disease and a lack of knowledge of how to deal with fish disease.

Published estimates of direct economic losses due to EUS mortalities in several affected countries are given, and further estimates are calculated from recent survey data. However, it may be the case that losses due to lower productivity may be of greater significance.

Reductions in aquaculture and fisheries production levels can be demonstrated during times of serious EUS outbreaks, although it cannot be positively determined that EUS was the factor that caused the decline. Anecdotal evidence suggests that during times of severe outbreaks these species may be difficult to locate at all. Long-term effects on aquatic ecology have not been investigated.

INTRODUCTION

There is a great deal of anecdotal information on EUS outbreaks and extrapolated data on EUS-related losses, but relatively little survey data that provide actual randomised counts of EUS-mortalities, or of fish with EUS-lesions. Even fewer studies define the EUS lesions and confirm the diagnosis in each case, or in a proportion of cases. Scientists at the Aquatic Animal Health Research Institute (AAHRI) in Thailand, the only OIE-approved EUS

diagnostic laboratory, define an EUS case as: A fish with necrotising granulomatous dermatitis and myositis associated with *Aphanomyces invadans* hyphae. This is a slightly modified definition of that given in Roberts *et al.* (1994), and requires H&E and Grocott histology of the sample in order to make a positive diagnosis.

The severity of EUS outbreaks has subsided in many areas, but there remain incidences of lesions on fish that do not fit with the conventional view of EUS, as they are not associated with large-scale fish kills. Nonetheless, a cross-sectional survey in Bangladesh conducted during the 1998-9 winter season, revealed that 80% of 471 fish with clinical lesions, sampled from 84 sites, were diagnosed as EUS-positive (Khan *et al.*, 1999). This indicates that EUS is still the largest cause of lesions on freshwater fish in Bangladesh, and studies that just examined fish with lesions were not grossly overestimating the impact of the disease.

A review of some previous EUS surveys in Bangladesh is given here, indicating whether a diagnosis fitting with that given above was provided. These surveys give information on social, economic and biodiversity impacts of EUS at that time.

EUS PREVALENCE SURVEYS IN BANGLADESH

The Bangladesh Flood Action Plan 17 Fisheries Studies and Pilot Project (FAP17) accumulated data on the occurrence of lesions on about 35,000 wild freshwater fish (excluding a similar number of crustaceans, reptiles, amphibians and aquatic mammals examined). Summary tables of this data (Figs 1-9) demonstrate a surprisingly high prevalence of lesions on these fish. More than half of the fish examined comprised the whole population of fish in the water body, which eliminates the danger of selecting sub-samples of less healthy fish (Fig.1). Whole population samples had only a slightly lower percentage of lesions (24%) than population sub-samples (28%).

The higher incidence of lesions in winter (Fig.2), and on species which are generally considered to be most EUS-susceptible (Figs 3 and 4), provide further evidence that the lesions are predominantly the result of EUS infections. It should be noted, however, that the majority of sampling took place in winter months (Fig.2). The prevalence data on individual species (Fig.4) equate well with data given by Khan *et al.* (1999) on species affected during the 1998-9 winter period. The latter data indicate which species were confirmed EUS affected.

The FAP17 database includes comments on the severity of the infections in each sample. These were coded from 1-5, as in Fig.5, and the average severity then plotted in Fig.6. Again, the most affected species were those recorded as highly susceptible to EUS (eg *Puntius* spp, *Channa* spp, *Anabas testinudineus*, *Mastacembelus* spp and *Mystus* spp). It is interesting to note any differences in susceptibility within genera for fish that are being considered as candidate species for aquaculture development.

Some regional differences in lesion occurrence are given in Fig.7, although these cannot be accounted for in terms of flooding, or other risk factors considered. Variations in lesion occurrence between habitat (Fig.8) support findings by Khan *et al.* (1999) that EUS is less likely to occur in actively flowing water bodies.

As with the analysis of whole- versus sub- sample analysis (Fig.1), the fish that were collected using less selective fishing methods (eg seine net) had a lower prevalence of lesions than fish collected using methods that were likely to select for weaker fish (eg spear, scoop net) (Fig.9).

An EUS prevalence study in 3 floodplain areas was undertaken by Subasinghe and Hossain (1997) using a histological diagnosis of the disease (Fig.10). They showed that disease prevalence was generally lower in artificially stocked fish sampled from the natural water bodies, than in wild fish. It is unlikely that fry reared in hatcheries within Bangladesh presently pose a significant EUS risk to wild fish, as there are few accounts of EUS in carp

hatcheries, and EUS has now been shown to be endemic in natural waterways throughout most of Bangladesh (Khan *et al.*, 1999). It is the wild fish themselves that are considered risk factors for EUS. Khan *et al.* (1999) also showed that sites that were artificially stocked showed no significant association with occurrence of EUS.

There has been a decreasing incidence of EUS in both wild (Fig.10) and farmed fish (Fig.11) in Bangladesh over the last 10 years. The reduced severity of outbreaks has been even more evident. EUS affected ponds netted during initial outbreaks commonly revealed 100% infected fish of particular species, with high rates of mortality (Barua *et al.*, 1989-91; Ahmed and Rab, 1995). These susceptible species now usually show lower rates of infection and lesions often heal as temperatures rise (Khan *et al.*, 1999). An ADB/NACA (1995) questionnaire survey of carp farmers in Bangladesh showed that 17% of extensive farmers and 53% of intensive and semi-intensive farmers reported resolution of the EUS problem from 1992-5 (Figs 12 and 13). Similarly, Hossain (1998) reported that 85% of Thana Fisheries Officers (TFOs) indicated that the general aquatic animal disease situation improved from 1994-6, and 91% TFOs indicated an improvement from 1996-8.

PRESENT STATUS OF EUS

Although EUS outbreaks have subsided in Bangladesh and other areas, new occurrences are being reported in previously unaffected areas, and in newly developed farming systems and fisheries management systems. A summary of recent confirmed outbreaks is given in Fig. 14.

The recent outbreaks show that EUS is not always strictly seasonal, or always causes high mortalities, but may be prevalent at a low level throughout the year. It may therefore have an effect on productivity, which cannot be measured in terms of mortalities.

This year, several occurrences of EUS in juvenile giant gouramies and climbing perch in Thailand have been confirmed at AAHRI. These have occurred at times outside the usual "EUS season".

EUS in Australia remains an important issue in estuarine wild fish and in cultured silver perch (*Bidyanus bidyanus*) in NSW, Queensland and Northern Territory. The disease has occurred almost all year round and at prevalences of 20-90% in farmed silver perch (Callinan, unpublished).

In 1998, EUS occurred for the first time in the Philippine island of Mindanao (BFAR, unpublished). Earlier, in Jan 1996, there was up to 30% prevalence in EUS susceptible fish from both Laguna Lake and Mangabol swamp in central Luzon.

SOCIAL IMPACTS

Particular communities that are heavily dependent on local fish catches have been most affected by outbreaks of EUS.

For example, Vinobaba *et al.* (1996) stated that 10,650 fishing families are dependant on fisheries production from Batticaloa lagoon in Eastern Sri Lanka, and any fluctuations in catch levels have severe socio-economic impact on the local community. Disease outbreaks have occurred in the lagoon in 1989, 1993 and 1994 and Vinobaba and Vinobaba (1999, abstract and unpublished notes) clearly demonstrated EUS mycotic granulomas in samples of a number of lagoon fish species. Vinobaba *et al.* (1996) implied that flooding and agricultural run-off may be risk factors for disease, and the authors conclude that there is an urgent need to protect and develop fisheries resources in Batticaloa district, which has 168,300 hectares (ha) of inland waterways, and should be capable of sustaining the local communities.

Similarly, Laguna Lake and Mangabol swamp in Luzon, Philippines support both capture fisheries and pond and cage aquaculture. 75,000 people depend on fish from these areas as a source of food and income. EUS first occurred there in 1986, and in 1992 the disease was considered the most important factor determining the size of the fish harvest. In 1989, over 50% of the harvest of susceptible fish was lost due to EUS; and in 1990, over 40% was lost (Callinan, unpublished).

In Kerala State, India, Kurup (1992) reported that between the first appearance of EUS in August 1991 and April 1992, the disease had caused serious loss of income to 25,000 full-time and 7,000 part-time fisherfolk.

Bhaumik *et al.* (1991) surveyed the effect of EUS on fish producers, traders and consumers in West Bengal and some of the results are reproduced in Fig. 15. Virtually all of the farmers interviewed (97%) were allocated as "marginal" or "small" farms. Of the EUS-affected farms, 48% were low-input, traditional farms, compared to 35% that were described as "semi-scientific" and 16% "scientific" farms.

The effect of EUS on traders and consumers was skewed towards poorer rural communities. A higher percentage (53%) of people from rural areas preferred the more susceptible snakehead and "miscellaneous fish species", compared with 18% of urban dwellers. Fewer rural people were able to eat fish often, but after an EUS outbreak, demand for fish was not much reduced in rural areas, largely because prices were reduced (Fig. 15). Similarly, despite high consumer resistance to diseased fish, they were more likely to be bought in rural areas. Das and Das (1993) reported that women fish vendors suffered particular hardship after EUS outbreaks and often had to seek alternative employment.

In contrast to the above situations, other communities that do not rely heavily on susceptible fish have not been badly affected by EUS outbreaks. For instance, snakeheads and other wild fishes in Punjab, Pakistan are not widely fished for local consumption. Of the freshwater fishes that are eaten in Pakistan, the preferred species are usually EUS-resistant common and Chinese carps.

Social impacts of EUS may extend beyond persons directly affected by fish losses. For example, one of the main constraints to aquaculture development in Nepal is an ongoing fear of disease, largely as a result of previous EUS outbreaks. People are reluctant to start aquaculture activities due to the perceived high risk of disease and a lack of knowledge of how to deal with fish disease (Callinan *et al.*, 1999). Conversely, Little *et al.* (1996) noted that in Thailand, the decimation of wild fish stocks, particularly snakeheads, due to EUS in the early 1980s, was a major stimulus to the culture of herbivorous fish.

In Thailand, a questionnaire survey of snakehead farms is currently being undertaken to determine the present impacts of the disease. Mortalities in intensive snakehead culture accounted for most of the recorded economic losses due to EUS in Thailand in the 1980s. It is suspected that losses are currently not high, but one early reply stated that the farmer had given up the business due to disease problems in 1994, when significant EUS outbreaks occurred.

With regard to control of diseases in rural aquaculture, the management of the risk of disease is usually a cheaper and more effective means of control than treatment. Gopal-Rao *et al.* (1992) pointed out that in Andhra Pradesh, an average of 10% of the production cost is spent on disease treatment. They called for an integrated approach to fish health by combining management techniques with chemotherapy.

ECONOMIC IMPACTS

Published estimates of direct economic losses due to EUS mortalities in several affected countries are listed by Lilley *et al.* (1998). Further estimates of economic loss during early outbreaks in Bangladesh are given by Hossain (1993), who reported that the 1988 EUS epizootic caused an average loss in each district of Tk 405,960 (US$8300); and Collis (1993),

who recorded the loss of 18 metric tonnes (mt) of fish at Tk 430,000 (US$8800) from 240 ponds in 6 thanas between 1991-3. More recent estimates of the economic loss to carp culture in the Region are given in a revised edition of the ADB/NACA (1995) data and are listed in Figs 12 and 13. Projecting future losses, the most conservative estimate of the cost of fish losses due to EUS in Australia, Philippines and Indonesia until the year 2027 has been calculated at US$63 million (ACIAR, 1998).

Freshwater aquaculture in Asia is generally not a major foreign exchange earner, and production is mainly for local or domestic consumption. Therefore, the more significant impacts of EUS on local micro-economies are probably not reflected within economic loss data.

As EUS lesions in most affected areas do not appear to be causing mortalities on the scale of previous outbreaks, it may also be the case that economic loss due to lower productivity may be of greater significance than direct mortalities. A survey in Andhra Pradesh in the early 1990s combined the effects of disease-induced growth loss with mortality, in an estimated annual loss due to disease of 40 million Indian rupees (Gopal-Rao *et al.*, 1992).

There are further concerns for the future, as several countries in the Region attempt to diversify the species used for aquaculture to include known EUS susceptible species. For example, snakehead culture is being developed in southern India, and the associated dangers of EUS should be considered during this process (Callinan *et al.*, 1999).

BIODIVERSITY IMPACTS

The idea that a pathogenic fungus can have significant biodiversity impacts on aquatic animals is not unique to the case of EUS. A very similar species of fungus, *Aphanomyces astaci*, devastated European crayfish populations at the end of the last century, and, when it hit Scandinavia in the early part of this century, Sweden was transformed from the world's largest crayfish exporter to the world's largest crayfish importer (Swahn, 1994). More recently, a chytridiomycete fungus has been identified as the cause of massive population declines and possible extinctions of amphibians. It is considered to be the single greatest cause of amphibian declines in the western hemisphere and Australia (Munkacsi, 1999).

Ulcerative mycosis (UM) outbreaks in the 1980s had a significant impact on the productivity of the estuarine fisheries of Eastern United States (Noga *et al.* 1988). Recent evidence has indicated that the invasive *Aphanomyces* involved in those outbreaks, may be *Aphanomyces invadans*, the EUS fungal pathogen (Blazer *et al.*, 1999).

Reductions in aquaculture and fisheries production can be demonstrated during times of serious EUS outbreaks. For example, within the past decade, India showed an increase in aquaculture production in every year other than 1990. Subasinghe (1999) speculated that this might be due to EUS losses in that year.

Data provided by Das (1994) of wild fish landings from the Brahmaputra River show massive declines in EUS susceptible species at the time of first EUS outbreaks in that water system. *Channa striata* landings went down 88% from 22 mt in 1987-8 to 3 mt in 1988-9 and remained at a similar level until 1991. *Channa punctata* declined by 85% from 30 mt to 5 mt over the same period, and further declined to 3 mt by 1991. Data on one species that is not generally considered susceptible are given (*Gadusia chapra*), and this showed an 880% rise in landings from 0.1 mt to 0.9 mt over the 1987 to 1989 period.

Similarly, scientists in Kerala are convinced that populations of susceptible fish have declined as a result of the EUS outbreaks, although no data were given (Callinan *et al.*, 1999). However, it cannot be positively determined that EUS was the major factor that caused declines in overall fish production.

Anecdotal evidence suggests that during times of severe outbreaks particular susceptible species disappeared from fish markets altogether. In Bangladesh, *Channa* spp, *Nandus* spp

and *Mastacembelus* spp were said to be difficult to locate in 1988-89, but Fig 4 indicates that by 1992-4, reasonable numbers were being caught. *Puntius sophore,* a highly susceptible fish, appears to have been present in large numbers throughout outbreaks.

CONCLUSIONS

- Randomisation and a pathological case diagnosis are essential for disease prevalence studies, and should be employed in studies monitoring EUS prevalence.
- There has been decreased severity and incidence, but continued widespread occurrence, of EUS in Bangladesh and many other affected areas.
- EUS has continued to affect new areas, and new systems.
- In Asia, EUS generally has more significant impacts in extensive, low input systems and wild fisheries, than in controlled intensive fish culture.
- In South and Southeast Asia, rural traders and consumers are more affected than urban/suburban communities.
- Fear of disease is an important constraint to aquaculture development in some areas.
- Managing risk of disease losses from EUS is usually a cheaper and more effective means of control than treatment.
- EUS has probably affected the diversity of species in certain areas, but the long-term effect of this is unknown.

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Fig. 1

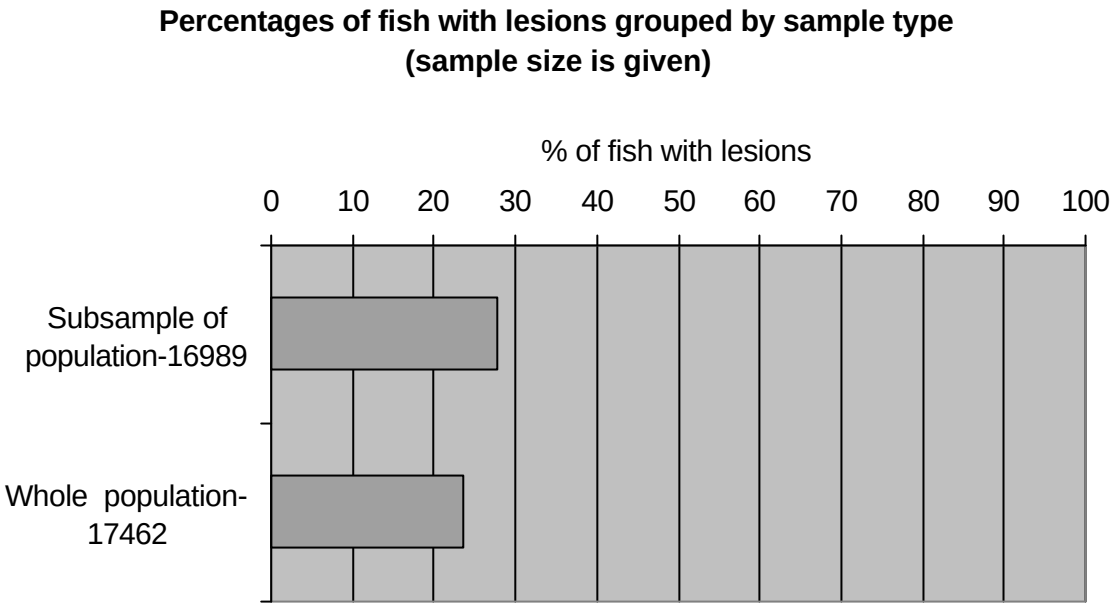
Percentages of fish with lesions grouped by sample type
(sample size is given)
% of fish with lesions
0
10
20
30
40
50
60
70
80
90
100
Subsample of population-16989
Whole population-17462

Fig. 2

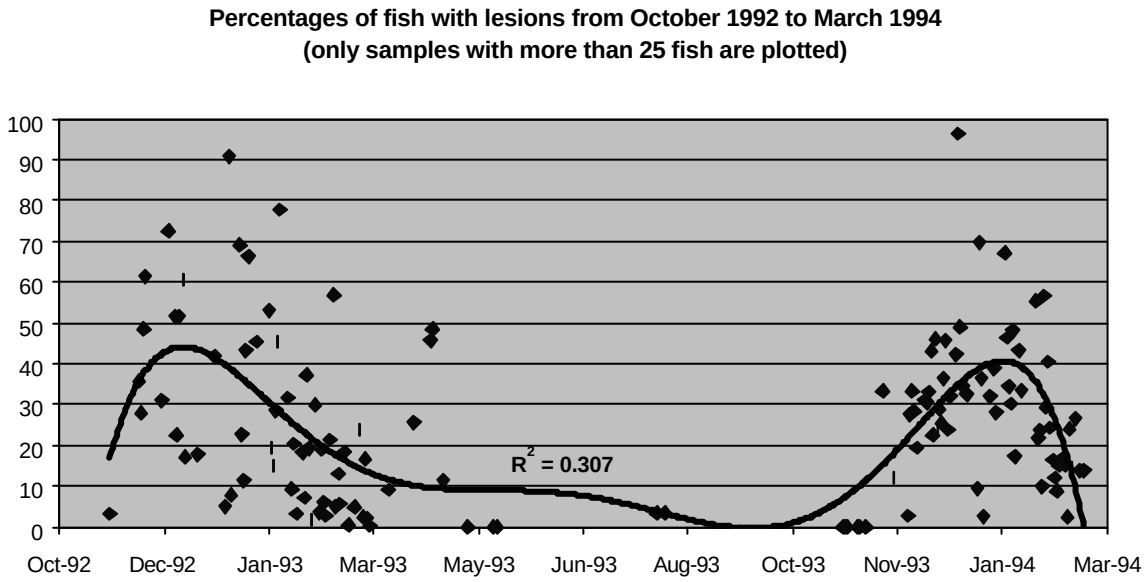
Percentages of fish with lesions from October 1992 to March 1994
(only samples with more than 25 fish are plotted)
100
90
80
70
60
50
40
30
20
10
0
R2 = 0.307
Oct-92
Dec-92
Jan-93
Mar-93
May-93
Jun-93
Aug-93
Oct-93
Nov-93
Jan-94
Mar-94

Fig. 3

Percentages of fish with lesions grouped by family
(sample size given, only samples with over 5 fish are plotted)

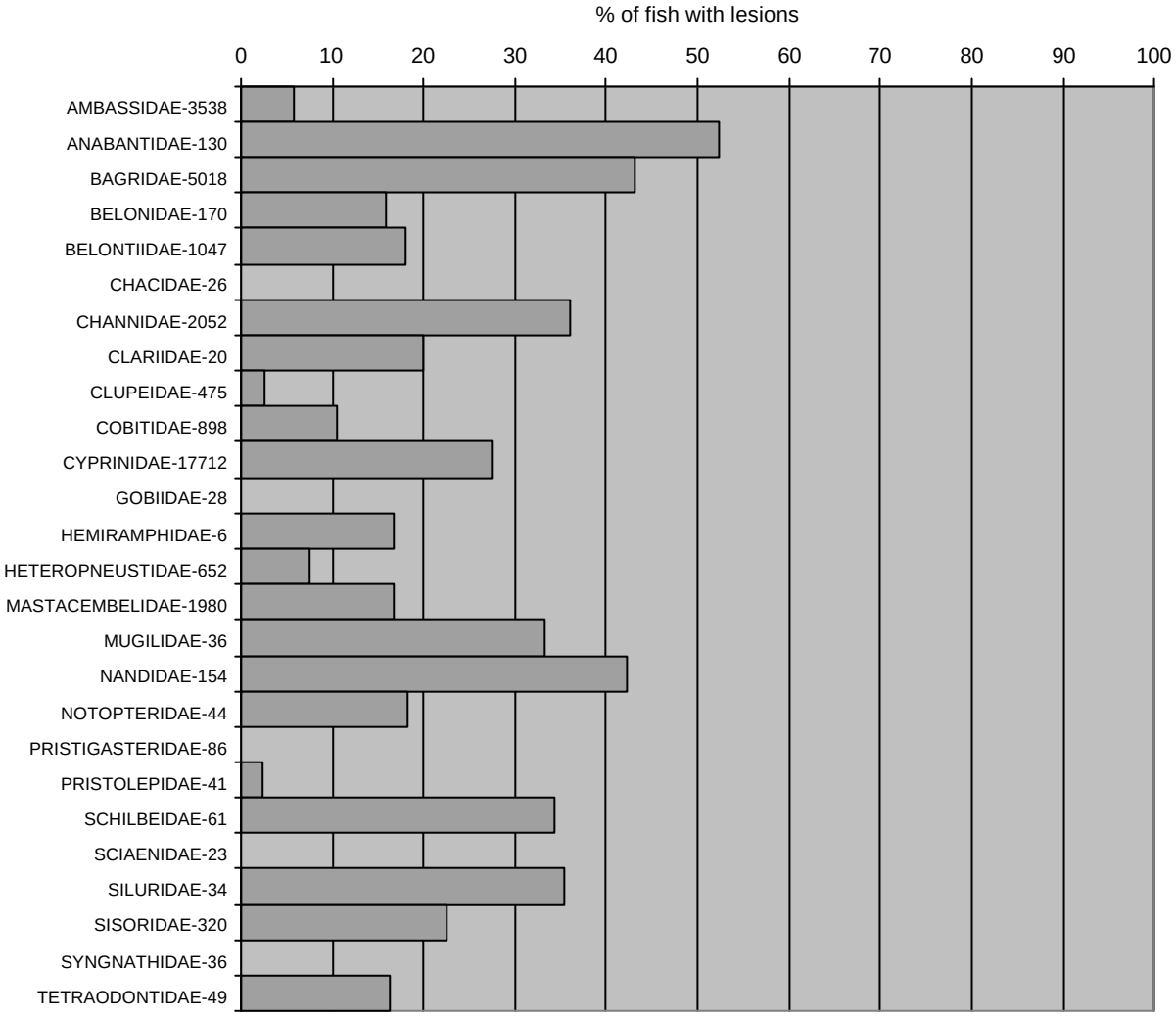

Fig. 4

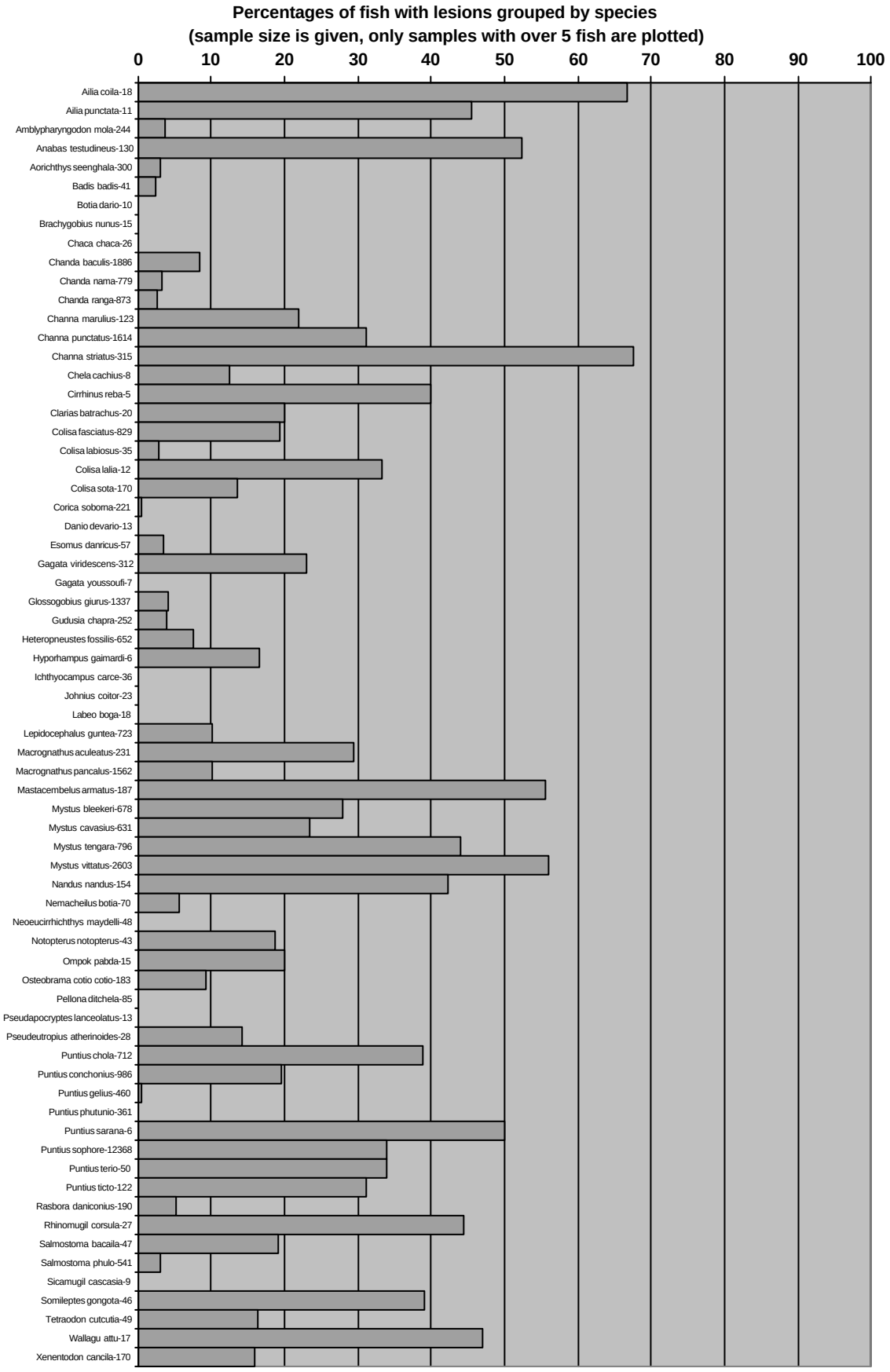
Percentages of fish with lesions grouped by species
(sample size is given, only samples with over 5 fish are plotted)
0
10
20
30
40
50
60
70
80
90
100
Ailia coila-18
Ailia punctata-11
Amblypharyngodon mola-244
Anabas testudineus-130
Aorichthys seenghala-300
Badis badis-41
Botia dario-10
Brachygobius nunus-15
Chaca chaca-26
Chanda baculis-1886
Chanda nama-779
Chanda ranga-873
Channa marulius-123
Channa punctatus-1614
Channa striatus-315
Chela cachius-8
Cirrhinus reba-5
Clarias batrachus-20
Colisa fasciatus-829
Colisa labiosus-35
Colisa lalia-12
Colisa sota-170
Corica soborna-221
Danio devario-13
Esomus danricus-57
Gagata viridescens-312
Gagata youssoufi-7
Glossogobius giurus-1337
Gudusia chapra-252
Heteropneustes fossilis-652
Hyporhampus gaimardi-6
Ichthyocampus carce-36
Johnius coitor-23
Labeo boga-18
Lepidocephalus guntea-723
Macrognathus aculeatus-231
Macrognathus pancalus-1562
Mastacembelus armatus-187
Mystus bleekeri-678
Mystus cavasius-631
Mystus tengara-796
Mystus vittatus-2603
Nandus nandus-154
Nemacheilus botia-70
Neoeucirrhichthys maydelli-48
Notopterus notopterus-43
Ompok pabda-15
Osteobrama cotio cotio-183
Pellona ditchela-85
Pseudapocryptes lanceolatus-13
Pseudeutropius atherinoides-28
Puntius chola-712
Puntius conchonius-986
Puntius gelius-460
Puntius phutunio-361
Puntius sarana-6
Puntius sophore-12368
Puntius terio-50
Puntius ticto-122
Rasbora daniconius-190
Rhinomugil corsula-27
Salmostoma bacaila-47
Salmostoma phulo-541
Sicamugil cascasia-9
Somileptes gongota-46
Tetraodon cutcutia-49
Wallagu attu-17
Xenentodon cancila-170

Fig.5 Coding of comments given in FAP17 database indicating severity of the lesion

Code	Comment
1	1-10% L, fin rot, first dorsal fin, initial, less, less and light spot, less infection, less severe, light, light in caudal fin, light on head and tail, low low severe, mild, minor minor infection, no very severe, not considerable, not heavy, spot found in lower jaw, very less
2	11-20%, L to M, dorsal site of the body, light to moderate, medium and light, ulcerative-light
3	21-40%, M, H to L, head and dorsal fin, head belly and tail medium spot, light to heavy, medium, medium severe, moderate, moderate infected, mouth and body less, partially infected, primary infection, tail
4	41-70%, M to H, head and mouth deeply, head and tail, heavy to moderate, moderate to heavy, mouth and tail deep spot, near caudal fin a big spot, on dorsal fin and head deeply, severe-medium, specially caudal fin also body, ulcerative syndrome, ulcerative-medium
5	71-100%, H, acute, badly affected, considerably, dominant, heavy, high, higher, highly, highly infected, highly severe major maximum serious, severe, severe/acute, severely infected, very severe

Fig. 6

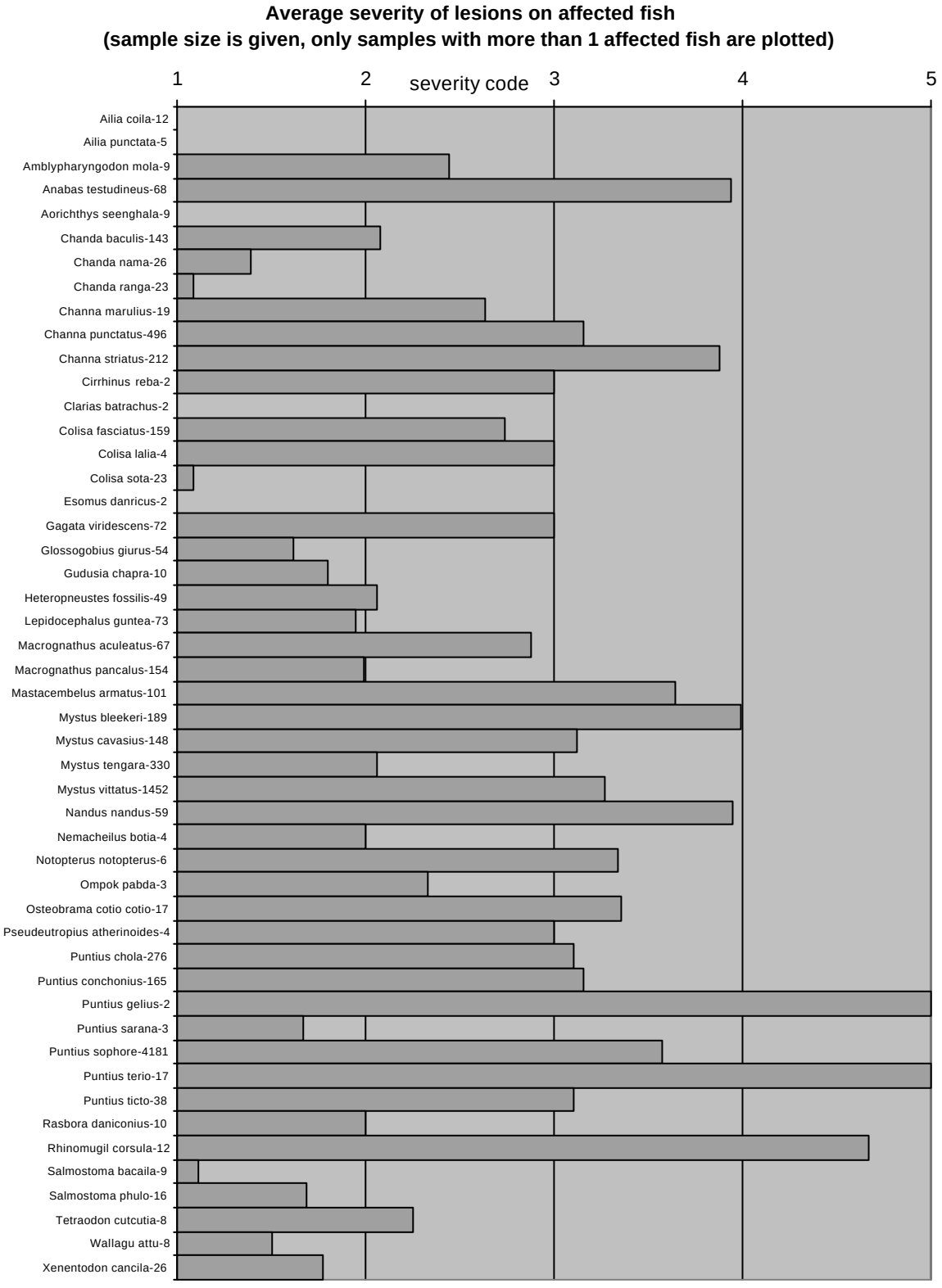
Average severity of lesions on affected fish
(sample size is given, only samples with more than 1 affected fish are plotted)
1
2
severity code
3
4
5
Ailia coila-12
Ailia punctata-5
Amblypharyngodon mola-9
Anabas testudineus-68
Aorichthys seenghala-9
Chanda baculis-143
Chanda nama-26
Chanda ranga-23
Channa marulius-19
Channa punctatus-496
Channa striatus-212
Cirrhinus reba-2
Clarias batrachus-2
Colisa fasciatus-159
Colisa lalia-4
Colisa sota-23
Esomus danricus-2
Gagata viridescens-72
Glossogobius giurus-54
Gudusia chapra-10
Heteropneustes fossilis-49
Lepidocephalus guntea-73
Macrognathus aculeatus-67
Macrognathus pancalus-154
Mastacembelus armatus-101
Mystus bleekeri-189
Mystus cavasius-148
Mystus tengara-330
Mystus vittatus-1452
Nandus nandus-59
Nemacheilus botia-4
Notopterus notopterus-6
Ompok pabda-3
Osteobrama cotio cotio-17
Pseudeutropius atherinoides-4
Puntius chola-276
Puntius conchonius-165
Puntius gelius-2
Puntius sarana-3
Puntius sophore-4181
Puntius terio-17
Puntius ticto-38
Rasbora daniconius-10
Rhinomugil corsula-12
Salmostoma bacaila-9
Salmostoma phulo-16
Tetraodon cutcutia-8
Wallagu attu-8
Xenentodon cancila-26

Fig.7

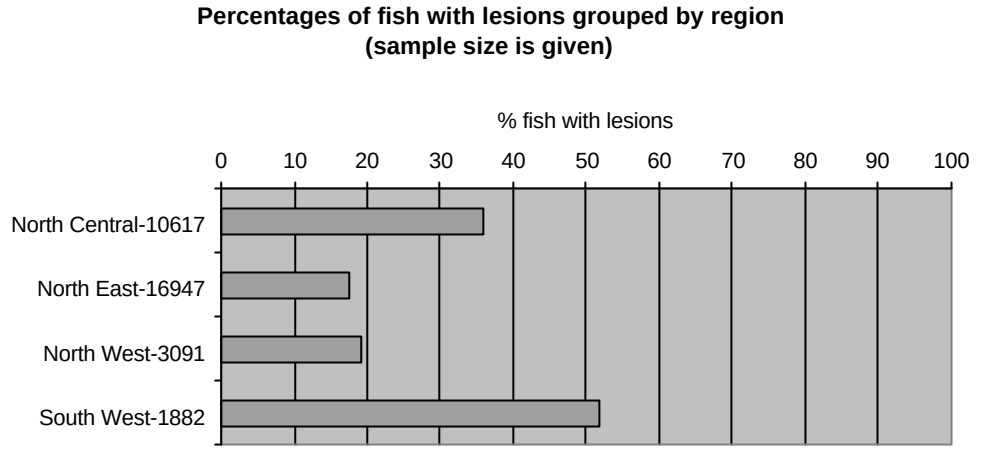

Fig.8

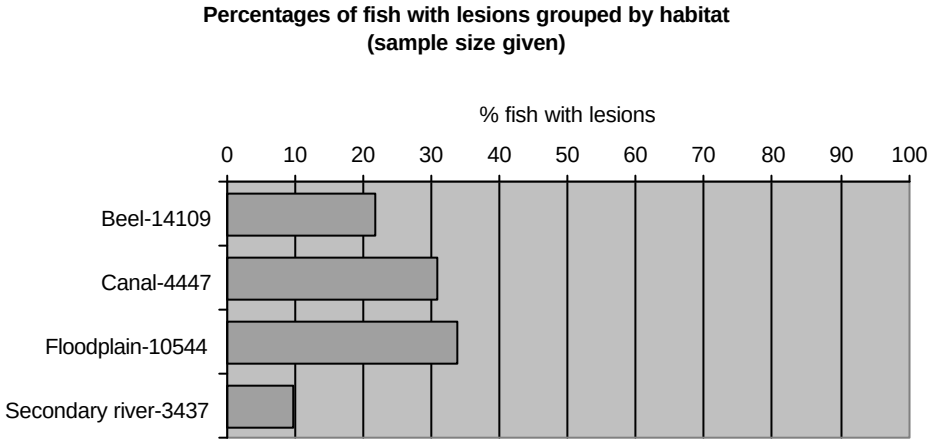

Fig.9

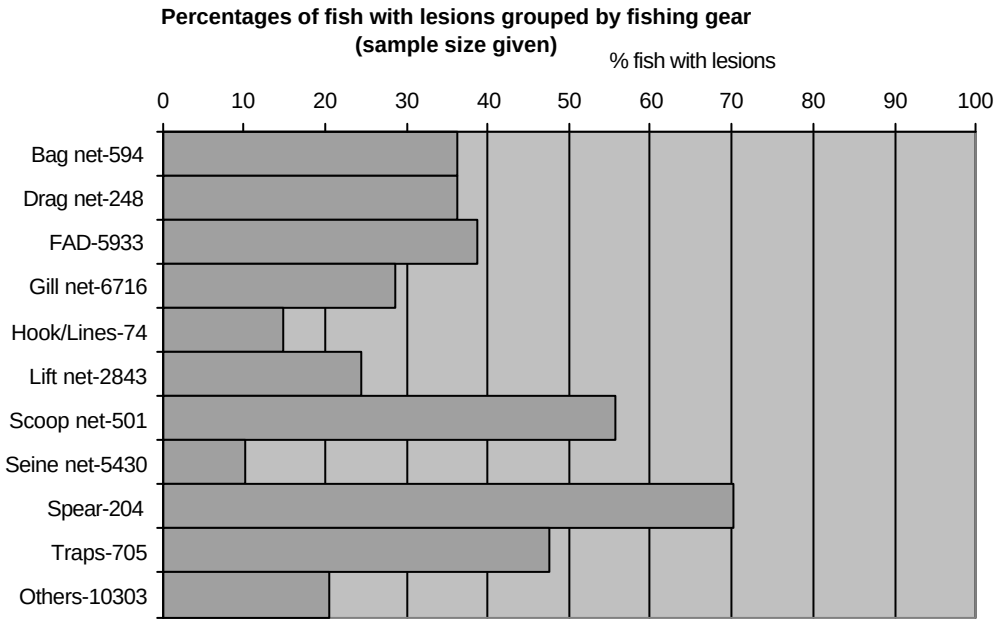

Fig.10 EUS surveys of wild fish in Bangladesh

- 26% of 34451 fish examined had lesions, Oct 92 – Mar 94 (FAP17)
- 0-23% of stocked fish and 2-37% of wild fish were affected by EUS in 3 floodplains, Dec 92-Mar 93. (Subasinghe & Hossain, 1997)*
- 0-9% of stocked fish and 0-11% of wild fish affected, Dec 93-Mar 94 (Subasinghe & Hossain, 1997)*
- 16% of 6408 wild fish had lesions Nov 98 – Mar 99. (Khan *et al.*, 1999)*

all studies used random or whole-populations samples

*indicates that a description of the pathology of EUS samples is also given

Fig.11 EUS surveys of farmed fish in Bangladesh

- 68% of 200 ponds in Chandpur district in Mar – Apr 1988 were affected, often severely (Hossain *et al.*, 1992)
- 50% of 234 carp ponds suffered EUS-type outbreak, 1991-2 (Ahmed & Rab, 1995)
- 13% of 96 extensive and 7% of 522 intensive/semi-intensive carp farmers reported EUS, 1992-5 (ADB/NACA, 1995)
- 15% of 6414 farmed fish had lesions Nov 98 – Mar 99 (Khan *et al.*, 1999)*

all studies used random or whole-populations samples

*indicates that a description of the pathology of EUS samples is also given

Fig. 12 Semi-intensive and intensive carp farms reporting EUS from 1992-5 (ADB/NACA, 1995, from later revised data)

	% of all farms reporting EUS	% of farms reporting disease that consider it to be EUS	% of farms reporting resolution of the EUS problem between 1992-4	National loss ($'000) due to disease	Calculated national loss ($'000) due to EUS
Bangladesh	7	29	53	4087	1185
Cambodia	1	9	50	2	0.18
China	0	0	-	17171	-
Hong Kong	0	0	-	89	-
India	7	37	77	7035	2603
Malaysia	0	0	-	1	-
Nepal	37	95	70	120	114
Pakistan	0	0	-	6	-
Thailand	0	0	-	49	-
Viet Nam	0	0	-	172	-
Average	5	17	63	2873	976

NB does not include losses from diseases categorised as “unknown” or fungal diseases, and does not include cage culture

Fig. 13 Extensive carp farms reporting EUS from 1992-5 (ADB/NACA, 1995, from later revised data)

	% of farms reporting EUS	% of farms reporting disease that consider it to be EUS	% farms with EUS reporting "resolution of problem"	Estimated national loss ($'000) due to disease	Calculated national loss ($'000) due to EUS
Bangladesh	13	42	17	1348	566
China	0	0	-	67201	-
Hong Kong	0	0	-	-	-
India	7	29	50	5684	1648
Malaysia	0	0	-	-	-
Nepal	18	100	35	14	14
Pakistan	0	0	-	21	-
Philippines	0	0	-	-	-
Thailand	0	0	-	-	-
Viet Nam	0	0	-	292	-
Average	4	17	34	12427	743

NB does not include losses from diseases categorised as “unknown” or fungal diseases, and does not include cage culture

Fig.14 Recent outbreaks

- Widespread, low severity occurrences in Bangladesh (1999)
- Snakeheads and cultured carp in Punjab (1996) & Sindh (1998), Pakistan
- Cultured giant gourami and climbing perch, Thailand (1999)
- Cultured silver perch, Australia (1998-9)
- Wild fish in Luzon (1996) and Mindanao (1998), Philippines
- Wild and cultured snakehead samples from Cambodia and Nepal (1999)
- Unconfirmed reports in cultured snakeheads in Southern Vietnam and cultured seabass in Southern Thailand

Fig.15 Study by Bhaumik *et al.* (1991)

- 73% of the 500 farms surveyed were affected by EUS
- 71% of affected farms suffered losses of 20-40%
- 78% of affected farms reported losses between Rs 1-10,000
- 96% of urban dwellers ate fish often before the EUS outbreak and 52% after the outbreak
- 59% of rural people ate fish often before the EUS outbreak and 47% after the outbreak
- 35% of rural people were prepared to eat diseased fish, but no urban dweller interviewed would.
- 77% of fish traders had decreased sales during the outbreak
- 89% would not trade in diseased fish due to the resistance from consumers

APPENDIX TWO

Paper two -

Lilley, J.H., and Chinabut, S. (2000) DNA-based studies on *Aphanomyces invadans,* the fungal pathogen of epizootic ulcerative syndrome (EUS). In: Walker, P. and Subasinghe, R. (Eds), DNA-based Molecular Diagnostic Techniques: Research Needs for Standardization and Validation of the Detection of Aquatic Animal Pathogens and Diseases. Pp. 83-87 FAO/NACA/ACIAR/CSIRO/DFID, Bangkok, Thailand.

DNA-based studies on *Aphanomyces invadans*, the fungal pathogen of epizootic ulcerative syndrome (EUS)

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Background

Epizootic ulcerative syndrome was defined at the DFID Regional Seminar on EUS in Bangkok in 1994 as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response" (Roberts *et al.*, 1994). Research requirements identified from the meeting included work to compare and speciate fungal isolates from EUS outbreaks, develop diagnostic tests, and study the factors that affect transmission of the disease. Attempts are being made to apply DNA-based techniques to each of these study recommendations, and these are discussed here.

Molecular characterisation of EUS-associated *Aphanomyces* isolates

Traditionally, Oomycete fungal isolates are speciated primarily on the basis of the morphology of sexual structures. However many strains, and the more pathogenic strains in particular (including *Aphanomyces astaci* and *Saprolegnia parasitica*), are reluctant to produce sexual structures in culture. Sexual structures have not been demonstrated for the EUS *Aphanomyces* pathogen, and therefore alternative methods of characterisation have been used (Hatai & Egusa, 1978; Willoughby *et al.*, 1995; Callinan *et al.*, 1995; Lilley, 1997). Recently, DNA-based methods have also been applied.

Molecular data sets are rapidly becoming an essential part of any detailed fungal taxonomic study. Previous studies on other *Aphanomyces* and *Saprolegnia* species have used restriction fragment length polymorphism (RFLP) analyses to demonstrate inter-specific relationships (Yeh, 1989; Molina *et al.*, 1995) and random amplification of polymorphic DNA (RAPD) studies have been used to show detailed intra-specific lineages (Huang *et al.*, 1994; Malvick *et al.*, 1998).

In studies comparing EUS-*Aphanomyces* isolates with isolates from mycotic granulomatosis (MG) and red spot disease (RSD) outbreaks, Hart (1997) analysed the ITS1-ITS4 region of the rRNA gene cluster using 10 enzymes (*Alu* I, *Dde* I, *Hae* III, *Hha* I, *Hinf* I, *Hpa* II, *Hsp*92 II, *Mbo* I, *Rsa* I and *Sau*96 I); and sequenced the NS5-NS6 and ITS1-ITS2 regions (Fig. 1). The isolates had all previously been shown to be slow-growing and pathogenic to snakehead fish when injected intramuscularly (Lilley & Roberts, 1997). These studies found no differences between any of the EUS, MG and RSD isolates.

A variety of saprophytic *Aphanomyces*, *Achlya* and *Saprolegnia* species isolated from the surface of EUS-affected fish or from infected waters, and Oomycete fungi involved in other diseases of aquatic animals, were also included in these analyses. These isolates had previously been shown to have very different temperature-growth profiles from the pathogens, and were incapable of growth within snakehead fish (Lilley & Roberts, 1997). The rRNA gene studies succeeded in

differentiating all of these isolates from the EUS, MG and RSD pathogens (Hart, 1997). Dendrograms constructed from the RFLP data showed that the *Aphanomyces* pathogens clustered most closely to European isolates of the crayfish plague fungus, *Aphanomyces astaci*.

Lilley *et al*. (1997) used RAPD-PCR of genomic DNA to investigate possible intra-specific differences between the isolates. Twenty pathogenic isolates from several localities in Bangladesh, Thailand, Indonesia, Philippines, Australia and Japan were compared using 14 ten-mer primers (Fig 2). Also included in the study were 6 *Aphanomyces* saprophytes, 4 *A. astaci* isolates, and 2 *Aphanomyces* isolates from fish affected by ulcerative mycosis (UM) off the eastern coast of the USA. A total of 321 bands were used for the analysis. The mean similarity (F ± SD) between all the pathogens was calculated at 0.95 ± 0.03, whereas the other *Aphanomyces* species had a mean similarity of only 0.14 ± 0.05 compared with the pathogens. These results show that the EUS, MG and RSD pathogens are not only con-specific (now listed in the Index of Fungi as *Aphanomyces invadans*), but also genetically very similar. This indicates that the isolates are not long-term residents in each locality, but have spread across Asia relatively recently. In comparison, RAPD studies on *A. astaci* yielded four distinguishable groups from 15 European isolates indicating that there have been several introductions of that fungus to Europe over a number of years (Huang *et al.*, 1994).

Fig 1. Structure of the rRNA gene cluster and positions of fungal PCR primers. The cluster is split into coding (18S, 5.8S and 28S genes) and non-coding (Internally Transcribed Spacer or ITS) regions. The positions of the PCR primers and their direction of synthesis are indicated by arrows.

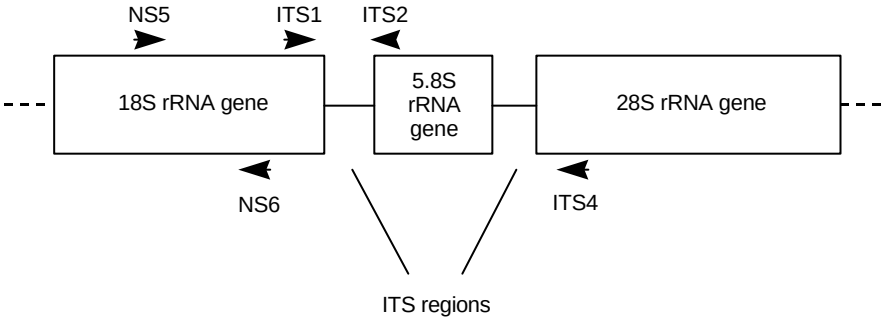

Fig 2. Sequence of 14 random 10-mer Operon primers used for RAPD analyses

A3 5'-AGTCAGCCAC
A4 5'-AATCGGGCTG
A6 5'-GGTCCCTGAC
A7 5'-GAAACGGGTG
A10 5'-GTGATCGCAG
A12 5'-TCGGCGATAG
A18 5'-AGGTGACCGT
A19 5'-CAAACGTCGG
A20 5'-GTTGCGATCC
B1 5'-GTTTCGCTCC
B2 5'-TGATCCCTGG
B4 5'-GGACTGGAGT
B5 5'-TGCGCCCTTC
B10 5'-CTGCTGGGAC

Diagnosis of EUS

Ulcerated fish are diagnosed as EUS-positive by histological demonstration of distinctive mycotic granulomas in underlying tissues. This is a reliable technique that yields a lot of information about the disease. As a result of the molecular characterisation work described above, a DNA probe for the specific detection of *Aphanomyces invadans* has been developed, and this could be used in a PCR-based diagnosis of EUS. However, the results of such a test would not give any information on the extent of infection, if indeed the fish is infected and not just carrying propagules of the fungus, or retaining fungal DNA from a past infection. PCR diagnoses also can suffer from problems of reliability and reproducibility, and in most EUS-affected areas it is a more expensive procedure than histology. Therefore, instead of PCR, attempts are being made to develop an *in situ* hybridisation technique using the probe. It is hoped this will enable histological sections to be further processed for the specific detection of *A. invadans*, and would compliment, rather than replace, histological diagnosis. The development and application of DNA probes for other agents associated with EUS (e.g. rhabdoviruses) would also provide further information about the involvement of these agents in EUS outbreaks.

PCR-based method to detect *Aphanomyces invadans* in the environment

To date, isolates of *A. invadans* have only been obtained from internal tissues of EUS-affected fish. Efforts have been made to recover *A. invadans* from natural water bodies in Thailand, but these have not succeeded due to colonisation of isolation media or fungus baits by faster-growing saprophytic fungi (Willoughby & Lilley, 1992). Fraser & Callinan (1996) used particular growth characteristics of *A. invadans* to devise a technique that excludes opportunist fungi. They were able to quantify *A. invadans*-like colonies on two occasions, but it has proved difficult to reproduce this technique reliably. As a result, important aspects of the natural ecology of *A. invadans* (e.g. persistence of the fungus in ponds outwith the EUS season, and fungus viability on resistant/carrier fish or on non-fish substrates) have yet to be studied.

Molecular detection techniques have been used to assay for Oomycete plant pathogens in environmental samples (Judelson & Messenger-Routh, 1996; Coelho *et al.,* 1997; Liew *et al.,* 1998) and DNA probes are presently being developed by researchers studying the toxin-producing dinoflagellate, *Pfiesteria* (Greer *et al.,* 1997). Ulcerative disease outbreaks in eastern USA have been associated with an invasive mycosis as well as with *Pfiesteria* toxins (Blazer *et al.,* 1998). In these cases, molecular techniques may be useful in detecting the various agents and determining their role in each outbreak.

A molecular assay technique has recently been devised to test for the presence of *A. invadans* DNA in water samples and other substrates. This is based on the PCR amplification of an *A. invadans*-specific 98 bp sequence, that was identified during the RFLP work described above. The particular problems of validating a PCR-based proxy detection method were identified by Hiney & Smith (1998) with regards to bacterial fish pathogens. They devised a study framework that evaluated quantitative, qualitative and reliability criteria at 4 levels of experimental complexity: (a) *in vitro*; (b) using a sterile seeded microcosm; (c) in a non-sterile incurred mesocosm; and (d) in non-sterile field samples. This approach can be applied to the study of *A. invadans* propagules in the environment. The planned study levels for this work are listed below.

(a) The *in vitro* study aims to assess the specificity and sensitivity (DNA low/high detection limits) of the PCR assay on DNA extracted from cultured fungal mycelium. A range of *A. invadans* isolates from different localities is being studied against a strain panel of related species recovered from EUS-affected areas.

(b) Sterile seeded mesocosm. A procedure is being developed to test the DNA extracted from fungal zoospores suspended in flasks of sterile distilled water. *A. invadans* spores will be tested among spores of fungi from the strain panel. Zoospore detection limits will be assessed by making haemocytometer counts of the zoospores present.

(c) Non-sterile incurred mesocosm. Fungal zoospore suspensions will be induced in tanks containing pond water. Zoospore detection limits cannot be accurately assessed at this level as fungal species cannot be identified during haemocytometer zoospore counts. It is hoped the tank study can be used to assess the effect of different variables on the persistence of fungal DNA in the water (e.g. water quality, presence/absence of fish and other potential substrates, different treatment regimes).

(d) The study of non-sterile field samples aims to test for *A. invadans* DNA in affected areas during non-outbreak periods, and in unaffected areas.

Conclusions

PCR-based techniques have provided valuable information in the study of the fungal pathogen involved in EUS outbreaks. In particular, research on the characterisation of *A. invadans* isolates has benefited from the use of these techniques, and it is envisaged that information on the ecology of the fungus will also be obtained with the use of PCR. It is hoped that the outputs from these latter studies will enable risk factors for the disease to be identified, so that recommendations can be made regarding control methods. It is not envisaged that PCR will be used in the routine diagnosis of EUS, but DNA-based methods may compliment histological diagnoses by providing *A. invadans*-specific stains using *in situ* hybridisation.

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APPENDIX THREE

Panyawachira, V., Lilley, J.H., Hart, D., and Kanchanakhan, S. (1999) A PCR-based technique for the identification of *Aphanomyces invadans*. Poster presentation at: Fourth Symposium on Diseases in Asian Aquaculture, Cebu City, Philippines, 22-26 November 1999. Asian Fisheries Society. (Reduced version)

A PCR-BASED TECHNIQUE FOR THE IDENTIFICATION OF *Aphanomyces invadans*

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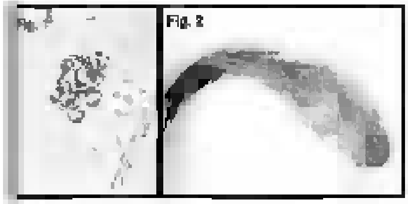

Fig.1 Typical Aphanomyces cluster of encysted primary zoospores.

Fig. 2 Lesion developing on striped snakehead (*Channa striata*) after intramuscular injection with *A. invadans* zoospores.

Introduction

Infection by the Oomycete fungus, *Aphanomyces invadans* (*A. piscicida*), and production of distinctive granulomas are the diagnostic features of EUS (epizootic ulcerative syndrome) (=MG: mycotic granulomatosis) in Asian fish. Identification of *A. invadans* isolates is based on demonstration of *Aphanomyces* sporulation morphology (Fig.1), slow growth rates in culture (Fig.3) and distinctive pathogenicity in EUS-susceptible fish (Fig.2). This poster describes the use of a polymerase chain reaction (PCR) based test to identify *A. invadans* cultures.

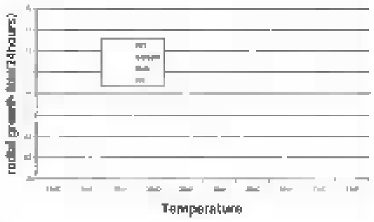

Fig. 3 Temperature growth profiles showing thermolabile characteristic of *A. invadans* isolates (PA7, NJM9701 & B99C) compared to *Aphanomyces* saprophyte (TF5)

Methodology

Primers

Primers were designed based on the DNA sequence of the internally transcribed spacer (ITS) region between the 18S and 5.8S rRNA genes of *A. invadans* (Fig.4). The nucleotide sequences of the primers are:

Aph3 forward 5' aca ggg ctt gtg ctg agc
Aph4 reverse 5' cat tte tgg tgg cta tag g

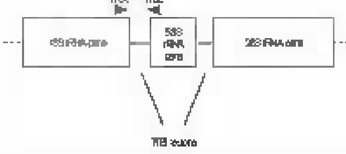

Fig.4 Structure of the rRNA gene cluster. The cluster is split into coding (18S, 5.8S and 28S genes) and non coding (ITS) regions. The position of the PCR primers and their direction of synthesis are indicated by arrows.

PCR reaction

Each 50 µl reaction tube contained the reagents listed in Fig.5. These were overlayed with 2 drops of mineral oil.

Reagent	Working concentration	Volume	Final concentration
H_2O		29µl	
Buffer	10x	5µl	1x
$MgCl_2$	25mM	5µl	2.5mM
dNTP	2.5mM	4µl	200µM
Primer 1	10pmol	2.5µl	25pmol
Primer 2	10pmol	2.5µl	25pmol
Taq	5U/µl	0.5µl	2.5U
DNA	[illegible]	1µl	10ng
Total		50µl	

Fig.5 Reagents used for PCR reactions

PCR reactions were performed in a Hybaid Omnigene thermocycler using the temperature cycle illustrated in Fig.6. Amplification products were separated on 1.5% agarose gels stained with ethidium bromide and visualised under UV illumination.

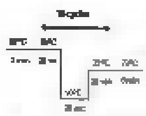

Fig.6 Temperature cycle for PCR reaction

Fungus Isolates

Six *A. invadans* isolates (previously shown to have *A. invadans* morphology, growth and pathogenicity characteristics) (Fig.7), and a strain panel of eleven other *Aphanomyces*, *Achlya*, *Saprolegnia* and *Phytophthora* species (Fig.8) were compared using the PCR test. DNA was extracted from fungal mycelium using the Nucleon BACC2 kit (Amersham Life Sciences, England).

Isolate	Host	Date	Location	Provider
RF6	*Channa striata*	Feb 97	Suphanburi, Thailand	L.G. Willoughby
PA7	*Channa striata*	Feb 95	Nakhon Nayok, Thailand	J.H. Lilley
B99C	[illegible]	May 98	Mymensingh, Bangladesh	J.H. Lilley & M.H. Khan
[illegible]	*Channa striata*	Dec 91	[illegible], Philippines	[illegible]
3P	*Mugil cephalus*	Mar 89	Queensland, Australia	A. Thomas
NJM9701	*Plecoglossus altivelis*	Aug 97	Shiga Prefecture, Japan	K. Hatai

Fig.7 Origin of *Aphanomyces invadans* isolates (all recovered from internal tissues of EUS/MG affected fish)

Species	Isolate	Isolated from	Disease	Date	Location	Provider
Aphanomyces astaci	[illegible]	[illegible]	crayfish plague	-	[illegible], UK	D.J. Alderman
Aphanomyces sp.	[illegible]	[illegible]	UM	May 91	[illegible], USA	[illegible]
Aphanomyces laevis	[illegible]	[illegible]		Jan 94	[illegible]	L.G. Willoughby
Aphanomyces sp.	[illegible]	*Channa striata*	EUS*	Jan 91	Thailand	S. Chinabut
Aphanomyces sp.	[illegible]	[illegible]	EUS**	Dec 91	[illegible], Thailand	L.G. Willoughby
Aphanomyces sp.	[illegible]	*Channa striata*	EUS**	[illegible]	[illegible], Thailand	J.H. Lilley
Aphanomyces sp.	[illegible]	*Channa striata*	external fungal infection	[illegible]	[illegible], Bangkok	J.H. Lilley
Aphanomyces sp.	[illegible]	[illegible]	[illegible]	-	Bangkok, Thailand	[illegible]
Saprolegnia [illegible]	[illegible]	[illegible]	EUS**	Dec 91	[illegible], Thailand	L.G. Willoughby
Achlya sp.	[illegible]	[illegible]	external fungal infection	[illegible]	AAHRI, Bangkok	J.H. Lilley
Phytophthora [illegible]	[illegible]	[illegible]	plant pathogen	[illegible]	[illegible]	[illegible]

Fig.8 Origin of other fungal isolates
*probably external fungal opportunist on ulcerative mycosis affected fish
**external fungal opportunist on EUS affected fish

Results and Discussion

The Aph3/Aph4 primers reacted with DNA extracted from mycelium of a range of *A. invadans* isolates from different countries and host fish species. A 98bp product was produced from each reaction. A few non-specific bands were also produced. The saprophytic *Aphanomyces* species and other Oomycete fungi tested also produced a range of band patterns, but none succeeded in producing the 98bp band. The PCR test is therefore a useful means of differentiating *A. invadans* cultures from other fungal species.

Fig.9 PCR products showing 98bp band amplified from all *A. invadans* isolates

Use of the probe to detect *A. invadans* in the environment

Efforts have been made to recover *A. invadans* from natural water bodies using culture-based techniques, but these have proved problematical due to colonisation of baits and culture medium by faster growing saprophytic fungi. As a result, important aspects of the natural ecology of *A. invadans* (e.g. persistence of the fungus in ponds and fungal viability on carrier fish and non-fish substrates) have not been adequately studied. Therefore, a technique was developed using the PCR primers for the proxy detection of *A. invadans* DNA in water samples and other substrates. Fungal zoospores were recovered on filter paper and DNA extracted using an ethanol based procedure. The technique was tested on *A. invadans* zoospore suspensions (i) in sterile water, (ii) in tank water containing EUS susceptible fish, and (iii) in pond water collected from active EUS outbreaks. The primers succeeded in detecting zoospores of *A. invadans* in experiment (i) but not in (ii) or (iii). This was probably due to the high levels of debris, and low levels of *A. invadans* DNA in the water.

Other applications of the PCR probe

* The PCR based test is being used to show the relationship to *A. invadans* of new invasive fungus isolates, obtained from ulcerative disease outbreaks on the east coast of USA.
* Attempts to use the DNA probe in an *A. invadans* specific histological stain using *in situ* hybridisation have not yet been successful.
* A procedure has been developed to test for *A. invadans* DNA in paraffin embedded tissues using the PCR primers. This has enabled examination of historical tissue samples (Blazer, pers. com.).

APPENDIX FOUR

Paper three - Lilley, J.H., Hart, D., Panyawachira, V., Kanchanakhan, S., Cerenius, L., and Söderhäll, K. (2001) Molecular characterization of the fish-pathogenic fungus *Aphanomyces invadans*. Mycological Research (draft, figures available from ARP Manager)

Molecular characterisation of the fish-pathogenic fungus *Aphanomyces invadans*

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Running title: Molecular characterisation of *Aphanomyces invadans*

ABSTRACT

Aphanomyces invadans (fam. Saprolegniaceae) is a peronosporomycete fungus associated with the serious Asian fish disease, epizootic ulcerative syndrome (EUS), that has been described primarily on the basis of growth and pathogenicity characteristics (Willoughby, Roberts & Chinabut, 1995). In the present study, inter-specific relationships were examined between *A. invadans* isolates and other aquatic animal saprolegniaceae pathogens, and saprophytic saprolegniaceae from EUS-affected areas. Restriction fragment length polymorphisms (RFLPs) and sequences of ribosomal DNA (rDNA) confirmed that *A. invadans* is distinct from all other species studied. A sequence from the internal transcribed spacer region ITS1, unique to *A. invadans,* was used to design primers for a PCR-based diagnostic test. Intra-specific relationships were also examined by random amplification of polymorphic DNA (RAPD) using 20 isolates of *A. invadans* from six countries. The isolates showed a high degree of genetic homogeneity using 14 random ten mer primers. This provides evidence that the fungus has spread across Asia in one, relatively rapid episode, which is consistent with reports of outbreaks of EUS. Physiological distinctions between *A. invadans* and other *Aphanomyces* species based on a data set of 16 growth parameters showed remarkable taxonomic congruence with the molecular phylogeny.

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is a disease that has affected wild and farmed fish in Asia and Australia over the last 30 years (Roberts *et al*., 1994). The first report of the disease came from Japan in 1971, where it is known as mycotic granulomatosis (MG) (Egusa & Masuda, 1971). In Australia, it was called red spot disease (RSD), and primarily affected estuarine mullet (McKenzie & Hall, 1976, Callinan, Fraser & Virgona, 1989). Since the early 1980's, EUS has spread westwards across Southeast and South Asia affecting over 100 species of freshwater and estuarine fish (Roberts *et al*., 1994, Vishwanath, Mohan, & Shankar, 1997). The disease is characterised by the presence of distinctive mycotic granulomas in internal tissues (Callinan *et al.,* 1989, Egusa & Masuda, 1971, McKenzie & Hall, 1976, Roberts *et al.,* 1994, Vishwanath *et al.,* 1997). Isolates of *Aphanomyces* have been recovered from affected fish in Japan (Hatai *et al.,* 1977), Australia (Fraser, Callinan & Calder, 1992), Philippines (Callinan *et al.,* 1995), Thailand, Bangladesh (Willoughby *et al.,* 1995) and

Indonesia. These isolates have all been shown to be unlike other saprolegniaceae, as they are capable of growing through fish muscle and other tissues, and inducing a granulomatous host response characteristic of EUS lesions (Lilley & Roberts, 1997). The pathogenic isolates have been recognised as a single species, and named as *A. piscicida* (Hatai, 1980) and *A. invaderis* (Willoughby *et al.,* 1995), but are now listed in the Index of Fungi as *A. invadans* (David & Kirk, 1997).

Sexual reproductive characters are fundamental to the identification of Peronosporomycete fungi (Dick, 1995), but such structures have not been observed in cultures of *A. invadans*. Therefore, aside from its pathogenic characteristics, it has been distinguished from saprophytic *Aphanomyces* species by growth characteristics (Willoughby *et al.,* 1995), gel electrophoresis (Callinan *et al.,* 1995; Lilley, Thompson & Adams, 1997a), pyrolysis mass spectrometry (Lilley, Beakes & Hetherington, 2001) and resistance to antimicrobial compounds (Lilley & Inglis, 1997). In these studies, *Aphanomyces* isolates from fish affected by ulcerative mycosis (UM) in Eastern USA (Dykstra *et al.,* 1986) were shown to be distinct from *A. invadans*. More recently, isolates recovered from internal tissues of UM-affected menhaden (*Brevoortia tyrannus*) in tributaries of Chesapeake Bay have been found to be indistinguishable from *A. invadans* (Blazer *et al.,* 1999).

In the present study, restriction fragment length polymorphism (RFLP) analyses and sequences of ribosomal DNA (rDNA) are used to compare *A. invadans* with related taxa, with the aim of developing a specific PCR-based diagnostic test for *A. invadans*. Previous studies have shown that analyses of the internal transcribed spacer (ITS) regions of rDNA provide a useful mean of differentiating species of the related genera *Saprolegnia* (Molina, Jong & Ma, 1995) and *Achlya* (Leclerc, Guillot & Deville, 2000). Random amplification of polymorphic DNA (RAPD) is used to study possible differences between geographically-diverse isolates.

MATERIALS AND METHODS

Fungi

A list of *A. invadans* isolates and other fungi used in these studies are given in Tables 1 and 2. Further details can be found in Lilley & Roberts (1997).

[TABLES 1 AND 2]

DNA preparation

Genomic DNA was extracted using the following procedure for all isolates except *Aphanomyces astaci*. About 50 mg of mycelium grown in GPY medium (3 g l^{-1} glucose, 1 g l^{-1} peptone, 0.5 g l^{-1} yeast, 0.13 g l^{-1} $MgSO_4.7H_2O$, 29 mg l^{-1} $CaCl_2.2H_2O$, 14 mg l^{-1} KH_2PO_4, 3.9 mg l^{-1} $CuSO_4.5H_2O$, 2.4 mg l^{-1} $FeCl_3.6H_2O$, 1.8 mg l^{-1} $MnCl_2.4H_2O$, 0.4 mg l^{-1} $ZnSO_4.7H_2O$), was homogenised in 11 ml lysis buffer (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 2 % SDS). Proteinase K was added to a final concentration of 1 mg ml^{-1} and incubated overnight at 37 ^{o}C with shaking. The sample was then chilled on ice for 10 min. Five millilitres of saturated NaCl was added to the tube which was mixed and then chilled for another 5 min. Precipitated protein was pelleted by centrifugation at 2000 g for 15 min at 4 ^{o}C. The supernatant was transferred to a fresh tube and centrifuged again to ensure removal of the precipitate. RNase A was added to a final concentration of 20 µg ml^{-1} and the tube incubated for 30 min at 37 ^{o}C. Two volumes of 100 % ethanol were added to the sample, which was then mixed and stored at –20 ^{o}C overnight. The sample was centrifuged at 2000 g for 15 min at 4 ^{o}C. The resulting DNA pellet was

washed with 10 ml ice-cold 75 % ethanol and centrifuged again for 5 min. The pellet was vacuum-dried and resuspended in 200 µl dH_2O.

DNA preparations of *A. astaci* isolates were made from cultures grown in PG-1 medium (3 g l^{-1} glucose, 6 g l^{1} peptone, 0.37 g l^{1} KCl, 0.17 g l^{1} $MgCl_2.6H_2O$, 0.15 g l^{1} $CaCl_2.2H_2O$, 20 mg l^{1} $FeCl_3.6H_2O$, 44 mg l^{-1} Na2 EDTA, 13 mM sodium phosphate buffer, pH 6.3) using the Nucleon II kit by following the supplied procedure for filamentous fungi (Scotlab, Strathclyde, UK, 1996). Briefly, mycelium was ground in liquid nitrogen and scraped into 2 ml lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA, 150 mM NaCl, 1 % SDS). 3 µl of 10 mg ml^{-1} RNase A was added and the sample incubated at 37 °C for 30 min. Then 1.5 ml 5 M sodium perchlorate was added to the sample, which was mixed for 15 min and incubated at 65 °C for 25 min. Ice-cold chloroform (5.5 ml) was then added and the solution mixed for 10 min and centrifuged at 800 g for 1 min. About 800 µl of a silica suspension was added and the tube centrifuged at 1,400 ***g*** for 3 min. The clear DNA-containing phase was recovered to which equal volume of 99 % cold ethanol was added and the resulting precipitate was centrifuged at 5000 g for 5 min. The DNA pellet was washed in 70 % ethanol and centrifuged again. The final pellet was vacuum-dried and resuspended in 100µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

PCR amplification of rDNA and RFLP

This work was undertaken at the Department of Veterinary Pathology, University of Glasgow. The region between the 18S and 28S rDNA subunits was amplified for each fungal isolate using the primers ITS1 and ITS4 (White *et al*., 1990). 100 ng of genomic DNA was amplified in a final volume of 100 µl containing 1x *Taq* buffer, 200 µM NTPs, 50 pmoles each of the primers ITS1 and ITS4, 1.5 mM $MgCl_2$ and 2 units of *Taq* polymerase. Amplifications were performed with an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 53 °C, and 45 sec at 72 °C, with a final extension of 7 min at 72 °C.

Approximately 100 ng of purified rDNA amplification product was digested in a final volume of 20 µl containing 5 units of enzyme. The reaction was incubated for 3 hours at the appropriate temperature. The enzymes used in the RFLP analyses were: Alu I, Dde I, Hae III, *Hha* I, *Hinf* I, *Hpa* II, *Hsp*92 II, *Mbo* I, *Rsa* I and *Sau*96 I. Digestion products were analysed by gel electrophoresis using a 1.5 % agarose gel. Digestion patterns were analysed and phylogenetic trees constructed using the "branch and bound" algorithm of Hendy & Penny (1982), using the PENNY program of the PHYLIP phylogenetic inference package.

PCR amplification of rDNA and sequencing

This work was undertaken at the Department of Veterinary Pathology, University of Glasgow and at the Aquatic Animal Health Research Institute (AAHRI), Department of Fisheries, Thailand. The ITS1 rDNA region was amplified for each fungal isolate using the primers ITS1 and ITS2 (White *et al*., 1990). 50 ng of genomic DNA was amplified in a final volume of 50 µl containing 1x *Taq* buffer, 200 µM NTPs, 25 pmoles each of the primers ITS1 and ITS2, 1.5 mM $MgCl_2$ and 2 units of *Taq* polymerase. Amplifications were performed with an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 55 °C, and 45 sec at 72 °C, with a final extension of 5 min at 72 °C. Products were isolated, purified and automatically sequenced on an ABI PRISM™ model 377. Sequences were submitted to GenBank, and accession numbers are given in Tables 1 and 2.

PCR diagnostic probe technique

Two sets of primers were designed from the ITS1 rDNA sequence for the specific amplification of *A. invadans* DNA. These were tested at AAHRI, Department of Fisheries, Thailand. The first set, FP1 5'-AAGGCTTGTGCTGAGCTCACACTC-3' and FP2 5'-GATGGCTAAGGTTTCAGTATGTAG-3', are located at nucleotide positions 50 to 73 and 124 to 147 of the ITS1 and ITS2 amplification product. The following PCR reagents were added to a 50 µl reaction tube: 1x *Taq* buffer, 200 µM NTPs, 25 pmoles each of the primers FP1 and FP2, 2.5 mM $MgCl_2$ and 1.25 units of *Taq* polymerase. Amplifications were performed with an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 68 °C, and 45 sec at 72 °C, with a final extension of 5 min at 72 °C.

The second set of diagnostic primers, APH3 5'-ATAAGGCTTGTGCTGAGC and APH4 5'-CATTTCTGATGGCTAAGG, are located at nucleotide positions 48 to 65 and 137 to 154 of the ITS1 and ITS2 amplification product. The following PCR reagents were added to a 50 µl reaction tube: 1x *Taq* buffer, 200 µM NTPs, 25 pmoles each of the primers APH3 and APH4, 2.5 mM $MgCl_2$ and 1.25 units of *Taq* polymerase. Amplifications were performed with an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 50 °C, and 30 sec at 72 °C, with a final extension of 5 min at 72 °C.

Random amplification of polymorphic DNA (RAPD)

The RAPD work was undertaken at the Division of Physiological Mycology, University of Uppsala, Sweden. Reactions were carried out in 50 µl volumes containing 5-50 ng genomic DNA, 20 pmol primer, 5 µl 10X USB buffer (AP Biotech, Uppsala), 1500 mM $MgCl_2$, 200 mM of each dNTP and 1.5 units of AmpliTaq® DNA polymerase. Fourteen random ten-mer primers were tested (A3, A4, A6, A7, A10, A12, A18, A19, A20, B1, B2, B4, B5 and B10; Operon Technologies). PCR reactions were performed in a Perkin Elmer GeneAmp® PCR System 2400 using the following temperature regime: 5 min at 95 ^{o}C, 2 min at 80 ^{o}C followed by addition of the enzyme and 45 cycles of 94 ^{o}C for 1 min, 36 ^{o}C for 1 min and 72 ^{o}C for 2 min. The reaction was then held at 72 ^{o}C for 7 min before cooling to 4 ^{o}C. Amplification products were separated on 14 % agarose gels stained with ethidium bromide and visualised under UV illumination. Similarity coefficients (F) were calculated according to the formula of Nei & Li (1979).

Growth data

Cluster analysis was also performed on data published in Lilley & Roberts (1997) comparing the growth rates of various *Aphanomyces* isolates at different temperatures and on different solidified media. A dendrogram was constructed by the agglomeration of a squared Euclidean dissimilarity matrix using the unweighted group-average method, UPGMA (Sneath & Sokal, 1973).

RESULTS

RFLP

Amplification of the ITS1, 5.8S rDNA coding region and ITS2 produced products of approximately 800 bp in size for each isolate. The products were confirmed as being of rDNA origin by DNA sequencing (data not shown). The PCR products were restriction enzyme digested using 4 bp target site enzymes. Ten enzymes used on a total of 27 fungal isolates produced 43 different restriction-banding patterns.

The data showed a division of the isolates into 14 distinct groups. All eight *A. invadans* isolates tested, including one from an MG outbreak in Japan (NJM9201), and two from red spot disease outbreaks in Australia (3P and 24P), displayed identical RFLP results. The data was used to construct a phylogenetic tree using the branch and bound algorithm (Fig. 1). The tree displays the single node containing the *A. invadans* isolates. Out of the other fungal isolates, *A. astaci* clustered most closely to the *A. invadans* group. The *Aphanomyces* isolate from menhaden (84-1240) was distinct from the *A. invadans* isolates. The unspeciated saprophytic *Aphanomyces* isolates could be divided into 6 separate groups based on the RFLP data.

[FIGURE 1] Dendrogram constructed from RFLP data

rDNA sequencing

DNA sequencing of the ITS1 region confirmed that the *A. invadans* isolates were identical to each other and to the isolates of red spot disease and mycotic granulomatosis (Fig. 2).

[FIGURE 2] Consensus sequences of ITS1 region of ribosomal DNA

PCR diagnostic probe technique

Amplification of genomic DNA from *A. invadans* fungal isolates using primers FP1 and FP2 produced a product of 98 bp (Fig 3A). Amplification of genomic DNA from the fungal isolates using primers APH3 and APH4 produced a product of 107 bp (Fig 3B). APH3 and APH4 also produced a number of non-specific bands in all isolates tested, but the primers had the advantage over FP1 And FP2 that a lower annealing temperature was required. FP1-2 and APH3-4 diagnostic tests have been used on 12 *A. invadans* isolates which have all tested positive; and 12 other fungi (9 *Aphanomyces* spp., 1 *Achlya* sp., 1 *Saprolegnia diclina* isolate and 1 *Phytopthora cinnamomi* isolate), which all tested negative (data not shown).

[FIGURE 3] PCR products using (A) primers FP1 and FP2 and (B) primers APH3 and APH4

RAPD

Two sample gels showing polymorphism fragments generated using two random primers are given in Fig. 4. A total of 321 bands, averaging 80.7 fragments per isolate, were used to calculate similarity coefficients (F). The mean similarity coefficient (F ± SD) comparing all 20 *A. invadans* isolates was 0.95 ± 0.03. The 6 saprophytic *Aphanomyces* spp, 6 *A. astaci* isolates and 2 UM-*Aphanomyces* isolates in combination gave an average similarity coefficient of 0.15 ± 0.05 compared with the *A. invadans* isolates. A dendrogram generated from this data using the neighbour joining method of Saitou and Nei (1987) is given in Fig. 5.

[FIGURE 4] RAPD banding pattern for *Aphanomyces* isolates using primer OP-A19 (Operon technologies)

[FIGURE 5] Dendrogram constructed from RAPD data

Growth data

A dendrogram constructed using 16 growth variables for each isolate is given in Fig. 6. All *A. invadans* isolates clustered together at similarity indices not less than 99.7. Of the other fungi, *A. astaci* was most similar to *A. invadans*, being separated at an index of 97.1. The Asian saprophytic *Aphanomyces* are shown to be a heterogenous group, separated from the other fungi at a similarity index of 40.3.

[FIGURE 6] Unweighted group-average cluster analysis of *Aphanomyces* growth data given in Lilley & Roberts (1997)

DISCUSSION

The study confirms that all the isolates that have been allocated to *A. invadans* do represent a single, fish-pathogenic species, which can be differentiated from other similar species by RFLP analyses of rDNA, sequencing the ITS1 region, and by RAPD analysis. Given that RAPDs have been shown to be very sensitive in distinguishing strains of saprolegniacean fungi (Huang *et al*, 1994; Diéguez-Uribeondo *et al*, 1995), the results of the present RAPD study indicate an extreme lack of genetic diversity between the *A. invadans* isolates. Lilley *et al*. (1997b) presented a summary of the RAPD study and concluded that, not only do the *A. invadans* isolates represent a single species, but they constitute a single clonal lineage. Fig. 5 shows that all the Philippine isolates clustered together, but from present data this cannot be considered a significant divergence from the other isolates, given the low level of variation involved

The genetic homogeneity between all the *A. invadans* isolates may be associated with observations that it lacks any sexual reproductive structures. However, RAPD studies on *A. astaci*, which is similarly asexual, yielded four distinguishable groups from 15 European isolates (Huang *et al.*, 1994, Diéguez-Uribeondo *et al.,* 1995). These showed an average between-group similarity of 0.25 ± 0.08, and the average within-group similarity (0.84 ± 0.11) was also lower than for the *A. invadans* isolates (data recalculated from Diéguez-Uribeondo *et al.*, 1995). This indicates that there have been several introductions of *A. astaci* to Europe over a number of years whereas *A. invadans* has achieved its colonisation of Asia in one relatively rapid episode. This correlates with the accounts of the spread of EUS outbreaks (Roberts *et al.,* 1994).

The time span over which *A. invadans* appears to have spread across Asia (from Japan in 1971 to Pakistan in 1996) is not unreasonable for a fungal pathogen. *A. astaci* spread from Italy to colonise most of Europe between the Netherlands and Russia over a similar time span in the last century (Alderman, 1996). In another study that employed RAPD-PCR to trace the origin of a pathogenic fungus, Hajek *et al.* (1996) showed that a single genotype of the weevil pathogen *Zoophthora phytonomi* appears to have spread to much of Eastern North America over a period of 8 years. Further comparisons can be drawn with the clonal genotypes of different *Phytophthora* spp that cause epidemics in particular host plant species. Hantula, Lilja & Parikka (1997) showed by means of random amplified microsatellites that all 20 isolates of *Phytophthora cactorum* originating from strawberry in 6 countries in Northern Europe were clonal. Likewise, Goodwin, Cohen & Fry (1994) used mating types, allozymes and Southern analyses of over 300 isolates of the Irish potato famine fungus, *Phytophthora infestans*, from 20 countries to demonstrate that of a number of original genotypes, it was probably a single genetic individual that was transported from Mexico to the United States, Europe and the rest of the world causing epidemics of potato blight from the 1840s onwards. Further work on different *P. infestans* genotypes showed that following migration, mutation rates at

pathogenicity loci are high, resulting in several pathotypes within each genotype investigated (Goodwin, Sujkowski & Fry, 1995).

In the case of EUS, it is not possible to determine whether *A. invadans* was introduced to Japan, or arose through changes in an endemic genotype. However, given the established nature of the fish importation industry in Japan, the former would seem the more plausible theory. Japan imports fish even from isolated areas, such as some Pacific islands, and it can be theorised that the pathogen may have evolved in such an environment alongside a natural, resistant host.

Of the data sets described here, the RFLP analyses provided the most useful information on inter-specific relationships of *A. invadans*. Yeh (1989) has previously shown RFLP analyses to be effective in distinguishing *A. astaci, A. stellatus, A. cochlioides* and *A. eutiches*. Out of the isolates studied here, the crayfish plague fungus, *A. astaci*, is the most closely related to *A. invadans*. Previous studies have shown that these two pathogens are unable to infect susceptible hosts of the other (Lilley & Roberts, 1997). The greater similarity that *A. invadans* shows with European *A. astaci* isolates than with the Asian saprophytic *Aphanomyces* spp. is important, as it shows that is clearly not derived from local saprophytes.

Sequencing the ITS1 region provided enough variability for the specific identification of *A. invadans*. The region of the 18S rDNA subunit between primers NS5 and NS6 (White *et al.,* 1990) was also sequenced (Genbank AF349602-9) but this is not described in detail here as it revealed few differences between the fungi tested. Dick *et al*. (1999) have shown that complete 18s DNA sequences are more useful in the investigation of the phylogenetic relationships of higher taxa within the class Peronosporormycetes. Riethmüeller, Weiß, & Oberwinkler (1999) have undertaken similar analyses of the 28s rDNA subunit.

Cluster analysis of the culture characteristics of the fungi demonstrated similar taxonomic relationships to those shown by the molecular studies. In culture and RAPD data sets, the Philippine *A. invadans* isolates clustered separately from the main group, but not at a level that would allow them to be distinguished from the other isolates. In culture and RFLP data sets *A. astaci* is the most closely related species to *A. invadans.* In the growth and RAPD data, the UM-*Aphanomyces* clustered most closely to *Aphanomyces laevis*, although RFLP analyses clearly distinguished the two. RFLP, RAPD and growth data sets indicated that the unspeciated saprophytic *Aphanomyces* isolates comprised of at least three species. Group 1 (where studied, comprising TF5, TF41, T1SA, A2SA, F3SA, SSA) was the largest group, and included isolates obtained from water, fish and turtles. TF33 and WSA formed a separate group (Group 2) and were also recovered from both water and fish. SA11 (Group 3) was isolated from a fish and was the most dissimilar from the main group in all analyses. TF54 was an exception in that, during culture studies, the growth rate changed slightly, and it became slower growing. This is reflected in the growth rate data (Fig. 6), but not in the RFLP data (Fig.1), which did not distinguish TF54 from three other saprophytic *Aphanomyces*. It is interesting to note that isolates from all three groups of saprophytic Aphanomyces were from fish, indicating the variety of species that colonise the surface of open wounds on fish. In contrast only *A. invadans* has been shown to be capable of infecting internal tissues.

Although previous isolates of *Aphanomyces* from ulcerative mycosis infected fish in USA are shown to be distinct from *A. invadans*, recently slow-growing cultures isolated from muscle tissue of ulcerated menhaden have shown similarities to *A. invadans* (Blazer *et al.,* 1999). Pathogenicity, growth and

molecular comparisons with Asian *A. invadans* isolates are currently underway. Aside from the UM infections, invasive *Aphanomyces* infections of fish have been reported recently in Egypt (Shaheen, El-Sayed & Faisal, 1999). Sequencing of the ITS1 region of such isolates, or use of the PCR diagnostic probe method described in this study, would help to determine the geographic limits of *A. invadans* beyond Asia.

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TABLE 1. List of fish-pathogenic *Aphanomyces invadans* isolates used in present studies

Isolate	Source	Location and date	RFLP	GenBank accession number	PCR test	RAPD	Growth data
TA1	Striped snakehead[1]	Thailand '91				X	X
RF6	Striped snakehead	Suphanburi ,Thailand '91	X		X	X	X
RF8	Striped snakehead	Suphanburi ,Thailand '91				X	X
S1PA	Striped snakehead	Bangkok, Thailand '91		AF349610			X
S3PA	Striped snakehead	Bangkok, Thailand '91				X	X
G2PA	Three spot gourami[2]	Bangkok, Thailand '91	X			X	X
PA1	Striped snakehead	Nonthaburi, Thailand '91				X	X
PA3	Striped snakehead	Nonthaburi, Thailand '91					X
PA4	Striped snakehead	Nonthaburi, Thailand '91				X	X
PA5	Striped snakehead	Nonthaburi, Thailand '91	X				X
PA7	Striped snakehead	Nonthaburi, Thailand '91			X		X
PA8	Striped snakehead	Nonthaburi, Thailand '91					X
PA10	Striped snakehead	Nonthaburi, Thailand '91					X
96PA	Snakehead	Pichit, Thailand '96				X	
BR	Rohu[3]	Parbatipur, Bangladesh '93				X	X
BH	Round-tailed garfish[4]	Srimangal, Bangladesh '93	X	AF349611		X	X
BS	Mud murrel[5]	Srimangal, Bangladesh '93					X
B99C	Reba[6]	Mymensingh, Bangladesh '99			X		
36/1P	Sand goby[7]	Java, Indonesia '93			X	X	X
30P	Striped snakehead	South Luzon, Philippines '91				X	X
33P	Striped snakehead	Central Luzon, Philippines '91			X	X	X
34P	Grey mullet[8]	North Luzon, Philippines '91				X	X
10D	"Snakehead"	Philippines '92	X			X	X
3P	Grey mullet	NSW, Australia '89	X				X
4P	Yellowfin bream[9]	NSW, Australia '89				X	X
10P	Sand whiting[10]	NSW, Australia '89				X	X
24P	Grey mullet	Queensland, Australia '90	X	AF349612	X	X	X
NJM9030	Ayu[11]	Schizuoka, Japan '90	X			X	X
NJM9201	Dwarf gourami[12]	Tokyo, Japan (imported from Singapore) '92				X	
NJM9701	Ayu	Shiga Prefecture, Japan '97		AF349613	X		

[1] *Channa striata* (Bloch)
[2] *Trichogaster trichopterus* (Pallas)
[3] *Labeo rohita* (Hamilton)
[4] *Xenentodon cancila* (Hamilton)
[5] *Channa punctata* (Bloch)
[6] *Cirrhinus ariza* (Hamilton)
[7] *Oxyeleotris marmoratus* (Bleeker)
[8] *Mugil cephalus* L.
[9] *Acanthopagrus australis* (Günther)
[10] *Sillago ciliata* Cuvier
[11] *Plecoglossus altivelis altivelis* (Temminck & Schlegel)
[12] *Colisa lalia* (Hamilton)

TABLE 2. List of non-fish pathogenic *Aphanomyces, Achlya,* and *Saprolegnia* isolates used in present studies

Species	Isolate	Source	Location and date	RFLP	GenBank accession number	PCR test	RAPD	Growth data
UM-*Aphanomyces*	84-1240 (ATCC 62427)	Menhaden[1]	Nth Carolina, USA '84	X		X	X	X
UM-*Aphanomyces*	84-1282	Menhaden	Nth Carolina, USA '84				X	
A. astaci	J1	Noble crayfish[2]	Östergötlands, Sweden '62				X	
A. astaci	PL	Signal crayfish[3]	L. Tahoe, USA '70				X	
A. astaci	KV	Signal crayfish	Sweden '78				X	
A. astaci	PC	Red swamp crayfish[4]	Spain ~'94				X	
A. astaci	FDL457	White-clawed crayfish[5]	Herefordshire, UK '90	X			X	X
A. astaci	FDL458	White-clawed crayfish	Herefordshire, UK '90	X		X	X	X
Aphanomyces sp.	TF5	Striped snakehead	Thailand '91	X		X		X
Aphanomyces sp.	TF33	Swamp eel[6]	Udon Thani, Thailand '91	X	AF349617			X
Aphanomyces sp.	TF41	Striped snakehead	Thailand '92	X			X	X
Aphanomyces sp.	TF54	Striped snakehead	Thailand '93	X			X	X
Aphanomyces sp.	T1SA	Soft shell turtle[7]	Bangkok, Thailand '93	X				X
Aphanomyces sp.	A2SA	Aquarium water	Bangkok, Thailand '94	X				X
Aphanomyces sp.	F3SA	Striped snakehead	Bangkok, Thailand '94	X			X	X
Aphanomyces sp.	SSA	Striped snakehead	Suphanburi, Thailand '94	X				X
Aphanomyces sp.	WSA	Fish pond	Suphanburi, Thailand '94	X			X	X
Aphanomyces sp.	SA11	Striped snakehead	Nonthaburi, Thailand '95	X			X	X
A. laevis	ASEAN1	Fish pond	Bangkok, Thailand '94	X	AF349615			X
A. laevis	ASEAN3	Fish pond	Bangkok, Thailand '94	X	AF349616	X	X	X
Achlya sp.	S2AC	Fish pond	Bangkok, Thailand '94	X				
Achlya diffusa	W2BAC	Fish pond	Nonthaburi, Thailand '95	X				
S. diclina	TF27	Striped snakehead	UdonThani, Thailand '91	X				
S. ferax	P32	Lake water	Windermere, UK '57	X				

[1]*Brevoortia tyrannus* (Latrobe)
[2]*Astacus astacus* L.
[3]*Pacifastacus leniusculus* Dana
[4]*Procambarus clarkii* Girard
[5]*Austropotamobius pallipes* Lereboullet
[6]*Fluta alba* (Zview)
[7]*Trionyx cartilagineus* (Boddaert)

Figure 1. Dendrogram constructed from RFLP data

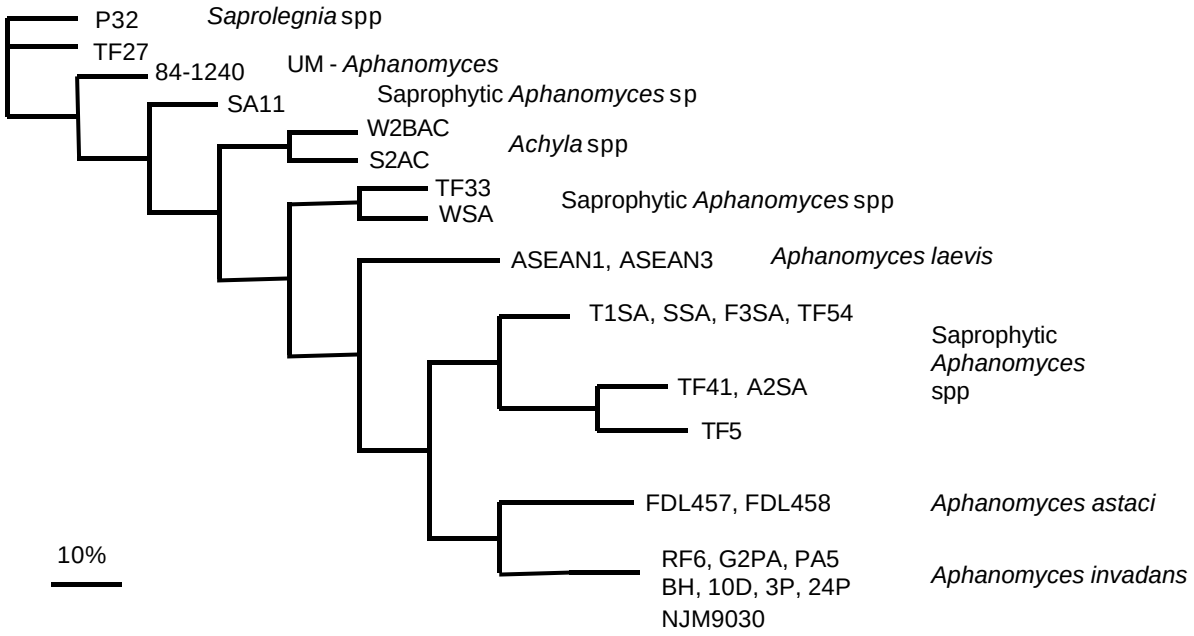


Figure 2. Consensus sequences of ITS1 region of ribosomal DNA (N=unknown nucleotide)

```
   S1PA  AAGGATCATT ACCACACCAA AAAAATATCC ACGTGAATGT ATTCTTTATA
     BH  AAGGATCATT ACCACACCAA AAAAATATCC ACGTGAATGT ATTCTTTATA
    24P  NNNNNNNNNN NNNNNNCCAA AAAAATATCC ACGTGAATGT ATTCTTTATA
 NJM9701  NAGGATCATT ACCACACCAA AAAAATATCC ACGTGAATGT ATTCTTTATA
  ASEAN1  AAGGATCATT ACCACACCAA AAAAATTTCC ACGTGAATGT ATTCTTTATT
  ASEAN3  AAGGATCATT ACCACACCAA AAAAATTTCC ACGTGAATGT ATTCTTTATT
    TF33  AAGGATCATT ACCACACCAA AAAAATATCC ACGTGAATGT ACTCTTTATG

   S1PA  AGGCTTGTGC TGAGCTCACA CTCGGCTAGC CGAAGGTTTC GCAAGAAACC
     BH  AGGCTTGTGC TGAGCTCACA CTCGGCTAGC CGAAGGTTTC GCAAGAAACC
    24P  AGGCTTGTGC TGAGCTCACA CTCGGCTAGC CGAAGGTTTC GCAAGAAACC
 NJM9701  AGGCTTGTGC TGAGCTCACA CTCGGCTAGC CGAAGGTTTC GCAAGAAACC
  ASEAN1  TGGCTTGTGC ...GGGTTCT GCCTGCTAGC CGAAGGTTTC GCAAGAAGCC
  ASEAN3  AAGGATCATT ACCACACCAA AAAAATTTCC ACGTGAATGT ATTCTTTATT
    TF33  AAGCCAAGTC ..AGGCGCAA GCTTGTAGGC AGAAGGTTTC GCAAGAAGCC

   S1PA  GATGTACTTT TAATCCC... TTTAAACTAC ATACTGAAAC CTTAGCCATC
     BH  GATGTACTTT TAATCCC... TTTAAACTAC ATACTGAAAC CTTAGCCATC
    24P  GATGTACTTT TAATCCC... TTTAAACTAC ATACTGAAAC CTTAGCCATC
 NJM9701  GATGTACTTT TAATCCC... TTTAAACTAC ATACTGAAAC CTTAGCCATC
  ASEAN1  GATGTACAAT TTAATCCCTT TTTAAACAAT ACACTGAAAC CTTAGCCATC
  ASEAN3  AAGGATCATT ACCACACCAA AAAAATTTCC ACGTGAATGT ATTCTTTATT
    TF33  GATGTGATTT CATCCCTTTT TTAATTGAAT GACTGATTGA GATAGCCATC

   S1PA  AGAAATGATA GCTTGTAATC AAATACAACT TTCAACAGTG GATGTCTAGG
     BH  AGAAATGATA GCTTGTAATC AAATACAACT TTCAACAGTG GATGTCTAGG
    24P  AGAAATGATA GCTTGTAATC AAATACAACT TTCAACAGTG GATGTCTAGG
 NJM9701  AGAAATGATA GCTTGTAATC AAATACAACT TTCAACAGTG GATGTCTAGG
  ASEAN1  AGAAATGATA GCTTGTAATA AATTACAACT TTCAACAGTG GATGTCTAGG
  ASEAN3  AAGGATCATT ACCACACCAA AAAAATTTCC ACGTGAATGT ATTCTTTATT
    TF33  AGAAATGATA GCTTGTAATA AAATACAACT TTCAACAGTG GATGTCTCGG

   S1PA  CTCGC
     BH  CTCGC
    24P  CTCGC
 NJM9701  CTCGC
  ASEAN1  CTCGC
  ASEAN3  CTCGC
    TF33  CTCGC
```

Figure 3. PCR products using (A) primers FP1 and FP2 and (B) primers APH3 and APH4

Lanes (1-7) correspond to *Aphanomyces invadans* isolates (1) RF6, (2) PA7, (3) B99C, (4) 36/1P, (5) 33P, (6) 24P, (7) NJM9701. Lane (8) *Aphanomyces astaci* FDL458, (9) UM-*Aphanomyces* 84-1240, (10) *Aphanomyces laevis* ASEAN3, (11) saprophytic *Aphanomyces* TF5. Markers denote 100 base-pairs.

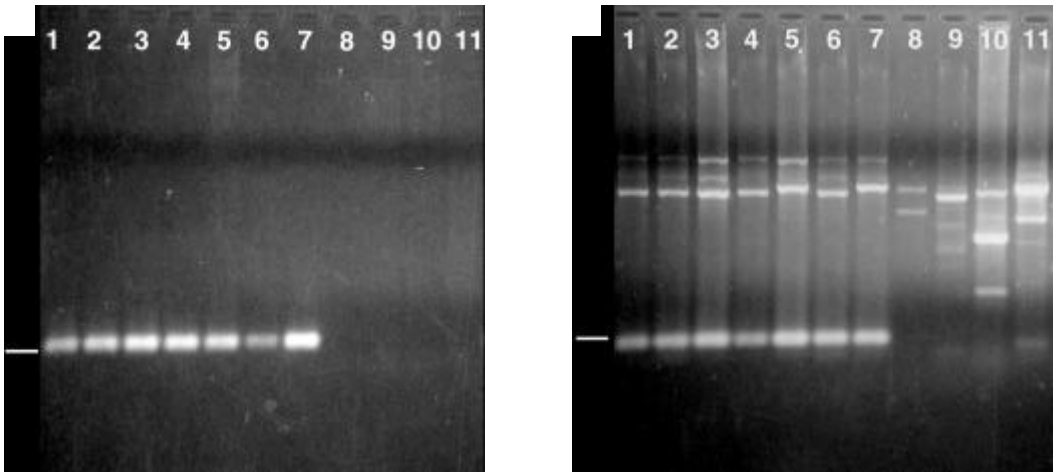


Figure 5. Dendrogram constructed from RAPD data

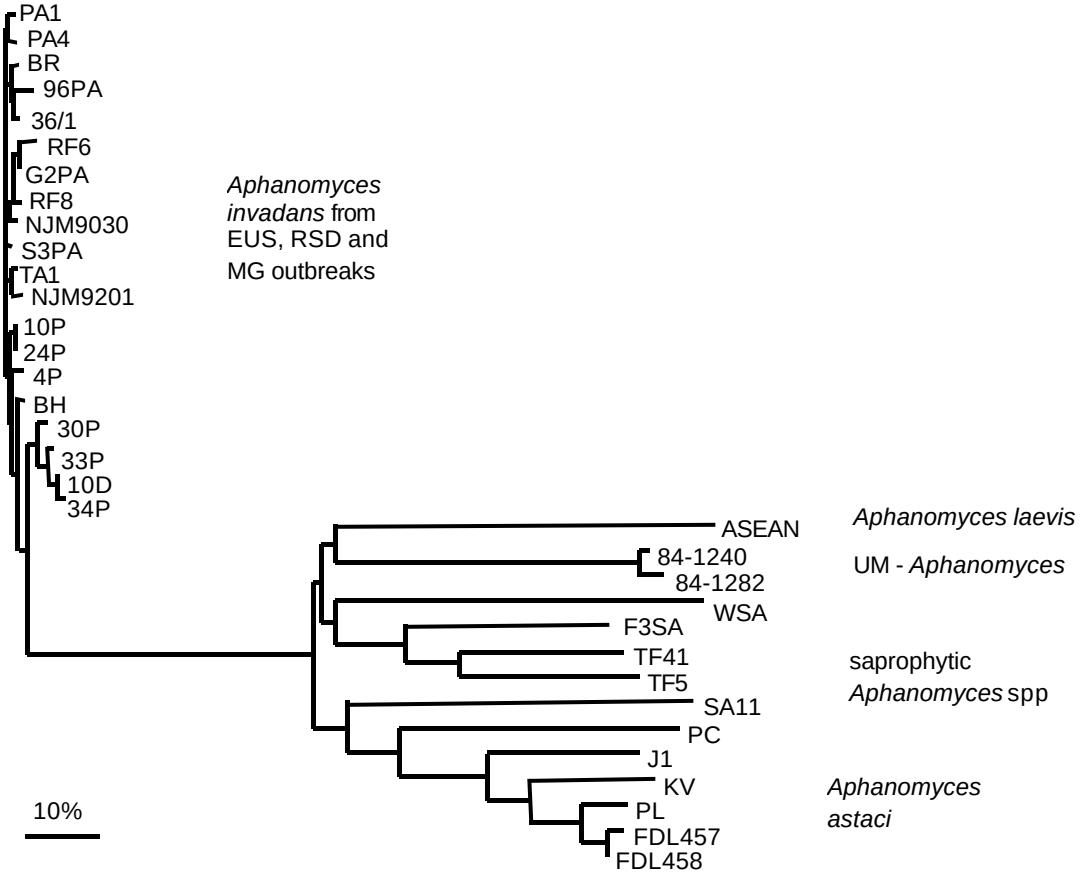


Figure 3. RAPD band profiles using primers (A) A19 and (B) A7

Markers denote 1200, 800 and 400 base-pairs. Lanes for (A) correspond to EUS pathogens: (1) TA1, (2) RF6, (3) RF8, (4) S3PA, (5) G2PA, (6) PA1, (7) PA4, (8) 96PA, (9) BR, (10) BH, (11) 36/1P, (12) 10D, (13) 30P, (14) 33P, (15) 34P; RSD pathogens: (16) 4P, (17) 10P, (18) 24P; MG pathogens: (19) NJM9030, (20) NJM9201; UM fungus: (21) 84-1240; saprophytic *Aphanomyces*: (22) TF41; and *A. astaci*: (23) FDL457, (24) PC. Lanes for (B) are the same except that (22) is saprophytic *Aphanomyces* isolate F3SA

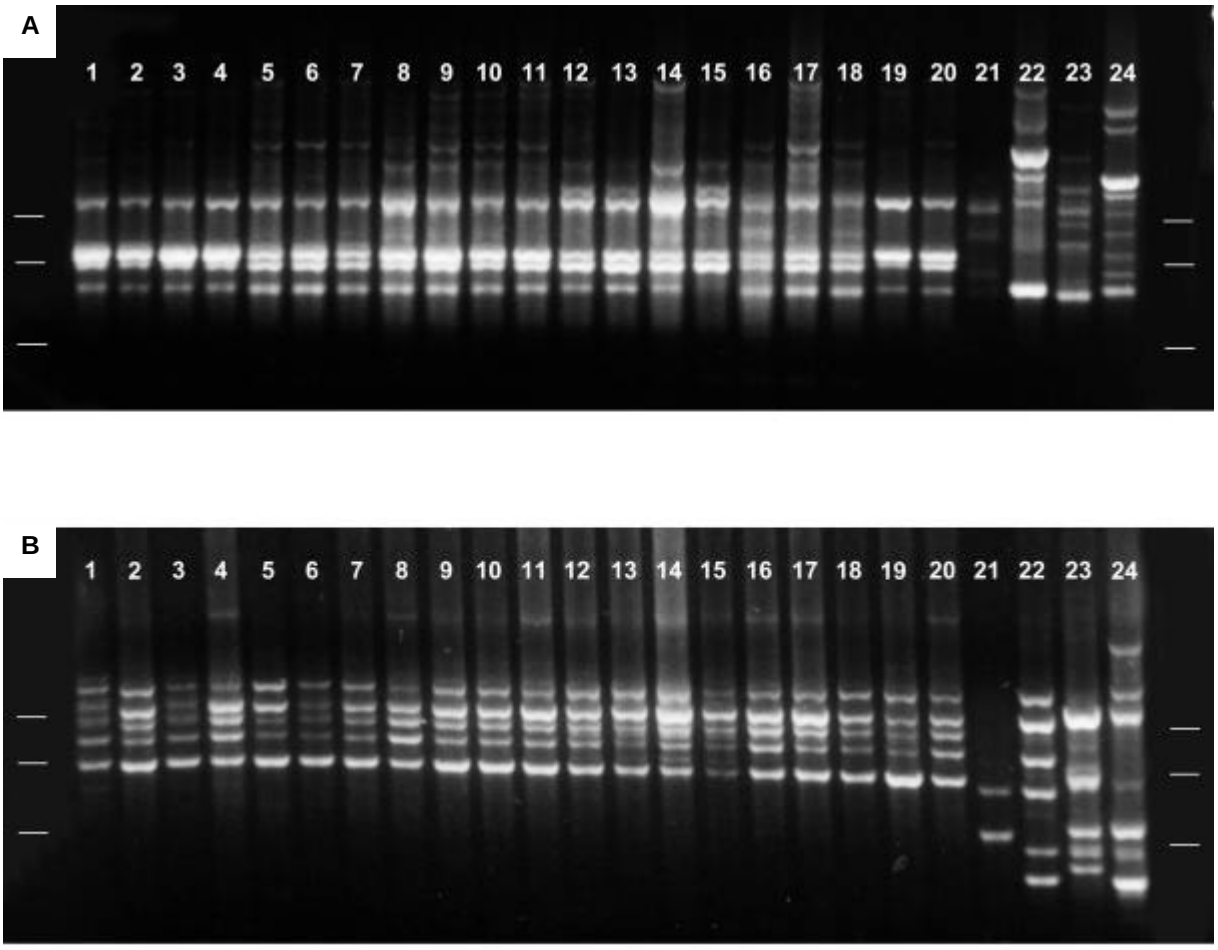
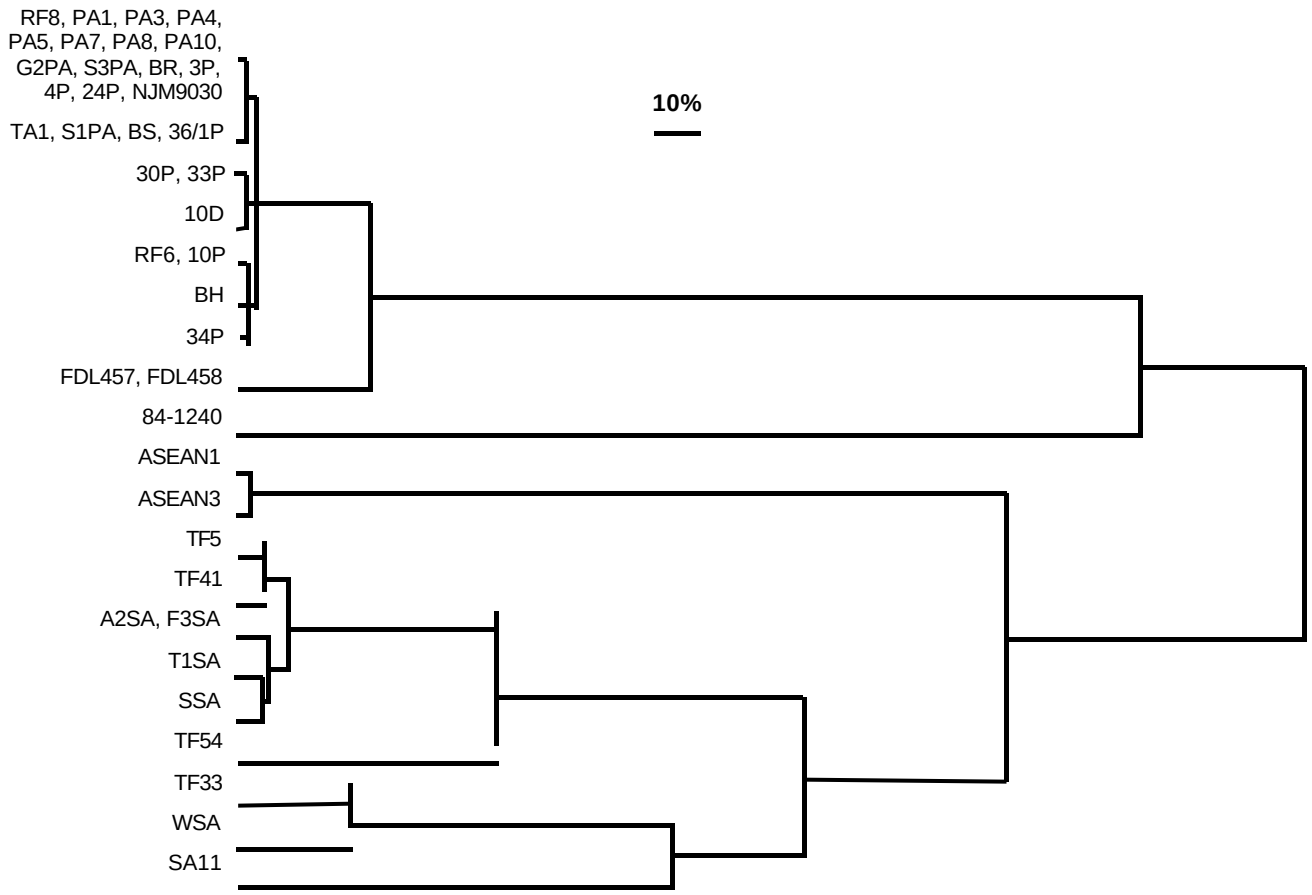


Figure 6. Unweighted group-average cluster analysis of *Aphanomyces* growth data given in Lilley & Roberts (1997)



APPENDIX FIVE

Paper four - Lilley, J.H., Beakes, G.W., and Hetherington, C.S. (2001) Characterization of *Aphanomyces invadans* using pyrolysis mass spectrometry (PyMS). Mycoses 3-4 (in press)

BRIEF COMMUNICATION

Characterisation of *Aphanomyces invadans* isolates using pyrolysis mass spectrometry (PyMS)

Charakterisierung von *Aphanomyces invadans* Isolaten mittels Pyrolysemassenspektrometrie (PyMS)

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Running title:
Pyrolysis mass spectrometry of *Aphanomyces*

Keywords:
Aphanomyces invadans, pyrolysis mass spectrometry, fish disease, epizootic ulcerative syndrome

Schlüsselwörter:
Aphanomyces invadans, Pyrolysemassenspektrometrie, Fischkrankheit, epizootisches ulzeratives Syndrom

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Summary

Twenty-one isolates of *Aphanomyces invadans,* the fungal pathogen associated with the Asian fish disease epizootic ulcerative syndrome (EUS), were compared with other Oomycete fungi in terms of their pyrolysis mass spectrometry (PyMS) profiles. Canonical variate analysis (CVA) of the pyrolysis mass spectra distinguished the *Aphanomyces* species from a wide scatter of *Achlya* and *Saprolegnia* isolates. Further CVA and hierarchal cluster analysis (HCA) separated the *Aphanomyces* species into two main groups. The first group clustered *A. invadans* isolates from EUS outbreaks in Thailand, Bangladesh, Indonesia, Philippines, Australia and Japan together. However, HCA also included the crayfish plague fungus *Aphanomyces astaci* within this group. Non-pathogenic *Aphanomyces* strains isolated from ulcerative mycosis (UM)-affected fish were shown to be distinct from *A. invadans,* and instead clustered with saprophytic *Aphanomyces* strains to form the second group. Recently, an invasive *Aphanomyces* pathogen has been isolated from UM-affected fish, but that was not tested here. This is the first report using PyMS in the study of Oomycete systematics. The technique was not sensitive enough to show any intra-specific differences, but it was considered a useful technique for the discrimination of species where taxonomic relationships are uncertain.

Zusammenfassung

Einundzwanzig Isolate von *Aphanomyces invadans*, einem pilzartigen Krankheitserreger, der mit dem epizootischen ulzerativen Syndrom der asiatischen Fischkrankheit (EUS) assoziiert ist, wurden mit anderen Oomycetenpilzen bezüglich ihrer Profile bei der Pyrolysemassenspektrometrie (PyMS) verglichen. Die Kanonische Varianzanalyse (CVA) der Pyrolysemassenspektren unterschied die Gattung *Aphanomyces* von einer breiten Streuung der Arten *Achlya* und *Saprolegnia*. Eine weitere CVA und eine Hierarchische Blockanalyse (HCA) trennten die Gattung *Aphanomyces* in zwei Hauptgruppen. Die erste Gruppe bündelte die *A. invadans*-Isolate aus EUS-Ausbrüchen in Thailand, Bangladesh, Indonesien, den Philippinen, Australien und Japan. Jedoch schloß die HCA auch den pilzartigen Erreger der Panzerkrebspest, *Aphanomyces astaci,* innerhalb dieser Gruppe mit ein. Es wurde gezeigt, dass nicht pathogene *Aphanomyces*-Stämme, die aus Fischen isoliert worden waren, die mit der ulzerativen Mykose (UM) infiziert waren, sich von *A. invadans* unterschieden. Anstatt zusammen mit saprophytischen *Aphanomyces*- Stämmen als Cluster zu erscheinen, bildeten sie die zweite Gruppe. Vor kurzem wurde ein invasiver *Aphanomyces*-Krankheitserreger aus UM-infizierten Fischen isoliert, aber dieser wurde hier nicht getestet. Dies ist der erste Bericht, der sich mit PyMS in der Erforschung der Oomycetensystematik befasst. Die Technik war nicht empfindlich genug, um zuverlässige intraspezifische Unterschiede aufzuzeigen, aber sie wurde als ein nützliches Instrument für die Unterscheidung der Gattung erachtet, wenn taxonomische Beziehungen ungewiss sind.

Introduction

Aphanomyces invadans (also known as *A. invaderis* and *A. piscicida*) is an Oomycete freshwater fish pathogen, which characterises cases of the Asian fish disease, epizootic ulcerative syndrome (EUS) [1]. As sexual stages have not been identified for this fungus, diagnosis relies on particular molecular, growth and asexual morphology characters [1, 2], and on its ability to invade internal tissues of EUS-

susceptible fish [3]. In this study, pyrolysis mass spectrometry was tested as a method of distinguishing a variety of isolates of *A. invadans* from saprophytic fungi obtained from EUS-affected areas, and from fungi involved in other diseases of aquatic animals.

Pyrolysis mass spectrometry (PyMS) is an analytical technique that can be used to obtain biochemical fingerprints of whole micro-organisms [4]. Briefly, the complex organic material of the sample is thermally degraded (pyrolyzed) in an inert atmosphere. Curie point PyMS, as used here, employs a ferro-magnetic foil as a sample carrier, which is heated and maintained at its Curie point by means of high-frequency alternating magnetic field. The resulting vapour or pyrolysate is bombarded with low-energy electrons, which generate molecular and fragment ions. These are separated by a quadrupole mass spectrometer on the basis of their mass : charge ratio (m/z) and displayed in the form of quantitative mass spectra.

PyMS has been increasingly used in bacterial systematics [5], both to show inter-specific [6] and inter-strain [7] relationships. Strains of a bacterial fish pathogen have also been characterised using this technique [8]. Fungal studies using PyMS have concentrated on yeasts [9, 10] and a few filamentous fungi [11, 12]. Nilsson *et al*. [11] used PyMs to classify *Penicilium* and *Aspergillus* species, although Law *et al*. [12] concluded that it was not a suitable typing method for *Aspergillus* spp. Apart from the taxonomic studies, Weijman *et al.* [13] evaluated PyMS as a method of diagnosing potato-gangrene caused by *Phoma*, and Niemann *et al.* [14] used this technique to demonstrate different levels of lignin degradation in carnations by *Fusarium* and *Phialophora*.

The present study aimed to evaluate PyMS as a method of discriminating different levels of Oomycete taxa: between strains of *A. invadans*, species of *Aphanomyces* and genera of Saprolegniaceae.

Materials and methods

Forty-five fungal isolates were tested as listed in Table 1. Full details of each isolate are given in Lilley & Roberts [2] except for 96PA, an isolate of *A. invadans* from an EUS-affected snakehead (*Channa striata*) sampled in Pichit, Thailand in January 1996; and 84-1249 and 84-1282 from UM-affected menhaden (*Brevoortia tyrannus*) from North Carolina, USA in 1984.

[TABLE 1]

Fungal colonies were grown in Petri dishes of glucose-peptone-yeast (GPY) liquid media [15]. The same batch of GPY was used throughout the experiment. Asian isolates were grown at room temperature (20-24^{o}C) and UK and USA isolates were grown at 12^{o}C. For each isolate, three squares of mycelium, approximately 2mm^2, were cut from the edges of actively growing colonies and used to inoculate a Petri dish containing GPY media. Halfway through the total growth period, the resulting three mycelial mats were again cut into 2mm^2 squares. The total incubation time was calculated from known growth rates of the isolates to produce end wet weights of approximately 0.3g, and ranged

between three days for the saprophytic *Aphanomyces* spp. to eight days for *A. astaci*. Ideally, identical growth conditions should be adopted for each isolate, but in this case, that would result in mycelium at very different stages of growth. The present regime was used so that each fungal culture was rich in actively growing hyphal tips.

The resulting mycelial mat was washed in sterile distilled water and filtered through cheesecloth. This was repeated four times. The mycelial mat was then homogenized in liquid nitrogen and stored at –20 ^{o}C. Preliminary work had shown that unhomogenized, washed mycelium produced less consistent results, possibly because of a lack of uniformity in the age of hyphae within the fungal preparation. Duplicate preparations of four isolates (S1PA, PA7, 10P and WSA) were prepared to check the reproducibility of different preparations of the same isolate.

Thawed homogenated material formed a paste, a small amount of which was smeared thinly on to alloy foils (50% iron : 50% nickel). The foils were inserted into pyrolysis tubes and oven-dried for a few minutes. It was found that excessive drying often resulted in the sample dropping off the foil. Three replicate tubes were prepared from each fungal homogenate. The samples were loaded on a RAPyD 400 pyrolysis mass spectrometer (Horizon Instruments Ltd, Heathfield, East Sussex, England) and pyrolyzed for three seconds at a Curie point of 530^{o}C. Preliminary runs were carried out to evaluate the amount of homogenated material required on the foil to give total ion counts of between three and ten million.

Results and Discussion

Sample mass spectra of two of the most distantly related isolates are given in Fig. 1. The spectra of all isolates showed similar peaks, but peak heights varied between isolates. A mixture of cell contents contribute to each peak, so as in most PyMS studies, no attempt is made to assign specific molecules to particular peaks. The spectra are not used as permanent type descriptions, and for this reason, PyMS is generally not considered a typing technique as such, but is used as a method to rapidly determine relationships of different isolates tested at the same time.

[FIG 1]

The most characteristic masses (i.e. those with the highest between-group variation and lowest within-group variation) were determined, and used for principle component (PC) analysis to maximise discrimination between groups. Canonical variate analysis (CVA) was then used to group the samples on the basis of the retained PCs. CVA generated eigenvectors for each of the three replicate samples. These were averaged and plotted on two 2-D scatter plots. Fig. 2 shows that the *Aphanomyces* species could be distinguished from most of the *Achlya* and *Saprolegnia* outgroup species. Further CVA using the *Aphanomyces* species alone (Fig. 2) succeeded in discriminating *A. invadans* from the saprophytic strains.

[FIGS 2 & 3]

A dendrogram produced by group-average hierarchal cluster analysis (HCA) (Fig. 4) gave a more clear-cut distinction between *A. invadans* and the saprophytes by forming two main groups separated at a similarity index of 61%.

[FIG. 4]

The first main group clustered all the *A. invadans* isolates from EUS/MG outbreaks together. Other recent studies have described similarities in morphology, culture characteristics, pathogenicity, genetic fingerprints and peptide banding profiles, which indicated that these fungi all represent a single species [2, 3, 16]. The PyMS evidence tended to support this hypothesis, although two isolates (33P and 4P) clustered with the main group at a much lower similarity index. Also of interest was the proximity that the crayfish plague fungus, *Aphanomyces astaci,* showed to the *A. invadans* group.

Aphanomyces strains associated with ulcerative mycosis (UM) were shown to be distinct from *A. invadans,* and instead clustered with saprophytic *Aphanomyces* to form the second group. This is consistent with the suggestion by Dykstra *et al.* [17] that these isolates may be *Aphanomyces laevis,* and with pathogenicity studies which have shown them to be non-invasive in EUS-susceptible fish [2]. Recently, an invasive *Aphanomyces* pathogen has been isolated from UM-affected fish [18], but that was not tested here.

Duplicate cultures of one isolate (WSA) clustered together at only 91% similarity, indicating that any relationships at this level or above were unreliable. Therefore, PyMS probably lacks the sensitivity to resolve intra-specific differences in these fungi. Indeed, the isolates representing some species, *A. astaci* and *A. laevis* in particular, failed to group convincingly. Law *et al.* [12] suggested that PyMS is less reproducible with filamentous fungi than with bacteria because of the non-homologous and more complex nature of fungal colonies. However, in the present study, the system was able to cluster the *A. invadans* isolates, and discriminate them from the non-pathogenic *Aphanomyces* species.

Acknowledgements

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Table 1. Description of fungal strains used in this study

Isolates	Description
TA1, RF6, RF8, S1PA, S3PA, G2PA, PA1, PA7, 96PA	*Aphanomyces invadans* from epizootic ulcerative disease (EUS)-affected fish in Thailand
BH, BR, BS	*A. invadans* from EUS-affected fish in Bangladesh
36/1P	*A. invadans* from EUS-affected fish in Indonesia
10D, 33P, 34P	*A. invadans* from EUS-affected fish in the Philippines
3P, 4P, 10P, 24P	*A. invadans* from EUS-affected fish in Australia
NJM9030	*A. invadans* (=*A. piscicida*) from EUS (=MG[1])-affected fish in Japan
84-1240 (=ATCC 62427), 84-1249, 84-1282	*Aphanomyces* sp. from ulcerative mycosis (UM)-affected fish in the USA
FDL457, FDL458	*Aphanomyces astaci* from plagued crayfish in the UK
TF5, TF33, TF41, F3SA, SSA, SA11	*Aphanomyces* sp. saprophytes from EUS-affected fish in Thailand
T1SA	*Aphanomyces* sp. saprophyte from a diseased turtle in Thailand
ASEAN1, ASEAN3	*Aphanomyces laevis* saprophytes from EUS-affected pond water in Thailand
A2SA, WSA	*Aphanomyces* sp. saprophytes from EUS-affected pond water in Thailand
W2BAC	*Achlya diffusa* saprophyte from EUS-affected pond water in Thailand
S2AC	*Achlya* sp. saprophytes from EUS-affected fish in Thailand
TF20S, TF29	*Saprolegnia* sp. saprophytes from EUS-affected fish in Thailand
S.AUST	*Saprolegnia australis* saprophyte from a fish in the UK
P32	*Saprolegnia ferax* saprophyte from lake water in the UK
E3 (=ATCC 36144)	*Saprolegnia diclina* saprophyte from lake water in the UK
TP41 (=ATCC 42062)	*Saprolegnia parasitica* from a diseased fish in the UK

[1]Mycotic granulomatosis

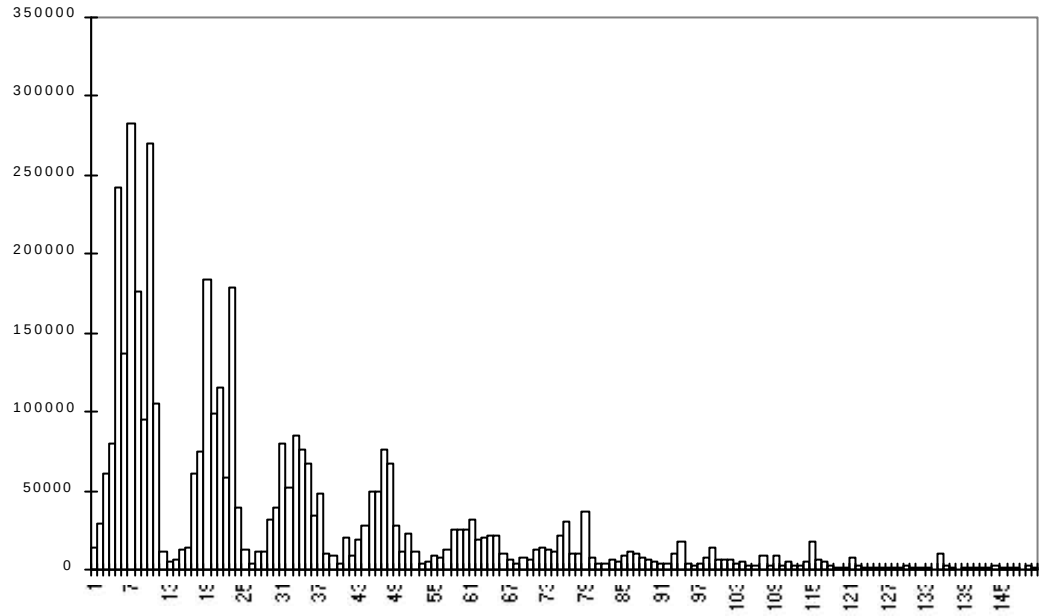
Fig 1 Sample mass spectra for (a) *Aphanomyces invadans* isolate RF8 and (b) *Saprolegnia diclina* isolate E3 (the averages of three replicates are shown)

Fig. 2 Canonical variate analysis (CVA) of pyrolysis mass spectra of all fungus isolates. Markers represent the average of three replicates.

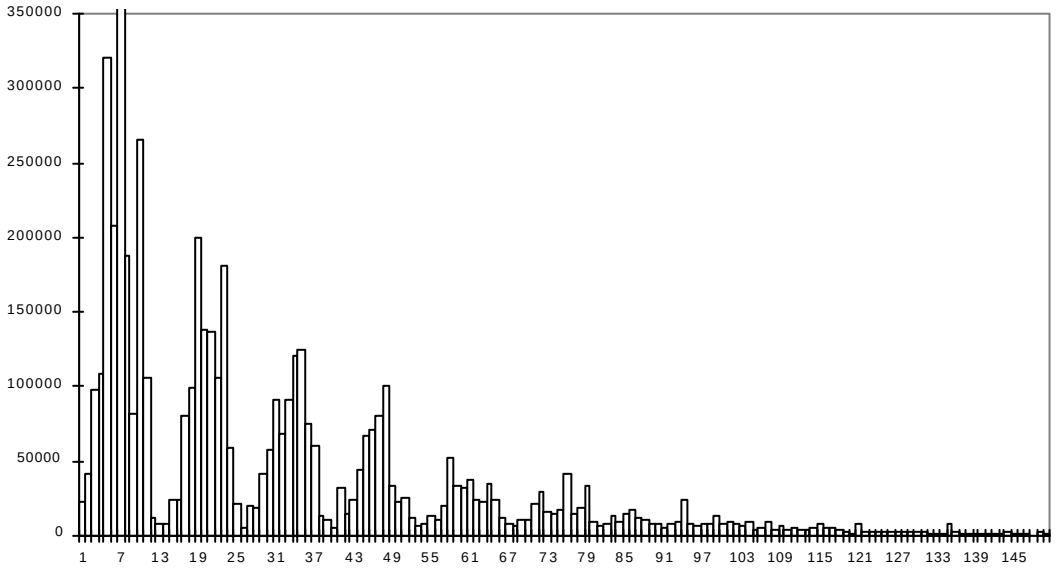
Fig. 3 Canonical variate analysis (CVA) of pyrolysis spectra of *Aphanomyces* species only. Markers represent the average of three replicates.

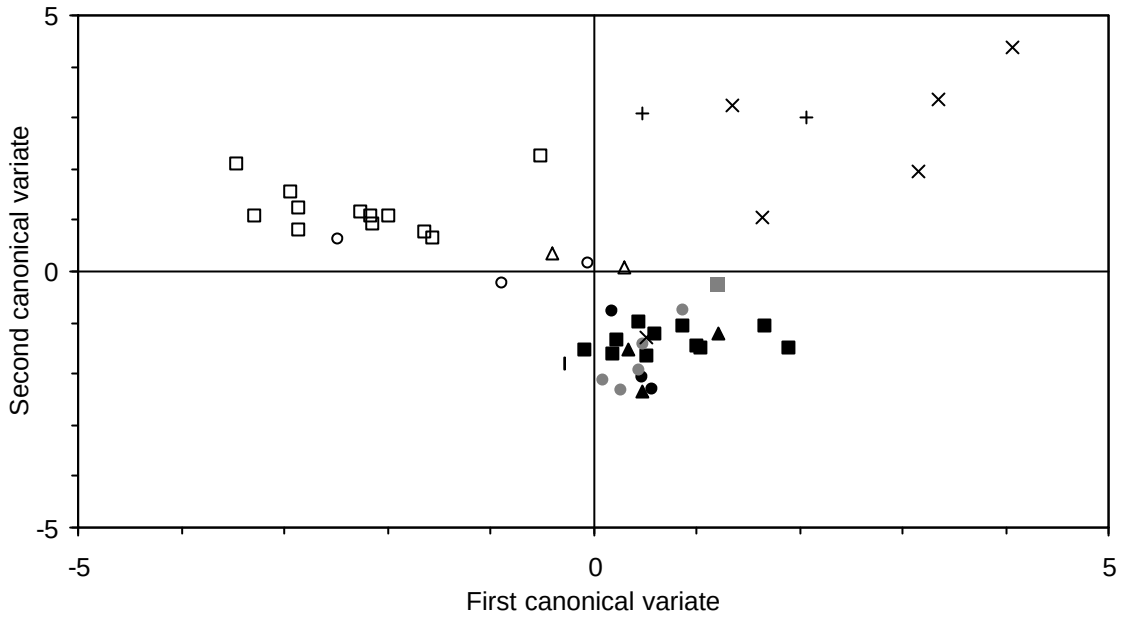
Fig. 4 Hierarchal cluster analysis (HCA) dendrogram of same data set as in Figure 3. Includes *Aphanomyces invadans* from EUS outbreaks in Thailand (EUS-Thai), Bangladesh (EUS-Bang), Indonesia (EUS-Indo), Philippines (EUS-Phil), Australia (EUS-Aus), and Japan (EUS/MG), *Aphanomyces astaci* (*astaci*), UM-*Aphanomyces* (UM) and various saprophytic *Aphanomyces* species (Sap.). S1PA[(2)], PA7[(2)], 10P[(2)] and WSA[(2)] are duplicate cultures of S1PA, PA7, 10P and WSA respectively.

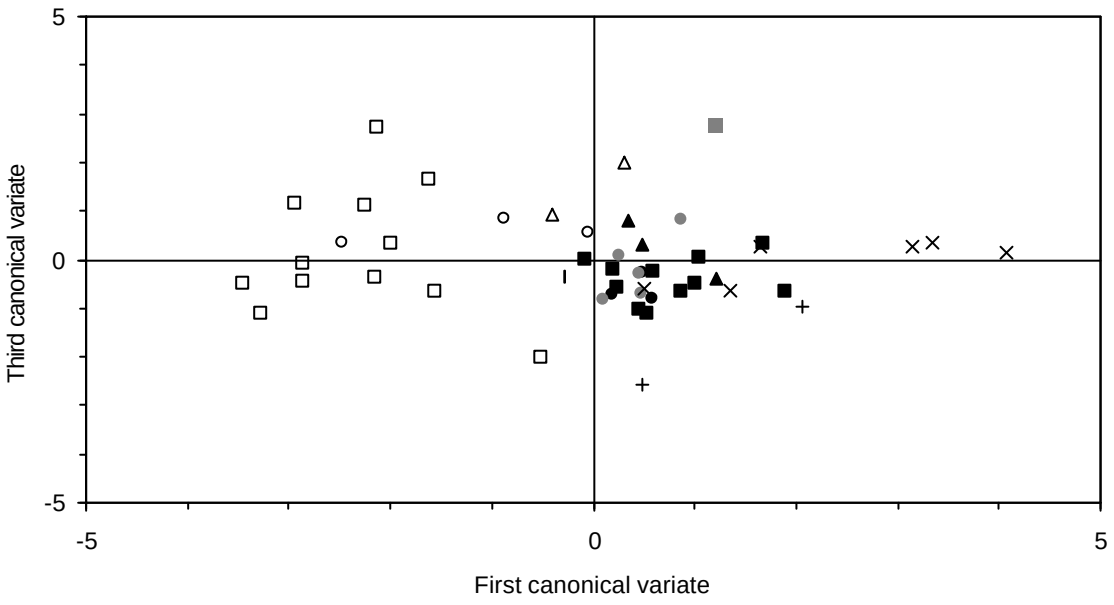
ion count



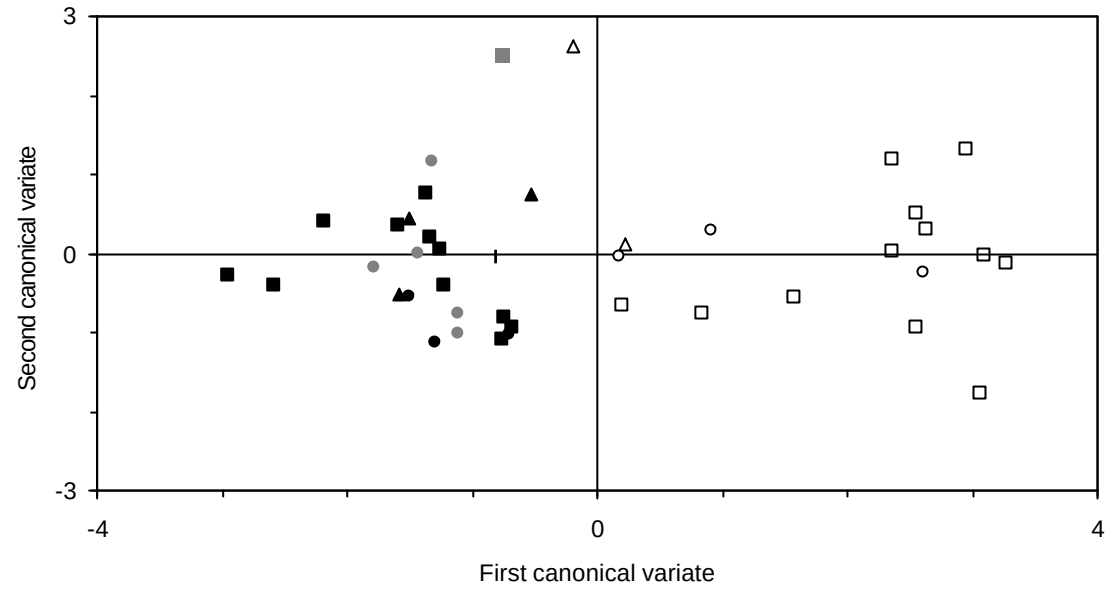
ion count

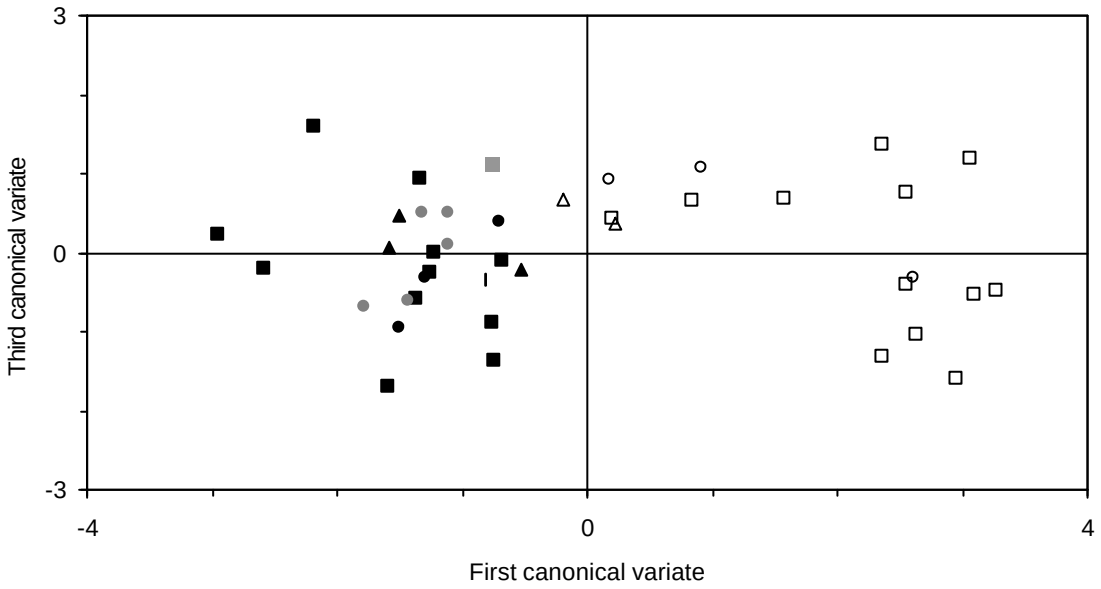






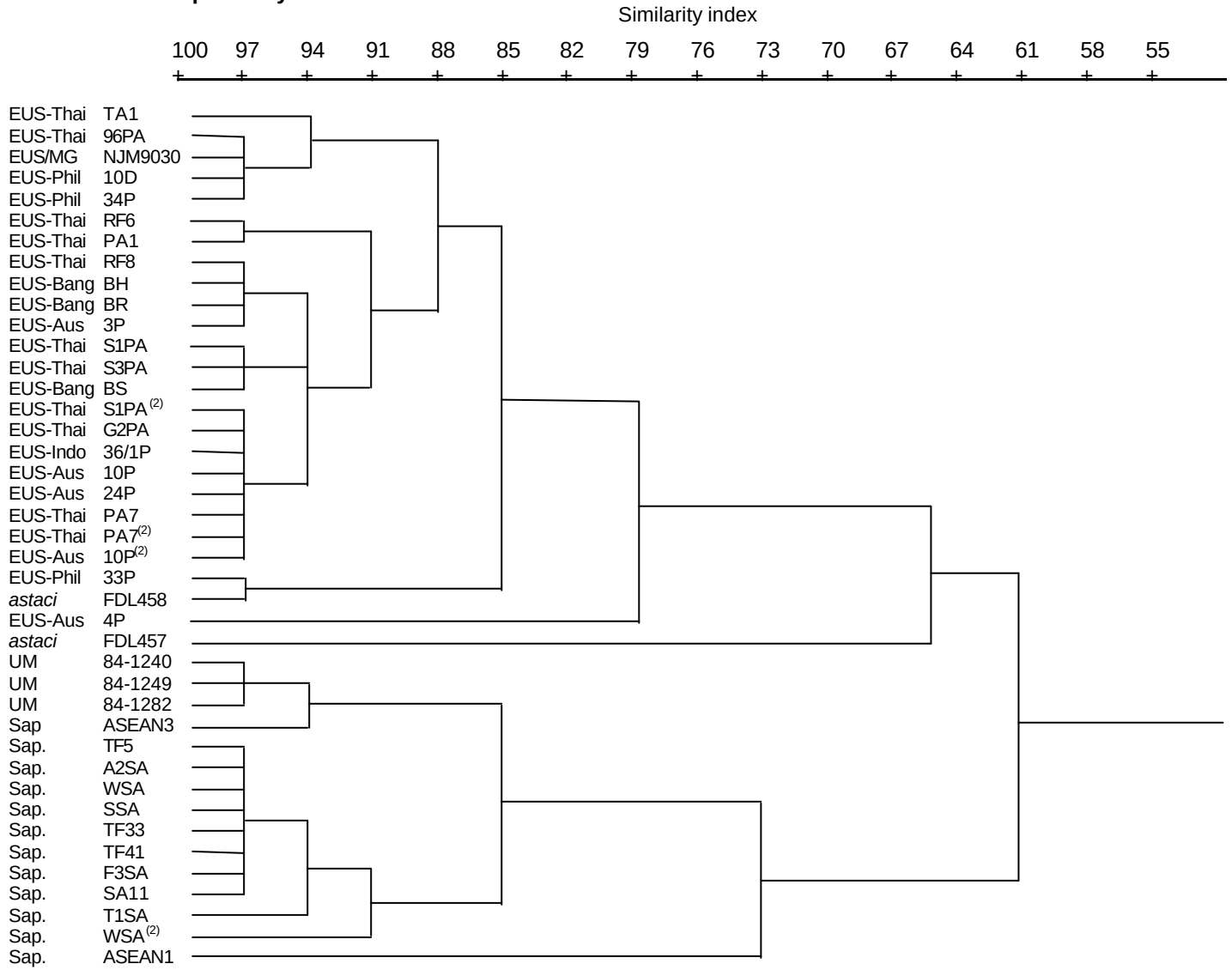
- EUS-Thailand
- EUS-Bangladesh
- EUS-Philippines
- EUS-Indonesia
- EUS-Australia
- EUS/MG-Japan
- A. astaci
- UM-Aphanomyces
- Aphanomyces saprophytes
- Achlya spp
- Saprolegnia spp





- EUS-Thailand
- EUS-Bangladesh
- EUS-Philippines
- EUS-Indonesia
- EUS-Australia
- EUS/MG-Japan
- A. astaci
- UM-Aphanomyces
- Aphanomyces saprophytes

Figure 4 Hierarchal cluster analysis (HCA) dendrogram of the same data set as in Figure 3. Includes *Aphanomyces invadans* from EUS outbreaks in Thailand (EUS-Thai), Bangladesh (EUS-Bang), Indonesia (EUS-Indo), Philippines (EUS-Phil), Australia (EUS-Aus), and Japan (EUS/MG); *Aphanomyces astaci* (*astaci*); UM-*Aphanomyces* (UM); and various saprophytic *Aphanomyces* species (Sap.). S1PA$^{(2)}$, PA7$^{(2)}$, 10P$^{(2)}$ and WSA$^{(2)}$ are duplicate cultures of S1PA, PA7, 10P and WSA respectively.



APPENDIX SIX

Paper five - Panyawachira, V., Lilley, J.H., and Chinabut, S. (2001) Comparison of histological techniques for the diagnosis of epizootic ulcerative syndrome (EUS). Journal of Fish Diseases. (in preparation, selected results shown, hard copy of figures available from ARP Manager)

TABLE 1 HISTOLOGY RESULTS FOR EUS-AFFECTED FISH

SAMPLE	natural EUS +ve gourami from Chainat, Thailand	natural EUS +ve Anabas from Chainat, Thailand	natural EUS +ve Anabas from Chainat, Thailand	artificial EUS +ve snakehead, Bangsai	artificial EUS +ve snakehead, Bangsai	artificial EUS +ve snakehead, Bangsai
CODE	D99006 1/1	D99009/3	D99010 4/4	R0004 (3,16)	R0004 (3,17)	R0004 (3,18)
SOURCE	AAHRI	AAHRI	AAHRI	AAHRI	AAHRI	AAHRI
Grocott	fungus✓✓ granuloma✓✓ staining intensity ✓✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓ granuloma✓✓ staining intensity ✓✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓
Uvitex + H&E	fungus✓✓ (mainly in the dermis) granuloma✓✓ staining intensity ✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓✓ (wide hyphae) granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓ (not deep in muscle) granuloma✓✓ staining intensity ✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓
Periodic-Acid-Schiff (PAS)	fungus✓ granuloma✓✓ staining intensity ✓✓	fungus✓✓ granuloma✓✓✓ staining intensity ✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓	fungus✓✓ granuloma✓✓ staining intensity ✓	fungus✓✓✓ granuloma✓✓✓ staining intensity ✓✓✓
PCR of *A. invadans* DNA from PET with APH3 & APH4, (Marchetti *et al*, 1998)	-	-	-	faint band✓	faint band✓	faint band✓
PCR of *A. invadans* DNA from PET with APH3 & APH4 (Qiagen kit)	-	band ✓✓	-	-	no band X	-
***In situ* hybridisation with APH3& APH4**	-	-	-	no reaction X	no reaction X	no reaction X
Polyclonal anti-*A.invadans* IHC	-	-	-	no reaction X	no reaction X	no reaction X
Monoclonal anti-*A.invadans* IHC	no reaction X	no reaction X	-	no reaction X	-	-

KEY

X	Negative
✓	Positive - low amount
✓✓	Positive - medium amount
✓✓✓	Positive - high amount
PET	Paraffin-embedded tissue
IHC	Immunohistochemistry
-	Not done

TABLE 2 HISTOLOGY RESULTS FOR NON-EUS-AFFECTED FISH

SAMPLE	Saprolegnia, channel catfish (other saprolegniaceae, fungus)	Lagenidium (non-saprolegniaceae, Oomycete fungus) infection of dog	Paeciliomyces farinosus (fungus only), Scotland (non-Oomycete fungus)	Exophiala, salmon, Shetlands, Scotland (non-Oomycete fungus)	Branchiomyces, channel catfish gills	Branchiomyces, channel catfish gills
CODE	**S00-007Skin**	**99R12821**	**D000096**	**R970036B**	**S99-1070-1**	**S99-1070-2**
SOURCE	**Dr Lester Khoo (MSU) via John Hawke & Amy Grooters, (LSU)**	**Amy Grooters via John Hawke, Louisiana State U.**	**Mark Freeman, Stirling**	**Richard Collins, Stirling**	**Lester Khoo, Mississippi State U.**	**Lester Khoo, Mississippi State U.**
Grocott	fungus ✓ (outside skin) granuloma X staining intensity ✓	fungus ✓✓✓ acute inflammation staining intensity ✓✓✓	staining intensity ✓	fungus ✓✓ (thin, septate) granuloma ✓✓✓ (filled in, unlike EUS) staining intensity ✓✓✓	fungus ✓✓ (in gills) granuloma X staining intensity ✓✓✓	fungus ✓✓✓ (in gills) granuloma X staining intensity ✓✓
Uvitex + H&E	fungus ✓✓ (only skin) granuloma X staining intensity ✓✓	fungus ✓✓✓ acute inflammation staining intensity ✓✓✓	staining intensity ✓	staining intensity X	staining intensity ✓	staining intensity ✓
Periodic-Acid-Schiff (PAS)	fungus ✓✓ granuloma X staining intensity ✓	fungus ✓✓ ✓ acute inflammation staining intensity ✓✓	staining intensity ✓✓✓	fungus ✓✓ granuloma ✓✓ staining intensity ✓✓	fungus ✓✓ (wide fungus) granuloma X staining intensity ✓✓✓	fungus ✓✓ granuloma X staining intensity ✓
Ziehl-Neelsen (for acid-fast bacteria)	-	-	negative X	negative X	-	-
Monoclonal anti-*A.invadans* IHC	-	-	-	+ve reaction with fungus ✓	-	-

KEY

X	Negative
✓	Positive - low amount
✓✓	Positive - medium amount
✓✓✓	Positive - high amount
PET	Paraffin-embedded tissue
IHC	Immunohistochemistry
-	Not done

TABLE 3 HISTOLOGY RESULTS FOR FISH WITH INFECTIONS OF UNCERTAIN RELATIONSHIP TO EUS

SAMPLE	UM +ve menhaden, Pocomoke River, USA, 1997	UM +ve menhaden	UM +ve menhaden	invasive Oomycete of channel catfish	invasive Oomycete of channel catfish	fungus infection, Murray cod, Australia	fungus infection, Murray cod, Australia
CODE	99-3 (1-1)	Z92-3067A	Z92-3070B	00F-127A	00F-127B	V001919B	V002170B
SOURCE	V. Blazer	E. Noga	E. Noga	J. Hawke & A. Grooters, Louisiana State U.	J. Hawke & A. Grooters, Louisiana State U.	Ruth Reuter, South Australia	Ruth Reuter, South Australia
Grocott	fungus ✓✓✓ granuloma ✓✓ staining intensity ✓✓✓	fungus ✓✓ granuloma ✓✓ staining intensity ✓✓✓	fungus ✓ granuloma ✓✓ (large) staining intensity ✓✓✓	fungus ✓✓✓ acute inflammation, giant cells staining intensity ✓✓✓	fungus ✓✓✓ acute inflammation, giant cells staining intensity ✓✓✓	fungus ✓✓✓ no inflammation staining intensity ✓✓	fungus X granuloma ✓ staining intensity X
Uvitex + H&E	fungus ✓✓✓ granuloma ✓✓✓ staining intensity ✓	fungus ✓✓ (not always in middle of granuloma) granuloma ✓✓✓ (very large) staining intensity ✓	fungus ✓✓ granuloma ✓✓✓ staining intensity ✓	fungus ✓✓✓ acute inflammation, giant cells staining intensity ✓✓✓	fungus ✓✓✓ acute inflammation, giant cells staining intensity ✓✓✓	fungus ✓✓✓ (internal & external) muscle degeneration, very little inflammation staining intensity ✓✓ (variable)	fungus X inflammation granuloma ✓
Periodic-Acid-Schiff (PAS)	fungus ✓✓ granuloma ✓✓ staining intensity ✓	fungus ✓✓ granuloma ✓✓ staining intensity ✓✓	fungus ✓ granuloma ✓✓ staining intensity ✓✓	fungus ✓✓✓ acute inflammation, giant cells staining intensity ✓✓✓	fungus ✓✓✓ acute inflammation, giant cells staining intensity ✓✓✓	fungus ✓✓ granuloma X staining intensity ✓	fungus X granuloma ✓ staining intensity X
Ziehl-Neelsen (for acid-fast bacteria)	negative X	negative X	negative X	-	-	negative X	negative X
PCR of *A. invadans* DNA from PET with APH3& APH4 (Qiagen kit)	no band X	-	-	-	-	no band X	-
Monoclonal anti-*A.invadans* IHC	-	-	-	fungus visible, but no Mab reaction X	fungus visible, but no Mab reaction X	-	-

KEY

X	Negative
✓	Positive - low amount
✓✓	Positive - medium amount
✓✓✓	Positive - high amount
PET	Paraffin-embedded tissue
IHC	Immunohistochemistry
-	Not done

TABLE 4 HISTOLOGY RESULTS FOR FISH WITH INFECTIONS OF UNCERTAIN RELATIONSHIP TO EUS

SAMPLE	Vietnamese redspot(?)	Vietnamese redspot(?)	Vietnamese redspot(?)	Vietnamese redspot(?)	Toxotes with fungal granuloma, Bangsai, Thailand
CODE	F00/4/1	F20/5/4	F20/15/1	F20/14/2	D00011
SOURCE	Phan Thi Van, RIA1	Phan Thi Van, RIA1	Phan Thi Van, RIA1	Phan Thi Van, RIA1	AAHRI
Grocott	fungus ✓ (only epidermis, not distinct) granuloma ✓ (not EUS) staining intensity ✓ muscle degeneration X	fungus X granuloma X staining intensity X muscle degeneration X	fungus ✓ granuloma? staining intensity ✓✓ muscle degeneration ✓	fungus ✓✓ granuloma ✓ staining intensity ✓✓✓ muscle degeneration ✓	-
Uvitex + H&E	fungus ✓ (only epidermis, not distinct) granuloma ✓ staining intensity ✓	fungus X granuloma X staining intensity X	fungus ✓ granuloma ✓ staining intensity ✓✓✓	fungus ✓✓ granuloma ✓ staining intensity ✓✓✓	fungus ✓✓ (not in all granulomas) granuloma ✓✓✓ (large, unlike EUS) staining intensity ✓✓✓
Periodic-Acid-Schiff (PAS)	fungus X granuloma ✓ staining intensity X	fungus X granuloma X staining intensity X	fungus ✓ granuloma X staining intensity ✓	fungus ✓✓ granuloma ✓ staining intensity ✓	fungus ✓✓ granuloma ✓✓✓ (large, unlike EUS) staining intensity ✓✓
Ziehl-Neelsen (for acid-fast bacteria)	-	-	-	-	negative X
Monoclonal anti-*A.invadans* IHC	-	-	negative X	-	-

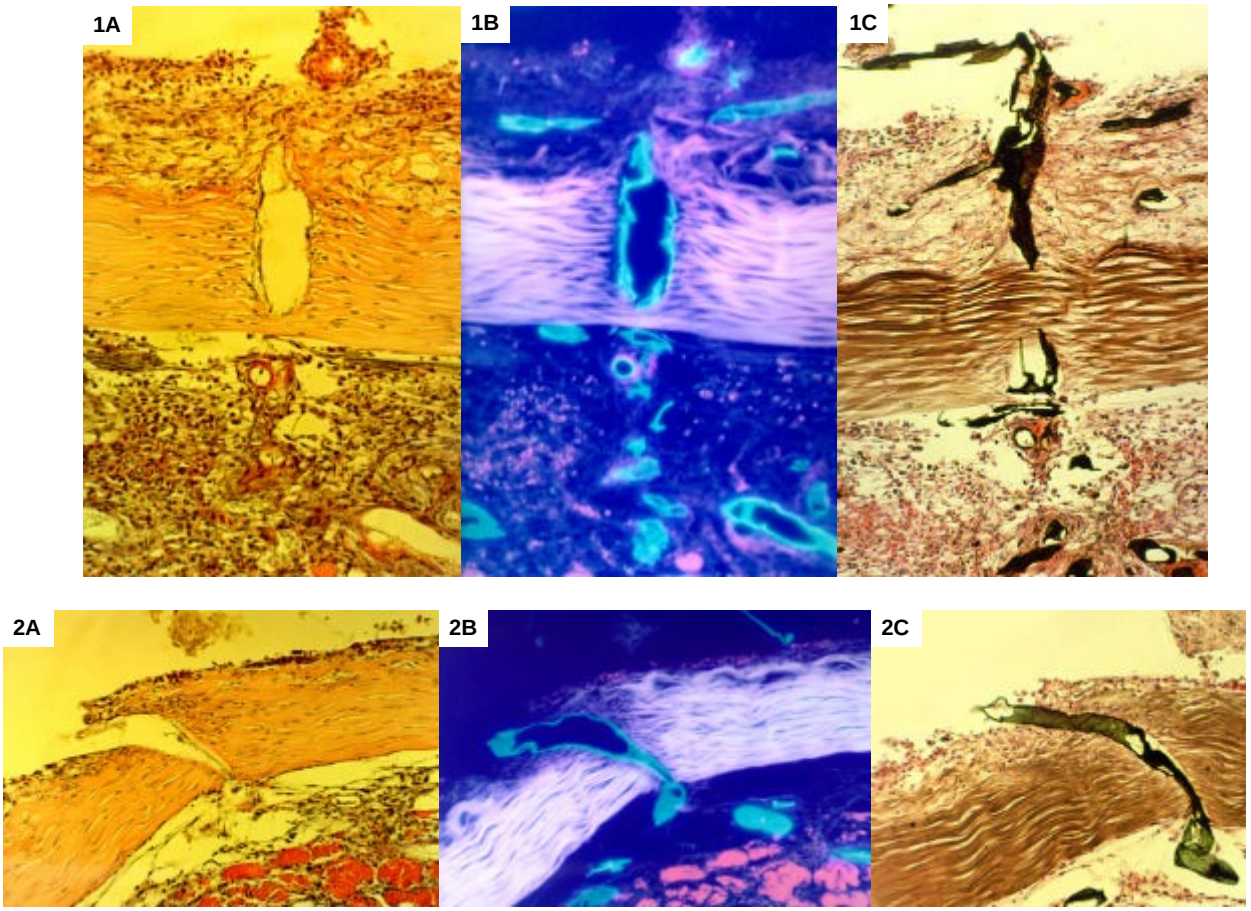
KEY

X	Negative
✓	Positive - low amount
✓✓	Positive - medium amount
✓✓✓	Positive - high amount
PET	Paraffin-embedded tissue
IHC	Immunohistochemistry
-	Not done

TABLE 5 ADVANTAGES AND DISADVANTAGES OF STAINING TECHNIQUES TESTED

TECHNIQUE	Advantages	Disadvantages
H&E	• Relatively easy • Widely available • Stains granulomas well	• Does not stain fungus
Grocott	• Good staining of fungi	• Expensive • Time consuming
Uvitex	• Good staining of fungi • Very rapid • Easily combined with H&E staining	• Not widely available • Requires fluorescent microscope
Periodic-Acid-Schiff (PAS)	• Relatively quick	• Poor staining of fungi
PCR of *A. invadans* DNA from PET with APH3 & APH4 (Marchetti *et al*., 1998)		• Not successful
PCR of *A. invadans* DNA from PET with APH3 & APH4 (Qiagen kit)	• Specific for *A. invadans*	• Requires PCR equipment • Rapidly uses up the entire histology block • Cannot visualise in situ • Not reliable • Cannot use with sample fixed with Bouin's or Davidson's, or with decalcified samples
***in situ* hybridisation with APH3& APH4**		• Not successful
Polyclonal anti-*A.invadans* IHC	• Fair staining of Oomycete fungi	• Limited supply • Can not be easily transported • Deteriorates over time
Monoclonal anti-*A.invadans* IHC	• Continuous supply of antibody	• Poor affinity to fungi • Can not be easily transported

DIAGNOSIS OF EUS USING H&E, UVITEX AND GROCOTT HISTOLOGY



Figs 1 & 2. *Aphanomyces invadans* penetrating skin of bath-challenged puntius
(A. H&E, B. Uvitex, C. Grocott's)

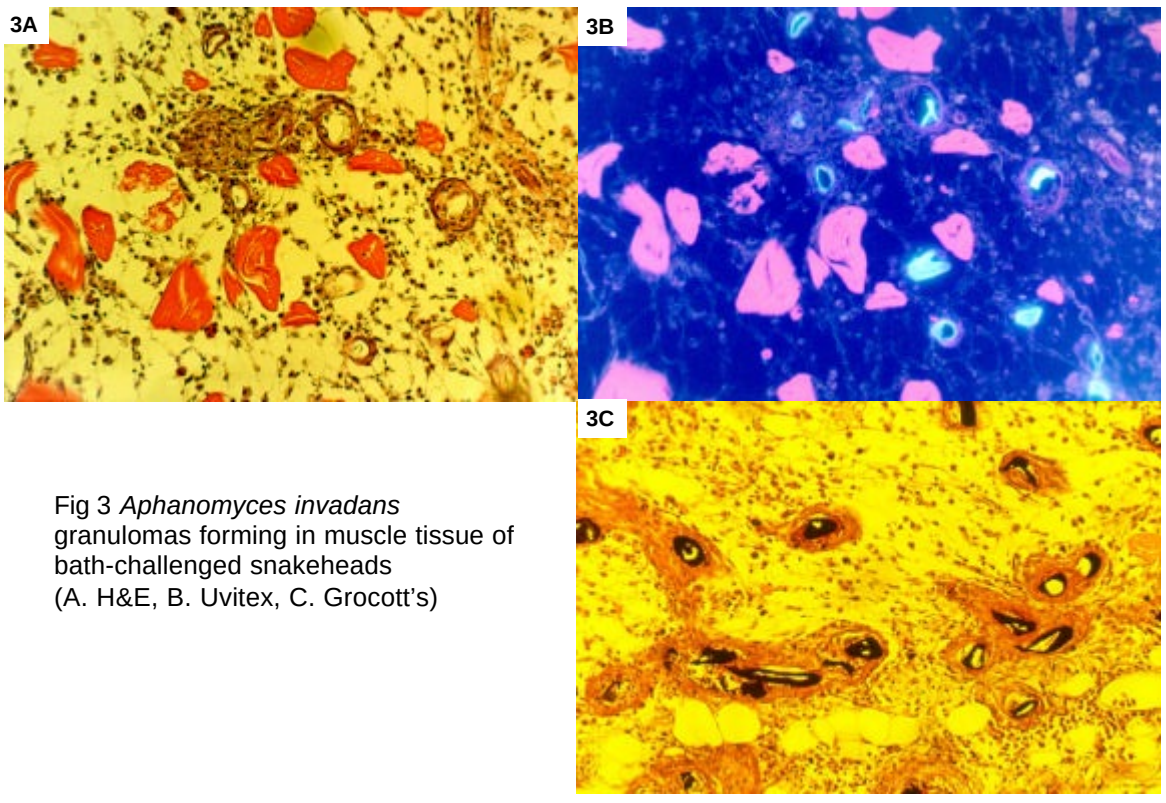


Fig 3 *Aphanomyces invadans*
granulomas forming in muscle tissue of
bath-challenged snakeheads
(A. H&E, B. Uvitex, C. Grocott's)

APPENDIX SEVEN

Paper six - Lilley, J.H., Khan, M.H. and Chinabut, S. (2001) Prevalence of epizootic ulcerative syndrome in wild and cultured fish in Bangladesh. Journal of Applied Ichthyology. (in preparation, selected results shown, hard copy of figures available from ARP Manager)

Table 1 Prevalence of lesions on 34612 wild fish in Bangladesh

(calculated from unpublished FAP17 survey data 1992-4, arranged in order of % with lesions)

Species	No. examined	% affected	Severity index
Ailia coila	18	66.7%	X
Channa striata	315	65.1%	XXXX
Mastacembelus armatus	187	55.6%	XXXX
Mystus vittatus	2603	53.9%	XXX
Anabas testudineus	130	52.3%	XXX
Puntius sarana	6	50.0%	*
Wallagu attu	17	47.1%	XX
Ailia punctata	11	45.5%	X
Rhinomugil corsula	27	44.4%	XXXX
Mystus tengara	796	44.1%	XXX
Nandus nandus	154	40.9%	XXXX
Cirrhinus ariza	5	40.0%	*
Puntius chola	712	38.8%	XXX
Puntius terio	50	34.0%	XXXXX
Puntius sophore	12368	33.4%	XXX
Colisa lalia	12	33.3%	XXX
Puntius ticto	122	31.1%	XXX
Channa punctata	1614	30.7%	XXX
Macrognathus aculeatus	231	26.4%	XXX
Mystus cavasius	631	23.5%	XX
Gangra viridescens	312	23.1%	XXX
Channa marulius	123	21.1%	XXX
Ompok pabda	15	20.0%	XXX
Clarias batrachus	20	20.0%	X
Puntius conchonius	986	19.5%	XXX
Colisa fasciatus	829	19.3%	XX
Salmostoma bacaila	47	19.1%	X
Notopterus notopterus	43	18.6%	XXX
Hyporhampus quoyi	6	16.7%	*
Tetraodon cutcutia	49	16.3%	XX
Xenentodon cancila	170	15.9%	XX
Pseudeutropius atherinoides	28	14.3%	XXX
Trichogaster chuna	170	13.5%	XX
Mystus bleekeri	678	13.3%	XXX
Chela cachius	8	12.5%	*
Lepidocephalichthys guntea	723	10.1%	XX
Osteobrama cotio cotio	183	9.3%	XXX
Parambassis baculis	1886	8.5%	XX
Heteropneustes fossilis	652	7.5%	XX
Macrognathus pancalus	1562	7.5%	XX
Acanthocobitis botia	70	5.7%	XX
Rasbora daniconius	190	5.3%	X
Glossogobius giurus	1337	4.1%	XX
Gudusia chapra	252	4.0%	XX
Amblypharyngodon mola	244	3.7%	XXX
Esomus danricus	57	3.5%	*
Chanda nama	779	3.3%	XX
Sperata seenghala	300	3.0%	X
Salmostoma phulo	541	3.0%	XX
Trichogaster labiosus	35	2.9%	*
Parambassis ranga	873	2.6%	X
Badis badis	41	2.4%	*
Corica soborna	221	0.5%	*
Puntius gelius	460	0.4%	*
Rama chandramara	5	0.0%	
Gagata cenia	7	0.0%	
Sicamugil cascasia	9	0.0%	
Botia dario	10	0.0%	
Danio devario	13	0.0%	
Pseudapocryptes elongatus	13	0.0%	
Brachygobius nunus	15	0.0%	
Labeo boga	18	0.0%	
Johnius coitor	23	0.0%	
Chaca chaca	26	0.0%	
Ichthyocampus carce	36	0.0%	
Somileptes gongota	44	0.0%	
Neoeucirrhichthys maydelli	48	0.0%	
Pellona ditchela	85	0.0%	
Puntius phutunio	361	0.0%	
Total / Average %	34612	17.5%	

*Too few infected samples (≤3) to calculate severity

Table 2 Prevalence of lesions and EUS on 6433 wild fish in Bangladesh
(from cross-sectional survey 1998-9, arranged in order of % with lesions)

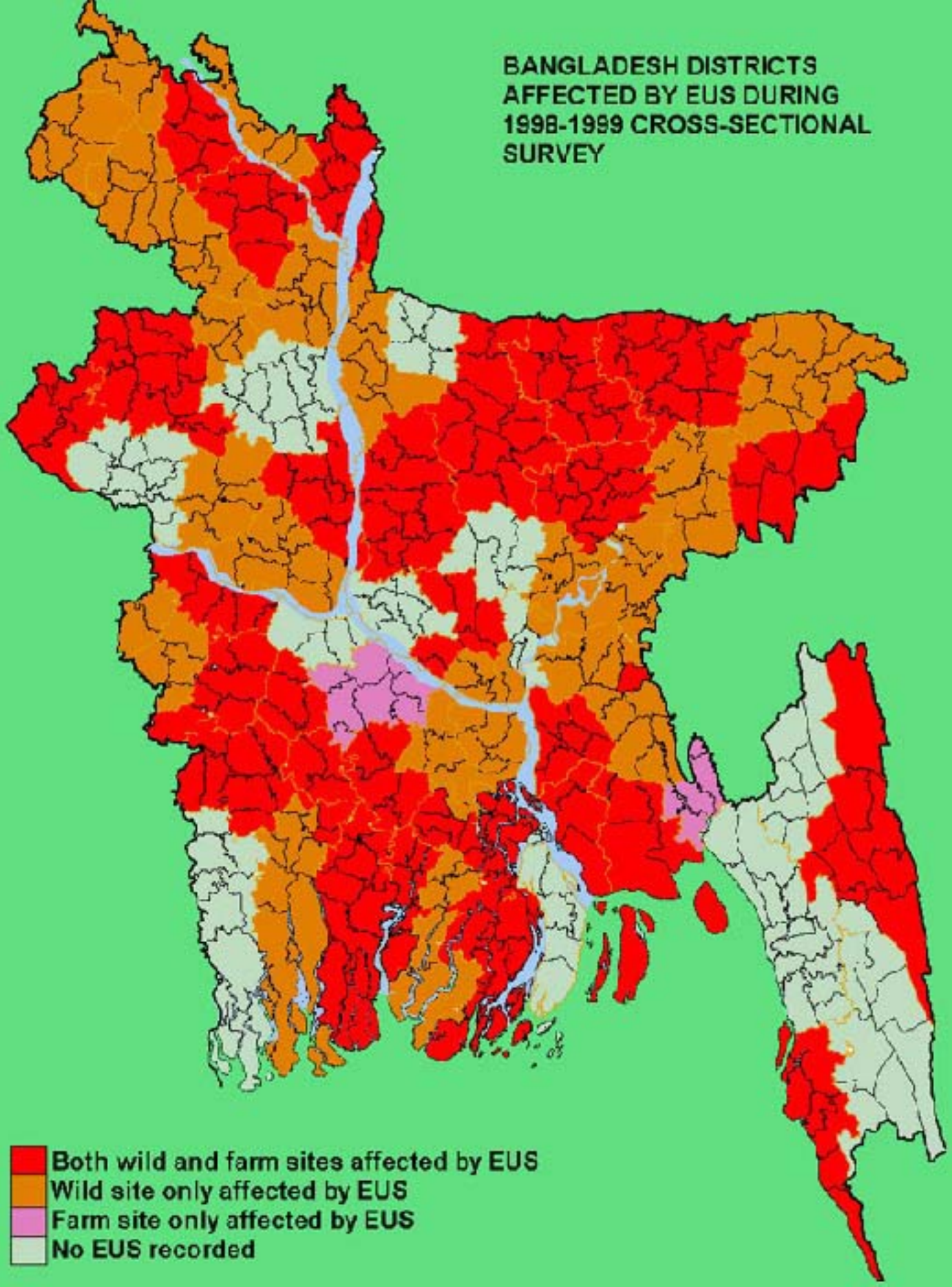
Species	Number (and %) of fish examined	Number (and %) of fish with lesions	No. EUS-positive / No. sampled for histology (% of sampled fish EUS-positive)
Tetraodon fluviatilis	13 (0.2%)	4 (30.8%)	0/1 (0%)
Channa marulius	20 (0.3%)	6 (30.0%)	5/5 (100%)
Glossogobius spp.	8 (0.1%)	2 (25.0%)	2/2 (100%)
Ctenopharyngodon idellus	8 (0.1%)	2 (25.0%)	0/1 (0%)
Channa striata	321 (5.0%)	80 (24.9%)	29/35 (82.9%)
Channa punctata	740 (11.5%)	175 (23.7%)	40/46 (87.0%)
Anabas testudeneus	329 (5.1%)	76 (23.1%)	25/28 (89.3%)
Rita rita	13 (0.2%)	3 (23.1%)	0/1 (0%)
Puntius ticto	834 (13.0%)	165 (19.8%)	28/32 (87.5%)
Wallago attu	41 (0.6%)	8 (19.5%)	5/5 (100%)
Channa orientalis	168 (2.61%)	31 (18.5%)	14/18 (77.8%)
Cirrhinus cirrhosus	180 (2.8%)	32 (17.8%)	13/16 (81.3%)
Trichogaster chuna	17 (0.3%)	3 (17.7%)	2/2 (100%)
Puntius sophore	672 (10.5%)	118 (17.6%)	22/24 (91.7%)
Mastacembelus armatus	342 (5.3%)	54 (15.8%)	15/19 (79.0%)
Colisa fasciatus	186 (2.9%)	29 (15.6%)	10/11 (90.9%)
Macrognathus aculeatus	179 (2.8%)	26 (14.6%)	11/15 (73.3%)
Glossogobius giuris	129 (2.0%)	18 (14.0%)	5/7 (71.4%)
Mystus vittatus	301 (4.7%)	40 (13.3%)	10/14 (71.4%)
Xenentodon cancila	76 (1.2%)	10 (13.2%)	0/3 (0%)
Notopterus notopterus	62 (1.0%)	8 (12.9%)	0/3 (0%)
Mystus cavasius	32 (0.5%)	4 (12.5%)	2/3 (66.7%)
Clarias batrachus	193 (3.0%)	24 (12.4%)	12/17 (70.6%)
Cirrhinus ariza	33 (0.5%)	4 (12.1%)	2/2 (100%)
Macrognathus pancalus	376 (5.8%)	42 (11.2%)	17/24 (70.8%)
Sperata aor	28 (0.4%)	3 (10.7%)	0/2 (0%)
Nandus nandus	65 (1.0%)	6 (9.2%)	6/6 (100%)
Parambassis. ranga	87 (1.4%)	8 (9.2%)	2/3 (66.7%)
Clarias gariepinus	11 (0.2%)	1 (9.1%)	1/1 (100%)
Morulius calbasu	11 (0.2%)	1 (9.1%)	1/1 (100%)
Barbodes gonionotus	45 (0.7%)	4 (8.9%)	2/2 (100%)
Lepidocephalichthys sp.	13 (0.2%)	1 (7.8%)	1/1 (100%)
Mystus tengara	280 (4.4%)	21 (7.5%)	8/11 (72.7%)
Heteropneustes fossilis	215 (3.3%)	15 (7.0%)	4/8 (50.0%)
Labeo gonius	31 (0.5%)	2 (6.5%)	0/1 (0%)
Labeo rohita	17 (0.3%)	1 (5.9%)	0/1 (0%)
Catla catla	46 (0.7%)	2 (4.4%)	1/2 (50%)
Lepidocephalichthys guntea	79 (1.2%)	2 (2.5%)	2/2 (100%)
Amblypharyngodon mola	67 (1.0%)	0 (0%)	-
Cyprinus carpio var communis	25 (0.4%)	0 (0%)	-
Cyprinus carpio var specularis	13 (0.2%)	0 (0%)	-
Esomus danricus	9 (0.1%)	0 (0%)	-
Gudusia chapra	37 (0.6%)	0 (0%)	-
Hypophthalmichthys molitrix	72 (1.1%)	0 (0%)	-
Oreochromis spp.	9 (0.1%)	0 (0%)	-
Total (average %)	6433 (100%)	1031 (16.0%)	297/375 (79.2%)

Table 3 Prevalence of lesions and EUS on 6401 farmed fish in Bangladesh
(from cross-sectional survey 1998-9, arranged in order of % with lesions)

Species	Number (and %) of fish examined	Number (and %) of fish with lesions	No. EUS-positive / No. sampled for histology (% of sampled fish EUS-positive)
Channa punctata	15 (0.2%)	8 (53.3%)	1/1 (100%)
Puntius ticto	51 (0.8%)	20 (39.2%)	3/3 (100%)
Cirrhinus cirrhosus	1563 (24.4%)	411 (26.2%)	30/32 (93.8%)
Morulius calbasu	19 (0.3%)	4 (21.1%)	1/1 (100%)
Barbodes gonionotus	1432 (22.4%)	295 (20.6%)	18/23 (78.3%)
Labeo rohita	1211 (18.9%)	182 (15.0%)	19/21 (90.5%)
Catla catla	964 (15.1%)	61 (6.8%)	9/12 (75.0%)
Ctenopharyngodon idellus	253 (4.0%)	10 (4.0%)	0/1 (0%)
Hypophthalmichthys molitrix	689 (10.8%)	2 (0.3%)	0/1 (0%)
Cyprinus carpio var communis	135 (2.11%)	0 (0%)	-
Cyprinus carpio var specularis	69 (1.1%)	0 (0%)	-
Total (average %)	6401 (100%)	993 (15.5%)	81/95 (85.3%)

Table 4 Prevalence of lesions and EUS on 9029 farmed fish in Mymensingh
(from case-control study 1998-00, arranged in order of % with lesions)

Species	No. (and %) of fish examined from control ponds	No. (and %) of fish examined from case ponds	No. (and %) of fish examined from case ponds with lesions	No. EUS-positive / No. sampled for histology (% of sampled fish EUS-positive)
Channa striata	0 (0%)	1 (0%)	1 (100%)	1/1 (100%)
Mastacembelus armatus	0 (0%)	1 (0%)	1 (100%)	1/1 (100%)
Lepidocephalichthys guntea	0 (0%)	5 (0.1%)	5 (100%)	0/1 (0%)
Monopterus cuchia	0 (0%)	1 (0%)	1 (100%)	0/1 (0%)
Wallago attu	0 (0%)	2 (0%)	2 (100%)	-
C. batrachus × *C. gariepinus*	0 (0%)	62 (1.2%)	56 (90.3%)	-
Channa orientalis	0 (0%)	11 (0.2%)	9 (81.8%)	0/1 (0%)
Colisa fasciata	0 (0%)	33 (0.6%)	22 (66.7%)	2/2 (100%)
Ctenopharyngodon idella	35 (0.9%)	19 (0.4%)	12 (63.2%)	-
Channa punctata	27 (0.7%)	235 (4.5%)	139 (59.2%)	5/6 (83.3%)
Puntius spp	37 (1.0%)	35 (0.7%)	20 (57.1%)	-
Macrognathus aculeatus	0 (0%)	14 (0.3%)	7 (50%)	-
Mystus tengara	0 (0%)	30 (0.6%)	15 (50%)	0/1 (0%)
Tetraodon cutcutia	0 (0%)	16 (0.3%)	8 (50%)	-
Anabas testudineus	7 (0.2%)	79 (1.5%)	33 (41.8%)	3/3 (100%)
Cirrhinus cirrhosa	904 (23.7%)	1431(27.5%)	576 (40.3%)	2 2/27 (81.5%)
Morulius calbasu	16 (0.4%)	11 (0.2%)	4 (36.4%)	1/1 (100%)
Clarias gariepinus	0 (0%)	109 (2.1%)	36 (33.0%)	2/4 (50%)
Macrognathus pancalus	0 (0%)	41 (0.8%)	12 (29.3%)	-
Mystus vittatus	0 (0%)	62 (1.2%)	18 (29.0%)	-
Barbodes gonionotus	231 (6.0%)	593 (11.4%)	170 (28.7%)	4/4 (100%)
Puntius sophore	144 (3.8%)	678 (13.0%)	182 (26.8%)	1/2 (50%)
Labeo gonius	12 (0.3%)	97 (1.9%)	26 (26.8%)	0/1 (0%)
Labeo rohita	1186 (31.0%)	847 (16.3%)	214 (25.3%)	8/10 (80%)
Clarias batrachus	0 (0%)	4 (0.1%)	1 (25%)	1/1 (100%)
Glossogobius giuris	41 (1.1%)	119 (2.3%)	27 (22.7%)	-
Catla catla	1102 (28.8%)	490 (9.4%)	100 (20.4%)	2/5 (40%)
Cirrhinus ariza	0 (0%)	31 (0.6%)	5 (16.1%)	-
Chanda nama	1 (0%)	21 (0.4%)	3 (14.3%)	-
Hypophthalmichthys molitrix	30 (0.8%)	12 (0.2%)	1 (8.3%)	-
Trichogaster chuna	25 (0.7%)	61 (1.2%)	5 (8.2%)	1/1 (100%)
Amblypharyngodon mola	25 (0.7%)	40 (0.8%)	2 (5%)	-
Heteropneustes fossilis	0 (0%)	3 (0.1%)	0 (0%)	-
Nandus nandus	0 (0%)	(0%)	0 (0%)	1/1 (100%)
Xenentodon cancila	0 (0%)	0 (0%)	0 (0%)	-
Pangasius hypophthalmus	0 (0%)	12 (0.2%)	0 (0%)	-
Total 34 species	**3823**	**5206**	**1713 (32.9%)**	**55/74 (74.3%)**


BANGLADESH DISTRICTS
AFFECTED BY EUS DURING
1998-1999 CROSS-SECTIONAL
SURVEY
Both wild and farm sites affected by EUS
Wild site only affected by EUS
Farm site only affected by EUS
No EUS recorded

APPENDIX EIGHT

Paper seven - Blazer, V.S., Vogelbein, W.K., Densmore, C.L., May, E.B., Lilley, J.H., and Zwerner, D.E. (1999) *Aphanomyces* as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. Journal of Aquatic Animal Health 11, 340-349. (hard copy available from ARP Manager)

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Aphanomyces as a Cause of Ulcerative Skin Lesions of Menhaden from Chesapeake Bay Tributaries

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Abstract.—During the summer and fall of 1997, an unusually high prevalence of skin lesions in fishes from Chesapeake Bay tributaries as well as two fish kills in the Pocomoke River stimulated significant public concern. Atlantic menhaden *Brevoortia tyrannus* were the most frequent target of the acute fish kills and displayed skin lesions that were attributed to the presence of the toxic dinoflagellate *Pfiesteria piscicida*. Hence, the penetrating skin ulcers so commonly found in this species are now widely viewed by the general public and some scientists as *Pfiesteria*-related and to be caused by exposure to *Pfiesteria* toxin. We examined, histologically, 121 menhaden with these ulcers collected from both Maryland and Virginia waters of the Chesapeake Bay in 1997 and 31 from the Pocomoke and Wicomico rivers in 1998. All of the deeply penetrating ulcers, as well as raised lesions (with or without eroded epithelium), were characterized by deeply penetrating fungal hyphae surrounded by chronic, granulomatous inflammation. These lesions had an appearance identical or similar to epizootic ulcerative syndrome (EUS), an ulcerative mycotic syndrome of fishes in other parts of the world caused by the fungal pathogen *Aphanomyces invadans*. They were also identical to ulcerative mycosis of menhaden previously reported along the Atlantic coast of the USA as associated with *Aphanomyces* spp. In 1998, using methods for isolation of *A. invadans*, we were able to culture from affected menhaden an *Aphanomyces* sp. that by preliminary tests is similar or identical to *A. invadans*. We believe these findings suggest that factors other than *Pfiesteria* toxin need to be considered as the cause or initiator of these lesions.

riety of fish species were involved as indicated by results of surveys conducted by several state and federal agencies during this period. In addition, two fish kills involving primarily juvenile Atlantic menhaden *Brevoortia tyrannus* occurred in the Pocomoke River during August 1997. The fish kills as well as the variety of fish lesions were attributed to the presence of the toxic dinoflagellate *Pfiesteria piscicida* or *Pfiesteria*-like (*Pfiesteria*-complex organisms) dinoflagellates. Because menhaden were the most frequent target of acute fish kills and episodes of fish lesions in the Chesapeake Bay, the penetrating ulcers so common in this species are now viewed by many as "*Pfiesteria*-related" and thought to be caused by exposure to *Pfiesteria* toxin (Burkholder and Glasgow 1997).

Worldwide there has been a reported increase in frequency and severity of harmful algal blooms or HABs (Lassus et al. 1995). Dinoflagellates contribute to these HABs with more than 40 species verified as producing toxins (Steidinger 1993). *Pfiesteria piscicida* (Steidinger et al. 1996), a recent addition to this list, was first observed in 1988 in an experimental culture system for blue tilapia *Tilapia aurea* in North Carolina (Smith et al. 1988, Noga et al. 1993). Acute mortality followed respiratory distress (shallow, rapid breathing), neurological signs (including hyperexcitability, lack of balance and spinning in the water column), and a cloudiness of the skin of experimentally exposed fish (Noga et al. 1993). Chronic ulcerative skin lesions similar to those found in wild menhaden were not reported among exposed tilapia. However, subsequent reports implicated *P. piscicida* as the cause of major estuarine fish kills in the Neuse and Pamlico estuaries in North Carolina specifically and of epidemic disease among estuarine fishes along the Atlantic Coast of the United States in general (Burkholder et al. 1992, 1995; Noga et al. 1996).

The objectives of our investigation were to (1) describe the lesions occurring in Chesapeake Bay menhaden obtained from acute fish kills and from episodes of fish lesions in which mortalities were not observed; (2) compare these lesions to those reported for other major epizootic ulcerative syndromes in fish from other parts of the world; and (3) examine the possible etiology of these lesions and the evidence supporting the hypothesis that toxins of *Pfiesteria* are the cause.

Methods

On August 6, 1997, hundreds of dead and dying fish, primarily juvenile menhaden, were found in the Pocomoke River near Shelltown, Maryland (Figure 1). The fish kill lasted 4 d and the state of Maryland closed a portion of the river from August 7 to 13. A second fish kill began in Pocomoke Sound on August 26, 1997. In this instance, fish with lesions were observed in the absence of significant mortality (Hughes et al. 1997). However, a portion of the river was closed until October 3. Kings Creek, a tributary of the Manokin River, was closed on September 10 because "significant" numbers of menhaden with lesions attributed to toxins from *Pfiesteria*-like organisms were observed. Four days later a portion of the Chicamacomico River was also closed for the same reason (Hughes et al. 1997). Menhaden were collected with cast nets, in trawls, or by dipnetting from boats in the Pocomoke River or Kings Creek by personnel from either Maryland Department of Natural Resources (MD DNR) or U.S. Geological Survey Fish Health Research Laboratory (FHL), Leetown, West Virginia, during the fish kills and episodes of fish lesions. A total of 82 menhaden with lesions, as illustrated in Figure 2, were processed for histology.

Menhaden were obtained from Virginia tributaries of Chesapeake Bay beginning on August 12 with the last sample obtained on December 8, 1997. Fish were collected in trawls, gill nets, and cast nets. Although no acute fish kill events were reported in Virginia waters during this time, 11 menhaden with characteristic ulcers were obtained on August 27 from the Virginia side of the Pocomoke River that might have been associated with the ongoing fish kill in Maryland. Menhaden ulcers not associated with acute mortality events were observed throughout summer and fall in several Virginia tributaries including the Great Wicomico, James, and Rappahannock rivers (Figure 1). A total of 39 menhaden with lesions (Figure 2) from these sites were processed for histological examination.

Tissues were placed into zinc formol fixative, 10% neutral-buffered formalin, Deitrich's fixative, or Bouin's solution. Samples were routinely processed for histopathology, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E), Giemsa, tissue Gram, or Grocott's methenamine silver methods (Luna 1992).

No acute fish kills occurred in 1998. However, menhaden with characteristic lesions were collected from the Pocomoke (20) and Wicomico (11) rivers in August with 10-min bottom trawls and beach seines. The muscle adjacent to the lesions was cultured using a modification of methods de-

FIGURE 1.—Map of lower Chesapeake Bay indicating locations in which menhaden with lesions were collected in 1997. Symbols indicate closure of river due to fish kill (■), closure of river due to menhaden lesion event (●) and no closures but high prevalence of lesions (▲).

scribed for the isolation of *Aphanomyces invadans*, the fungus causing epizootic ulcerative syndrome or EUS (Willoughby and Roberts 1994). Briefly, the external surface of the periphery of lesions was swabbed with alcohol, seared, and the epidermis and dermis were removed. Very thin slices of affected muscle were aseptically removed and placed in liquid growth media, glucose–peptone plus penicillin, and oxolinic acid. Media was changed every 8 h until hyphal growth (usually in 2–3 d) was observed. Small pieces of supporting muscle with new growth were aseptically transferred to new liquid media and again observed for growth (1–2 d) before transfer onto glucose–peptone–yeast extract (GPY) agar with penicillin and streptomycin sulfate (Willoughby and Roberts 1994). Cultures were incubated at 22°C. Presumptive identification was based on colony–hyphal morphology, temperature growth curves as described by Lilley and Roberts (1997), and immunohistochemical staining of selected paraffin sections with a polyclonal antibody raised against *A. invadans* as described by Lilley et al. (1997b). For the temperature growth curves, our isolate was compared with PA7, an isolate of *A. invadans* from Thailand (Lilley and Roberts 1997), and NJM9701, a Japanese *A. invadans* isolate from ayu *Plecoglossus altivelis*; TF5, a Thai saprophytic *Aphanomyces* sp. (Lilley and Roberts 1997) was also included.

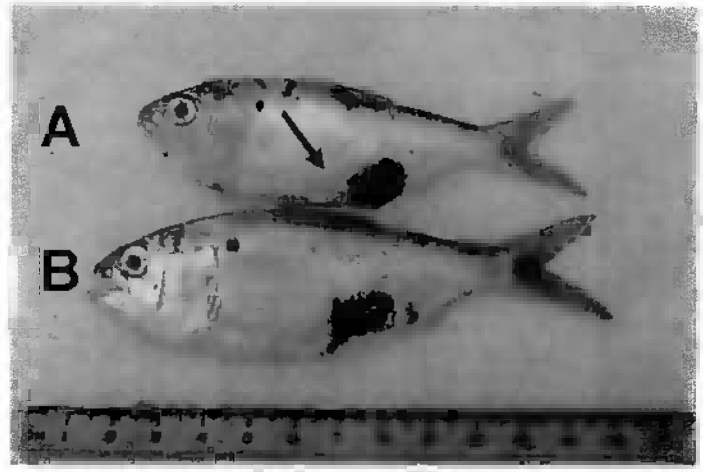

FIGURE 2.—The two most common lesion manifestations observed on menhaden collected in 1997. (A) Raised, grayish-white to reddish area with eroded skin. The periphery of the eroded area (arrow) is also raised although it remains covered by intact skin. (B) Deep ulceration of both skin and muscle forms a bloody lesion extending through the skeletal muscle into the peritoneal cavity. Rule bar is in centimeters.

Results

Lesion Prevalence and Gross Pathology

Twenty-five of 29 menhaden (90–100 mm total length) collected by FHL personnel from the Pocomoke River during August 26–28, 1997, had gross lesions. Of these, 24 fish had lesions that were the deep ulcers or raised lesions illustrated in Figure 2. The remaining fish with lesions had a deep linear abrasion along the caudal margin of the operculum. Gross and histologic appearance of this opercular lesion suggested it was traumatic in origin. The locations of the ulcerative lesions were the ventral, perianal region (in 11 of 24 fish with lesions), dorsally over the epaxial muscle (4 fish), laterally over the hypaxial muscle (3 fish), and along the caudal peduncle and caudal fin (2 fish). Four fish had lesions in multiple locations. Most of the lesions were deep circumscribed erosions of skin and muscle (Figure 2b) with or without erythema. However, a few lesions appeared as raised, friable, white to gray areas (Figure 2a), some of which had the epithelium eroded and some in which the epithelium was intact. The lesions in menhaden collected and processed by MD DNR personnel from the Pocomoke River and King's Creek (throughout August and September) were characterized as circumscribed, deep ulcers most often in the anal area and sometimes penetrating into the abdominal cavity.

In Virginia waters during September 1997, sampling efforts found 92% of the 24 menhaden collected in the Pocomoke Sound, 66% of 56 menhaden collected in the Rappahannock River, and 95% of 95 collected in the Great Wicomico River had skin lesions like those we described. Nine menhaden were collected in the James River in October with 78% exhibiting ulcers. The James River was again sampled in November with 100% of 10 menhaden exhibiting ulcers. Two samples from the Rappahannock River in December resulted in collections of 51 and 19 menhaden, with lesion prevalences of 49% and 89%, respectively. Most fish exhibited only a single ulcer either perianally, anterioventrally, dorsally, laterally, or associated with the caudal peduncle. Several fish had multiple ulcers. Most lesions were deeply penetrating, involving underlying body muscle and sometimes visceral organs. Some lesions exhibited peripheral erythema, others did not. In some lesions necrotic tissue within the area was friable and raised above the surface of the skin.

Microscopic Pathology of Ulcerative Lesions

All of the ulcerative lesions collected in both Virginia and Maryland waters contained a chronic, granulomatous reaction around highly invasive and deeply penetrating fungal hyphae. The histologic appearance was identical to the previously well-described lesions of EUS (Hatai et al. 1977; Callinan et al. 1989; Wada et al. 1996; Lilley et

FIGURE 3.—An area of infected skeletal muscle. Tracks of granulomatous inflammation surrounding fungal hyphae (thin arrows) penetrate through and around muscle bundles (a) under intact skin (large arrow) and muscle. Bar = 40 μm.

al. 1998) and ulcerative mycosis (Noga et al. 1988) and will only be briefly reviewed here. The infection was highly invasive, with "fingers" of this chronic inflammatory reaction around hyphae penetrating almost completely through the body of some animals. In fish with raised lesions (Figure 2a) the hyphae and associated host response extended into muscle underlying intact dermis and epidermis (Figure 3). In the more advanced cases such as seen in Figure 2b, much of the inflammation and necrotic skin and muscle tissue was sloughed and the fungi had penetrated into visceral organs including liver, pancreas, spleen, and intestine and was accompanied by inflammation and necrosis.

In many fish, the chronic granulomatous reaction around fungal hyphae was evident deep in the muscle. However, on the surface of the lesion was a demarcated area of massive necrosis (Figure 4). Remnants of the chronic granulomatous response around hyphae could still be seen within this area. However, fungal hyphae not eliciting a granulomatous response and presumably representing saprophytic species were also present within the necrotic areas. Many bacterial cells were also observed among the necrotic host tissue.

Isolation of Fungi from Ulcerative Lesions

The fungal isolates from lesions of menhaden collected in the Wicomico and Pocomoke rivers displayed typical *Aphanomyces* morphology and sporulation characteristics (Willoughby et al. 1995). Hyphae were variable in width (8–15 μm across) and sporangia were terminal with primary zoospores emerging laterally and encysting. The primary zoospore cysts contained spores held together in a circular array. Growth was slow at all temperatures between 10°C and 31°C with no growth at 37°C. The temperature–growth curve was similar to those for *A. invadans* isolates PA7 and NJM9701 (Figure 5). Fungal elements in paraffin sections of menhaden lesions collected in the Pocomoke (1997 and 1998) and Wicomico (1998) rivers showed a positive reaction using anti-*A. invadans* polyclonal antibodies as shown in Lilley et al. (1997b).

Discussion

Skin lesions in fishes can be caused by a variety of infectious and noninfectious insults. Sindermann (1988) suggested that presence of skin ulcers is one of the most useful biomarkers of polluted or otherwise stressful aquatic environments. Often these lesions are caused by opportunistic, facultative pathogens that infect weakened or stressed hosts. These facultative pathogens, which include viruses, bacteria, fungi, and other parasites, may gain entry because of impaired immune function or disease resistance factors or because the natural defense mechanisms of the skin are impaired or breached.

The skin lesions we observed in menhaden col-

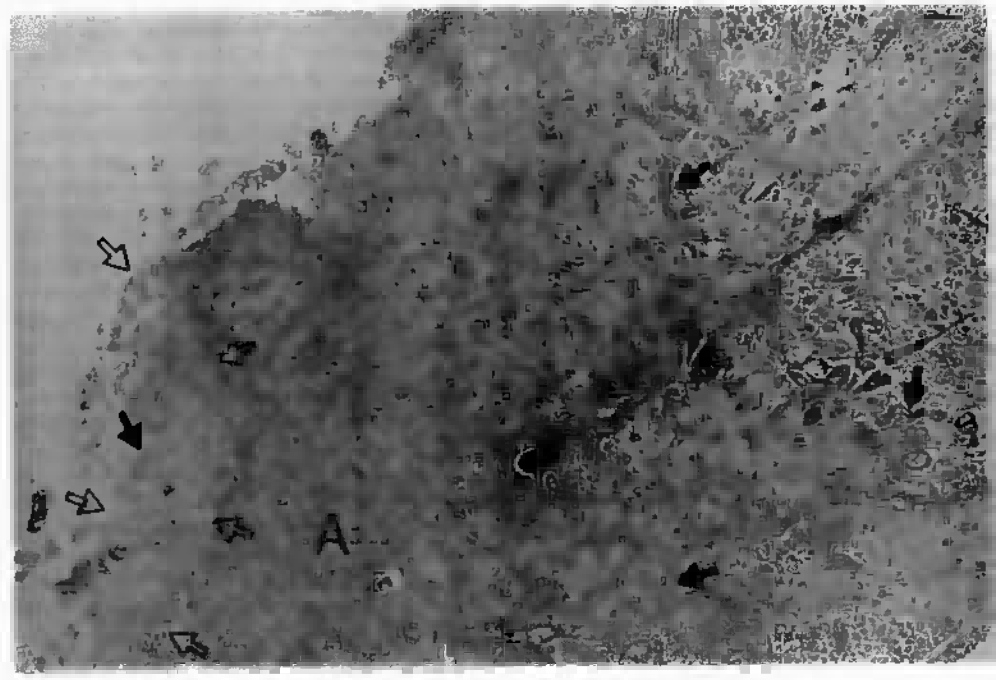

FIGURE 4.—Deep ulcerative lesion of menhaden. The periphery of the lesion (bound by open arrows) has undergone massive necrosis. The deeper regions of the lesion (A) contain acute and chronic inflammation with the typical tracks of granulomatous reaction around fungal hyphae (closed arrows). Hyphae (closed arrow on left) also remain in the necrotic area of the lesion (between open arrows). Bar = 60 μm.

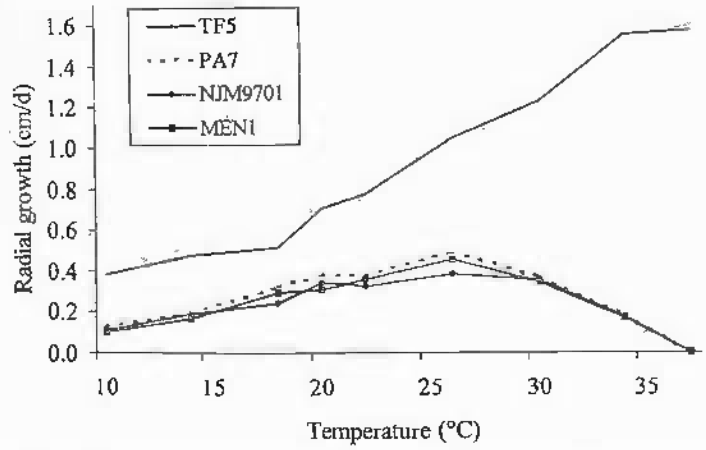

FIGURE 5.—Temperature–growth curves of our fungal isolate from menhaden (MEN1), two isolates of *A. invadans* (PA7 from Thailand and NJM9701 from Japan), and a saprophytic *Aphanomyces* isolate (TF5) from Thailand.

lected from the Chesapeake Bay in 1997–1998 were identical to the lesions observed in EUS, red spot disease, and mycotic granulomatosis—the latter two now also considered to be EUS (Lilley et al. 1998). These are cutaneous ulcerative syndromes, characterized histologically by granulomatous dermatitis and myositis associated with highly invasive aseptate fungi belonging to the genus *Aphanomyces*. These syndromes have occurred in wild and cultured freshwater and estuarine fishes in the Asia–Pacific region since the 1970s (Callinan 1994; Vishwanath et al. 1998). They are characterized by gross external lesions of varied appearance and size ranging from small hemorrhagic foci in the early stage to large, deep necrotic ulcers in more advanced cases.

A common feature of EUS is the variety of aquatic fungi (including species of *Aphanomyces*, *Achlya*, and *Saprolegnia*) growing saprophytically on the surface of the lesions. This has led to confusion about which fungus is responsible for the characteristic granulomas and lesion formation (Lilley and Inglis 1997). However, recent studies have confirmed *A. invadans* as the cause of these lesions. Pathogenicity of isolates from wild fish was tested by inserting a small mass of mycelia under the dermis of healthy chevron snakeheads *Channa striata* via an incision on the left shoulder (Roberts et al. 1993). When the slow-growing, thermolabile strains of *Aphanomyces* were inserted, there was initially a mild, local inflammatory lesion followed by severe myonecrosis extending deep into the muscle (within 5 d). Within 15–20 d at water temperature of 22°C the lesion progressed to resemble the typical chronic granulomatous response seen in EUS-infected wild fish. When the same infectivity studies were conducted with strains of *Achlya*, *Saprolegnia*, and fast-growing *Aphanomyces*, a localized foreign-body-type response occurred and the inoculation site healed (Roberts et al. 1993).

Recent work has confirmed that a single species of *Aphanomyces* is a necessary cause of EUS—it occurs in all outbreaks and in some outbreaks it may be the only biological factor required for disease to occur (Lilley et al. 1998). The pathogenic isolate was described and initially named *Aphanomyces invaderis* (Willoughby et al. 1995) and now is known as *A. invadans* (IMI 1997). This species is a slow-growing, aquatic fungus with wide, aseptate mycelia. It grows best between 24°C and 30°C, will grow at 31°C, but dies at 37°C. Lilley et al. (1997a) used random amplified polymorphic DNA (RAPD) analysis based on the polymerase chain reaction (PCR) to compare isolates of *Aphanomyces* from various locations in Bangladesh, Thailand, Indonesia, the Philippines, Australia, and Japan. They found that the isolates were conspecific and probably constitute a single clonal genotype spread throughout the Indo-Pacific area. It was speculated that both floods and massive cross-border movement of fish for aquaculture and ornamental fish industries caused this spread.

The fungal isolates made from lesions of menhaden collected in the Wicomico and Pocomoke rivers show typical *Aphanomyces* sporulation characteristics, slow growth on GPY at 22°C, and no growth at 37°C as with *A. invadans* (Figure 5). Fungal elements present in paraffin sections of menhaden lesions collected in the Pocomoke (1997, 1998) and Wicomico rivers (1998) stained positively using anti-*A. invadans* polyclonal antibody. This preliminary information suggests the fungal isolates may be *A. invadans*, however infectivity experiments and genetic comparisons are needed for confirmation.

The skin lesions we describe are also similar, both grossly and histologically, to ulcerative mycosis described in the 1980s from menhaden along the east coast of the United States (reviewed by Noga 1993) and to those in menhaden collected from the Chicamacomico River in September 1997 by Kane et al. (1998). The fungal pathogen *Aphanomyces* was reported to be involved with ulcerative mycosis by Dykstra et al. (1986). However, isolations were made on corn meal agar (Dykstra et al. 1986, 1989). These *Aphanomyces* isolates were of three distinct growth and morphology patterns: (1) isolates that grew vigorously, produced zoospores copiously, and formed oospores (*Aphanomyces* isolate ATCC 62427: American Type Culture Collection, Rockville, Maryland); (2) isolates that produced asexual zoospores abundantly and grew vigorously; and (3) isolates that produced scant mycelium and few zoospores (Dykstra et al. 1989). *Aphanomyces* isolate ATCC 62427 was the isolate identified with ulcerative mycosis in subsequent comparisons of this disease with EUS (Lilley and Inglis 1997; Lilley and Roberts 1997) and found to be distinct from *A. invadans*. Attempts to reproduce the disease by challenge with this isolate failed to induce ulcerative mycosis; however, fish challenged with lesion material did develop typical lesions (Noga 1993). Hence, based on what we now know about the difficulty in culturing *A. invadans* (Willoughby and Roberts 1994), lack of growth on corn meal agar, and growth characteristics (Willoughby et al. 1995), perhaps the actual pathogen of ulcerative mycosis in menhaden was never isolated.

Most cases of this fungal infection from our study sites were observed in menhaden, although we noted it in a few other fish species (one Atlantic croaker *Micropogonias undulatus* and one spot *Leiostomus xanthurus*). However, it must be recognized that this study was not directed toward determining the infection prevalence or species distribution of these lesions. In a study directed toward species distribution, menhaden were also the predominantly affected species in the Tar–Pamlico river estuary, North Carolina, although other species of affected fish were occasionally found (Levine et al. 1990). Atlantic menhaden are found in temperate waters of the Atlantic from Nova Scotia to Florida. They are a migratory, schooling fish of commercial importance as well as prey for other fish species and seasonally important components of estuarine fish assemblages (Rogers and Van Den Avyle 1989). Larvae migrate into estuaries from May through October in the North Atlantic region and from October to June in the mid-Atlantic (Reintjes and Pacheco 1966). A number of studies have shown high abundance of young menhaden in portions of the estuaries with the lowest salinities. Rogers et al. (1984) provided evidence that prejuveniles select tidewater areas that are fresh or low salinity. A comparison of EUS *Aphanomyces* isolates showed that these organisms were killed by exposure to 20‰ sodium chloride for 1 h whereas the saprophytic isolates survived (Lilley and Inglis 1997). Dykstra et al. (1986) also found a salinity preference in Oomycetes isolated from menhaden with ulcerative mycosis in the Pamlico River estuary. One isolate of *Aphanomyces* exhibited enhanced growth when 2–4‰ NaCl was added to the medium but was somewhat inhibited at higher concentrations (20–26‰). Perhaps we see the lesions on menhaden because of their schooling or feeding behavior and primarily in the rivers because of low-salinity preference of the causative agent. However, once the infection is established and deep in the muscle it may be somewhat protected from higher salinities, as shown for other Oomycetes securely established on suitable substrates (Padgett 1984).

More recently, it was suggested that the fungal infections seen in menhaden and other estuarine fishes in North Carolina are secondary, opportunistic infections that occur subsequent to exposure of the fish to toxins from *Pfiesteria* (Noga et al. 1996; Burkholder and Glasgow 1997). However, scientific evidence for the relationship of these lesions with toxic dinoflagellates is lacking. Laboratory exposures to *Pfiesteria* have not produced similar lesions. In the initial laboratory mortalities, respiratory distress, neurological signs, and a cloudiness of the skin with acute mortality were reported (Smith et al. 1988; Noga et al. 1993). More recently described were dermatological lesions including intra- and extracellular edema and necrosis of the epithelium resulting in erosions extending to the basement membrane (Noga et al. 1996). The same study also examined fish that survived an acute sublethal exposure to *P. piscicida* and were placed into clean water. One of 10 fish subsequently developed lesions containing fungal hyphae. However, the hyphae appeared typical of secondary, saprophytic fungi growing in necrotic tissue. The fungus did not elicit the typical granulomatous response described in wild menhaden. The remaining fish had bacterial infections (Noga et al. 1996). Burkholder and Glasgow (1997) stated that "the fungi are opportunists; their hyphae generally do not penetrate to the lesion base, suggesting they do not form the lesions; rather, they colonize lesions formed by toxin(s) from *P. piscicida*." This is not the manifestation we observed. In all instances the fungal hyphae and surrounding chronic inflammation extended deep into muscle, often under intact, normal skin (Figure 3). We believe the raised lesions caused by the massive inflammatory response and hyphae may be an earlier manifestation of the deep ulcerated lesions. As the infection progresses, necrosis results from the effects of secondary invaders colonizing the lesions and the associated intense inflammatory response. Necrotic skin and muscle ultimately slough to form the more advanced, deeply penetrating, lesions. This was previously suggested as the progression of ulcerative mycosis (Noga 1993).

Epizootic ulcerative syndrome is considered a multifactorial problem because a variety of predisposing factors may be involved—the only required factor being the presence of *A. invadans*. Hence, EUS has been experimentally reproduced in susceptible species by mechanical damage of epidermis, intramuscular injection of a rhabdovirus, or sublethal exposure to acid sulfate soil runoff water followed by exposure to *A. invadans* propagules (Lilley et al. 1998). Toxic dinoflagellates may play a role in the initiation of some menhaden skin ulcers. Sublethal levels of toxin could damage the skin or cause immunosuppression, thus allowing the fungi to invade. However, it seems unlikely that the same histopathological presentation would be so consistently observed if

these were simply opportunistic invaders. Rather, we would expect to see a variety of infections such as acute, subacute, or chronic bacterial infections, lesions with only saprophytic fungi and bacteria growing in the damaged tissue, and opportunistic parasitic infections. Based on these observations and the numerous reports of similar epizootics in other parts of the world, we believe factors other than toxic dinoflagellates need to be considered as causes or initiators of these lesions.

The presence of deeply penetrating skin ulcers on fish, particularly menhaden, is currently used in Maryland and Virginia waters of the Chesapeake Bay as one indicator of local activity by *P. piscicida* or other *Pfiesteria*-like dinoflagellates. Hence, the observation of these lesions during 1997 led to the closure of stretches of certain tributaries in Maryland for recreational, commercial, and research uses for weeks at a time. However, the chronic nature of the lesions in a migratory fish and the consistent presence of an invasive fungal pathogen raise questions about the usefulness of current criteria for the local activity of toxic dinoflagellates.

Further research is needed to study the development of this fungal disease in menhaden and to understand the variety of underlying stressors that may initiate infection, increase susceptibility of the host, and allow the fungus to penetrate and grow within the host. Additional investigations pursuing what role, if any, toxic dinoflagellates play in the occurrence or progression of these lesions is critical. Lastly, and perhaps most importantly, we must understand the environmental factors that encourage proliferation of the fungus in the environment.

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APPENDIX NINE

Paper eight - Blazer V., Vogelbein W.K., Densmore C., Kator H., Zwerner D., and Lilley J. (2000) Etiology and pathogenesis of skin ulcers in menhaden, *Brevoortia tyrannis*: does *Pfiesteria piscicida* play a role? Marine Environmental Research 50(1), 487-488. (abstract given)

Blazer V., Vogelbein W.K., Densmore C., Kator H., Zwerner D., and Lilley J. (2000) Etiology and pathogenesis of skin ulcers in menhaden, *Brevoortia tyrannis*: does *Pfiesteria piscicida* play a role? Marine Environmental Research 50(1), 487-488

The toxic dinoflagellate, *Pfiesteria piscicida*, is widely blamed for adverse human health effects, acute fish kills and skin lesion events in fishes, particularly menhaden, *Brevoortia tyrannis*, inhabiting coastal waters from Delaware to North Carolina, USA. In response, we initiated studies to clarify the etiology and pathogenesis of presumed *'Pfiesteria*-specific' menhaden skin lesions. Histopathologically, all lesions (>150 fish examined) were associated with a highly invasive and pathogenic fungus eliciting severe tissue necrosis and intense granulomatous inflammation. Severity and extent of the host response indicates that ulcers were at least 1 week old or older. Maryland and Virginia currently use menhaden ulcers as one of several indicators of local *Pfiesteria* activity. However, their chronic nature, advanced age, and consistent fungal involvement suggest that their use for this purpose may not be valid. We recently isolated an *Aphanomyces* sp. from the menhaden lesions which by appearance in culture, temperature growth curves, pathogenicity studies in snakehead and positive immunohistochemical staining with polyclonal antibodies suggest the infectious agent is *A. invadans* (cause of epizootic ulcerative syndrome in Asia, Japan and Australia) or a very closely related species. Ongoing research will address pathogenicity of the fungus in menhaden, genetic comparisons of isolates, and the role of environmental stressors, including *P. piscicida*, in initiation of the infection.

APPENDIX TEN

Paper nine - PAPER NINE - Blazer, V.S, Lilley, J.H., Schill, W.B., Kiryu, Y., Densmore, C.L. Panyawachira, V. and Chinabut, S. (2001) *Aphanomyces invadans* along the East Coast of the United States. Journal of Aquatic Animal Health (in preparation, project contribution shown, hard copy of figures available from ARP Manager)

MATERIALS AND METHODS

The following work was undertaken at the Aquatic Animal Health Research Institute, Department of Fisheries, Thailand.

Fungus

American isolate UM3 was compared to various Asian *Aphanomyces invadans* isolates and saprophytic *Aphanomyces* species listed in Table 1.

Table 1 Fungi isolates used in present experiments

Species	Isolate	Origin
UM-*Aphanomyces*	UM3	Ulcerated menhaden, *Brevoortia tyrannic,* Wicomico River, 1998
A. invadans	PA7	EUS-affected snakehead, *Channa striata*, Nonthaburi, Thailand, 1995
A. invadans	NJM9701	EUS-affected ayu, *Plecoglossus altivelis*, Shiga, Japan, 1997
A. invadans	B99C	EUS-affected *Cirrhinus reba*, Mymensingh, Bangladesh 1999
A. invadans	24P	EUS-affected mullet, *Mugil cephalus*, Queensland, Australia, 1990
Aphanomyces saprophyte	TF5	Surface of an ulcerated snakehead, *Channa striata*, Thailand, 1991
Aphanomyces saprophyte	ASEAN3	Pond water, Thailand, 1994
Aphanomyces saprophyte	TF33	Surface of an ulcerated swamp eel, *Fluta alba*, Thailand, 91

Challenge

Three fungus isolates were used in the challenge (UM3, PA7 and NJM9701). Fungi were maintained on glucose-peptone (GP) agar (Roberts *et al,* 1993). Suspensions of motile secondary zoospores were produced using a similar method to that of Willoughby and Roberts (1994). Briefly, 4mm agar plugs of fungus were incubated in Petri dishes containing GP broth for 3 days at 22°C, and then washed in autoclaved pond water (APW), and incubated overnight at 22^{o}C. The resulting sporulating cultures showed typical *Aphanomyces* zoosporangia and achlyoid spore clusters (Fig. 1). Counts of motile zoospores were made using a haemocytometer and concentrations were adjusted with APW to 500 zoospores/ml.

Twenty eight clinically-normal juvenile striped snakeheads (*Channa striata)*, 10-15 cm in length, were obtained from a farm in Suphanburi, Thailand. They were acclimatised in seven 20-litre tanks, each containing four fish, at 20-23^{o}C for 10 days. Snakeheads are air-breathers and no aeration or continuous water exchange was required. Two thirds of the water was changed every 3-4 days. Fish were fed pelleted feed once daily to satiation.

Two tanks were randomly allocated to each of the three fungus challenges and the fish in the remaining tank were injected with APW. Fungi were sporulated as described above, and 0.1ml of the zoospore suspension was injected intramuscularly into each fish, on the left flank just below the anterior part of the dorsal fin.

Two fish from each tank were sampled for histology on days 9 and 18. Transverse sections were made through the site of injection and at least one slide stained with H&E, and one with Grocott's silver stain.

Growth studies

Temperature growth studies were conducted on GP agar medium using UM3, 3 *A. invadans* isolates (PA7, NJM9701, B99C) and 1 saprophytic *Aphanomyces* isolate (TF5). A 4mm plug of GP agar containing fungus was placed on GP agar, growth measured after 3 days and the colony radius increase per 24 hr calculated.

PCR

Primers FP1 and FP2, designed from the internal transcribed spacer (ITS1) regions of ribosomal DNA (rDNA) were used for the specific amplification of *A. invadans* DNA. Two DNA extractions of UM3 were made and compared with DNA from *A. invadans* isolates PA7, 24P and NJM9701 and saprophytic *Aphanomyces* isolates ASEAN3 and TF33. The following PCR reagents were added to a 50 µl reaction tube: 1x *Taq* buffer, 200 µM NTPs, 25 pmoles each of the primers FP1 and FP2, 2.5

mM $MgCl_2$ and 1.25 units of *Taq* polymerase. Amplifications were performed with an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 68 °C, and 45 sec at 72 °C, with a final extension of 5 min at 72 °C.

Sequencing

The ITS1 rDNA region was amplified for isolate UM3 using the primers ITS1 and ITS2 (White *et al*., 1990). 50 ng of genomic DNA was amplified in a final volume of 50 µl containing 1x *Taq* buffer, 200 µM NTPs, 25 pmoles each of the primers ITS1 and ITS2, 1.5 mM $MgCl_2$ and 2 units of *Taq* polymerase. Amplifications were performed with an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 55 °C, and 45 sec at 72 °C, with a final extension of 5 min at 72 °C. Products were isolated, purified and automatically sequenced on an ABI PRISM™ model 377. The sequences was submitted to GenBank.

RESULTS

Challenge

No mortalities were recorded during the experiment. Fish injected with UM3 started to develop clinical lesions by day 9 (Fig. 2a) and by day 18, fungus was seen erupting from lesions at the site of injection (Fig. 2b). Fish injected with PA7 and NJM9701 showed similar clinical signs, although lesion development was slower, and no external fungus was observed. APW-injected fish did not develop lesions and no significant pathological signs were observed.

Invasive fungal growth was observed in silver stained sections of all fungus-injected fish sampled on day 9. By day 18, fungal hyphae had extended to the opposite side of the fish to the site of injection and typical EUS-like granulomas were found to be investing fungal hyphae (Fig. 3). Hyphal penetration was also associated with extensive floccular muscle degeneration.

The only consistent difference observed between sections of fish injected with UM3, and those injected with the two *Aphanomyces invadans* isolates, was the width of hyphae in tissues. The UM3 hyphae ranged from 6-15µm in width, whereas the PA7 and NJM9701 hyphae measured 13-30µm in width.

Growth studies

All the *A. invadans* isolates, including UM3, produced the distinctive slow-growing, thermo-labile growth profile, in contrast to the saprophytic *Aphanomyces* isolate (Fig. 4).

PCR

Amplification with *A. invadans*-specific primers produced the diagnostic 98bp product when genomic DNA from UM3 and the A. invadans isolates were tested, but no band was produced by the the saprophytic *Aphanomyces* isolates (Fig. 5).

Sequencing

Sequencing of the entire ITS1 region of UM3 produced a 202bp segment (GenBank AF349614) that showed 100% homology with Asian *A. invadans* isolates (GenBank AF349610-3).

DISCUSSION

The UM3 isolate from ulcerative disease outbreaks in the United States was identified as *Aphanomyces invadans*, the causal pathogen of the Asian fish disease, epizootic ulcerative syndrome (EUS). Identification was made on the basis of pathoegenicity in snakehead fish, growth rates at ten different temperatures, use of an *A. invadans*-specific DNA probe, and sequencing of the ITS1 region of the isolate's rDNA.

Pathogenicity in snakehead fish has been listed as one of the distinguishing features of *Aphanomyces invadans* (Willoughby *et al*, 1995). Lilley and Roberts (1997) tested a number of Asian *A. invadans* isolates (including PA7) and showed them all to be invasive in snakeheads, whereas a variety of other species of *Aphanomyces*, *Achlya* and *Saprolegnia* were found to be incapable of sustained growth in snakehead muscle.

The invasiveness of UM3, and its ability to induce a granulomatous host response in snakeheads strongly supports the idea that this isolate is *A. invadans*. The differences in hyphal width between

UM3 and the Asian isolates in fish tissue demonstrate that there are some strain differences, however all three isolates fall into the ranges recorded for *A. invadans* infections in fish.

Lesion development was found to be more rapid with the UM3 isolate, probably due to the fact that it is a much more recent isolate. Repeated subculture is known to reduce virulence in *Aphanomyces* species (Unestam and Svensson, 1971) and may have affected the older two isolates, PA7 and NJM9701.

In conclusion, it seems apparent that *A. invadans* is present in environments along the East Coast of the U.S., and in some inland areas. It is an important pathogen of menhaden and has the potential to affect other fish species as well.

ACKNOWLEDGEMENTS

Thanks to Professor K. Hatai, Nippon Veterinary and Animal Science University, Tokyo, for isolate NJM9701.

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FIGURES

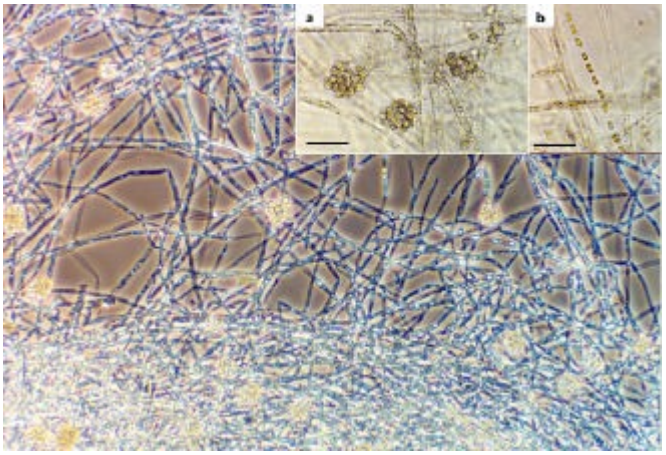
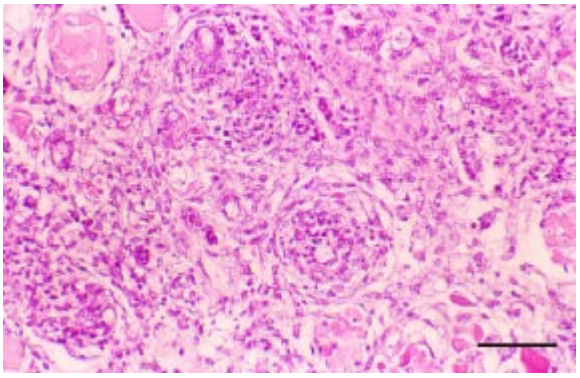


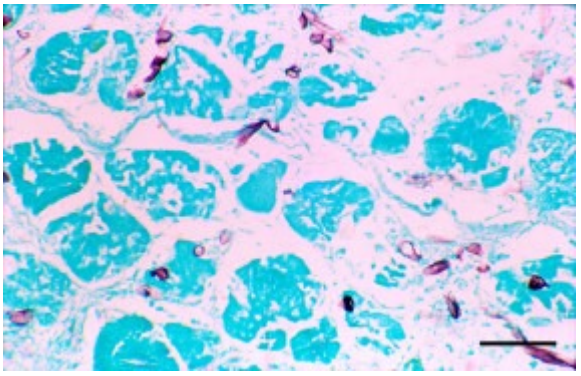
Fig. 1 Sporulating culture of UM3. Insert a: Clusters of encysted primary zoospores. Insert b: Primary zoospores within a zoosporangium

2a UM3-injected snakehead, day 9

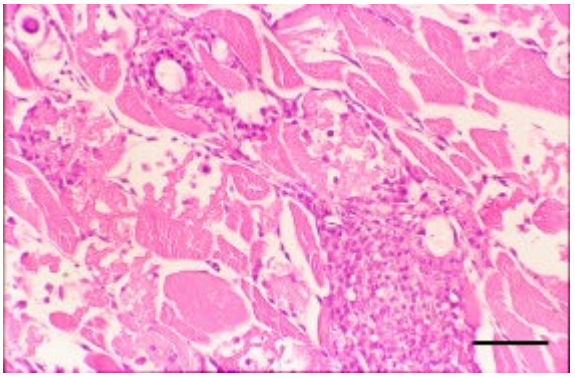
2b UM3-injected snakehead, day 18



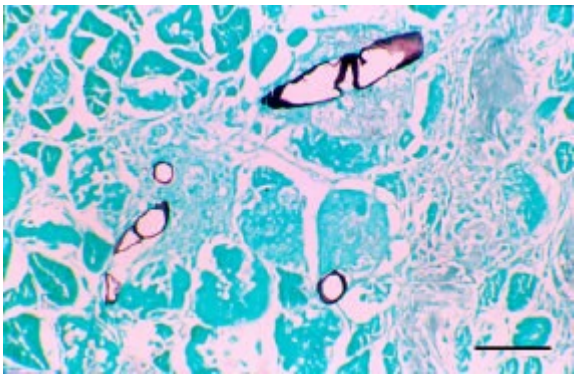
3a Mycotic granulomas forming in the muscle of UM3-injected snakehead, day 18 (H&E, bar = 50µm)



3b Extensive invasive fungal growth in the muscle of UM3-injected snakehead, day 18 (Grocott's, bar = 50µm)

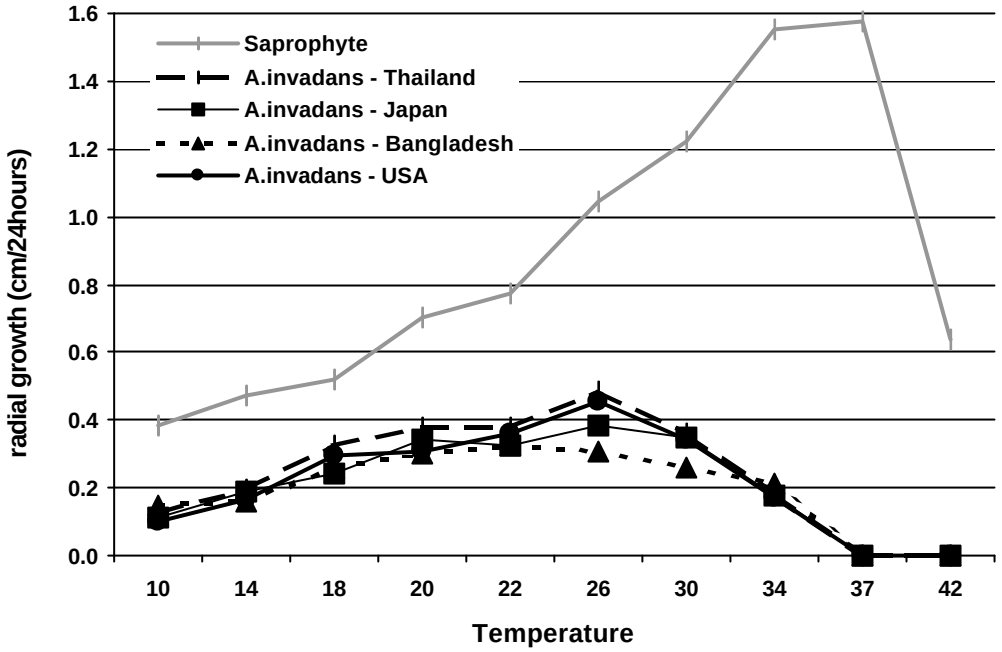


3c Mycotic granulomas forming in the muscle of PA7-injected snakehead, day 18 (H&E, bar = 50µm)



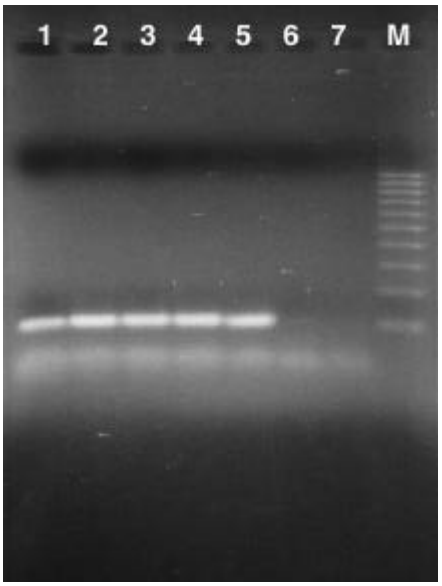
3d Extensive invasive fungal growth in the muscle of PA7-injected snakehead, day 18 (Grocott's, bar = 50µm)

Fig.4 Temperature-growth curves



PCR of fungal DNA extraction using specific *A. invadans* primers FP1 and FP2

Lanes 1-5 are *A. invadans* isolates and 6-7 are saprophytic *Aphanomyces*
(1) UM3 extraction 1 (2) UM3 extraction 2 (3) PA7 (4) 24P (5) NJM9701
(6) ASEAN3 (7) TF33 (M) 100bp Biorad molecular marker



APPENDIX ELEVEN

Article one - Lilley, J., Bangyeekhun, E., Panyawachira, V., and Cerenius, L. (1999) Zoospore physiology of *Aphanomyces invadans* 1. Polyplanetism. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok. 8(2), 6-8. (hard copy available from ARP Manager)

Zoospore physiology of *Aphanomyces invadans* 1. Polyplanetism

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Motile zoospores provide Oomycete fungi with a capacity for dispersal (Lange and Olson, 1983) and host/substrate location (Deacon and Donaldson, 1993). Therefore, the study of adaptations in the structure, physiology and behaviour of zoospores are likely to provide information on the processes by which pathogenic fungi locate and infect hosts.

Polyplanetism is a phenomenon of some Oomycete fungi whereby several successive tertiary generations of zoospores may be produced from the secondary cyst (Fig.1). This repeated emergence of zoospores was considered to be an adaptation to parasitism among particular species of *Aphanomyces* (Cerenius and Söderhäll, 1985).

In two experiments, polyplanetism was studied in isolates of *Aphanomyces invadans* (also known as *A. invaderis* and *A. piscicida*), the invasive fungal pathogen that characterises epizootic ulcerative syndrome (EUS) infections of freshwater fish.

Experiment 1: Determining the number of polyplanetic generations that can be induced

Three isolates of *A. invadans* from EUS-affected fish in Thailand (96PA[1]), Indonesia (36/1P[2]) and Philippines (33P[2]) were compared with 2 isolates of *Aphanomyces laevis* (107-52[3] and ASEAN3[2]), 2 saprophytic *Aphanomyces* spp (F3SA[2] and WSA[2]) and 1 isolate of *Saprolegnia parasitica* (SPT[4]).

Suspensions of secondary zoospores in autoclaved lake water were produced as described by Diéguez-Uribeondo *et al* (1994). These were pipetted into test tubes and the zoospores induced to encyst by agitating on a vortex mixer for 45 seconds. The resulting cyst suspensions were kept at 22°C and examined periodically until motile zoospores were observed (the time taken for cysts to release zoospores varied between species). This next generation of zoospores was pipetted off, and again encysted by vortexing. This procedure was continued until no subsequent zoospore generations were released. At each stage prior to vortexing, the proportion of germinated cysts, empty cyst coats (each representing a released motile zoospore) and undifferentiated cysts (i.e. neither germinated nor empty), were counted by examining 200-300 cells under a microscope.

A. invadans isolates were shown to be capable of producing 1 additional generation of motile zoospores from artificially encysted secondary zoospores, but no further generations could be induced (Fig. 2). There was however, a fairly high proportion of undifferentiated cysts that neither germinated nor released zoospores. Bangyeekhun (unpublished) has compared isolates of *A. astaci* and shown that those kept in culture for several years have a much reduced capability for producing successive zoospore generations, and this may also be the case for the *A. invadans* isolates tested

[1] From *Channa striata*, January 1996, Pichit, Thailand.
[2] See Lilley and Robert (1997)
[3] See Cerenius and Söderhäll (1985)
[4] See Diéguez-Uribeondo *et al* (1994)

here. *A. astaci* isolates have been shown to be capable of producing between 1 and 3 successive generations depending of the age of the isolate (Cerenius and Söderhäll, 1984; Bangyeekhun unpublished).

A different method of agitating *A. invadans* zoospore suspensions was used in studies by Willoughby and Roberts (1994). They reported that the zoospores lost and regained motility without an intervening encystment stage. In the present study, empty "ghost" cysts left by the tertiary zoospore generation of *A. invadans* were considered evidence of an intervening encystment stage.

In the current study, both *A. laevis* isolates produced 2 generations, but zoospore release from the second generation was poor. The two other saprophytic *Aphanomyces* isolates showed a high level of polyplanetism, each producing up to 4 successive generations of zoospores. The saprophytes are of similar age to the *A. invadans* cultures, and therefore are shown to retain a greater facility for polyplanetism than *A. invadans*. In contrast, the more parasitic *Aphanomyces* species studied by Cerenius and Söderhäll (1985) showed higher levels of polyplanetism. Of the saprophytes studied here, F3SA could be considered to be a wound parasite as it was isolated from a surface lesion of a fish, but WSA and the *A. laevis* isolates were derived from water samples.

Saprolegnia parasitica had a higher level of polyplanetism than any of the *Aphanomyces* species. Up to 6 successive generations of *S. parasitica* zoospores could be induced, as was previously reported by Diéguez-Uribeondo *et al* (1994) for the same isolate.

Experiment 2: Polyplanetism in a general nutrient background

This study assessed the relative tendency of encysted zoospores to germinate, or produce a further zoospore generation, given a general nutrient background, glucose-peptone-yeast broth (GPY: Willoughby and Roberts, 1994). All the isolates described above were used in the experiment, except that *A. astaci* isolate An[5] replaced *S. parasitica*. Motile zoospore suspensions were obtained and encysted as in experiment 1. An equal volume of GPY broth was added immediately after encystment. After 2 hours, the percentages of cysts that had germinated, released zoospores (indicated by empty cyst or empty "mini-sporangia"), or were undifferentiated (neither germinated nor empty), were determined by examining 200-300 cells under a microscope.

The addition of nutrient media to cyst suspensions resulted in a clear difference in the response of *A. invadans* from the other isolates (Fig. 3). In all three *A. invadans* isolates, zoospores were observed swimming after 2 hours and the number of zoospores was estimated by counting empty cysts or empty mini-sporangia. Zoospores were observed emerging from both cysts and mini-sporangia (Fig. 4). Of the isolates studied here, mini-sporangia were only observed in *A. invadans* cultures in the presence of GPY, but they have also been observed in suspensions of the *Aphanomyces* saprophyte, WSA, kept in sterile lake water.

Although a proportion of the *A. invadans* cells released zoospores, the majority germinated. In the other isolates however, all of the viable cysts germinated. Germlings of *A. astaci* were slower in forming, and after 6 hours a much greater proportion of cells were identified as germlings (97%). This is partly because the *A. astaci* isolate (An) was incubated at a lower temperature (14^{o}C) than the other isolates (22^{o}C). Other studies have shown the specific nature and timing of stimuli required by

[5] From *Pacifastacus leniusculus*, 1998, River Arakil, Navarra, Spain (Diéguez-Uribeondo)

A. astaci for germination to take place. Svensson and Unestam (1975) demonstrated particular chemical germination triggers for *A. astaci,* and Cerenius and Söderhäll (1984) and Persson and Söderhäll (1986) showed that cysts had to be exposed to these germination triggers within 15 minutes of encystment for the germination response to occur. Cerenius and Bangyeekhun (unpublished) have also showed that *A. astaci* cysts retain the ability to produce further zoospore generations if, after exposure to nutrient media, the media is replaced with sterile lake water.

In comparison to the *Aphanomyces* isolates, Diéguez-Uribeondo *et al* (1994) showed that almost 100% of each of 5 generations of zoospores of *Saprolegnia parasitica* would germinate in a nutrient background (PG-1).

Conclusions

1. *Aphanomyces invadans* is capable of polyplanetism in autoclaved lake water, but this ability is limited compared to polyplanetism in the other *Aphanomyces* strains studied here.
2. In the presence of a general nutrient background, some *A. invadans* cysts germinate and some produce a further zoospore generation. This is presumably an adaptation to aid the location of a specific host.
3. The production of a "mini-sporangium" is a distinctive feature of *A. invadans,* but it is not unique to the species.

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Figure 1

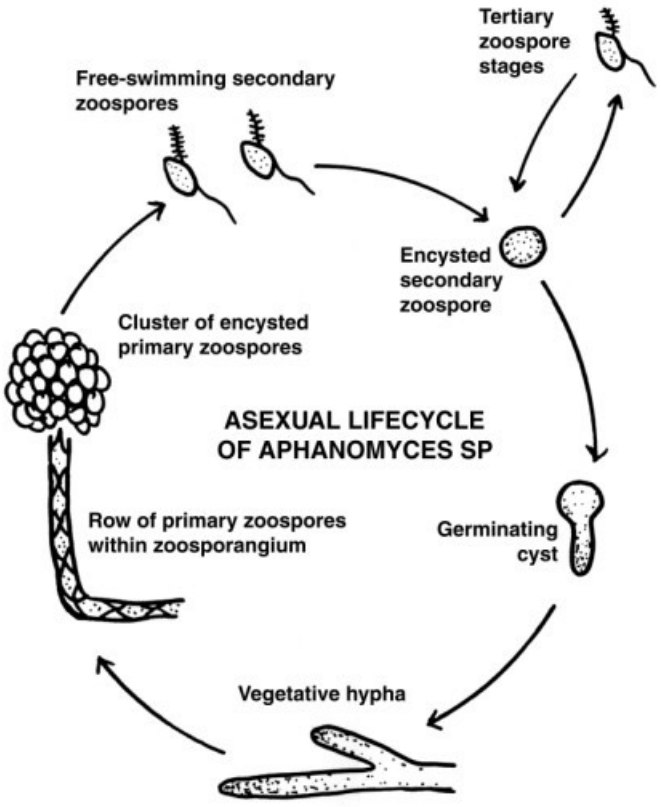

Tertiary zoospore stages
Free-swimming secondary zoospores
Encysted secondary zoospore
Cluster of encysted primary zoospores
ASEXUAL LIFECYCLE OF APHANOMYCES SP
Row of primary zoospores within zoosporangium
Germinating cyst
Vegetative hypha

Figure 2 Number of zoospore generations induced in fungal isolates

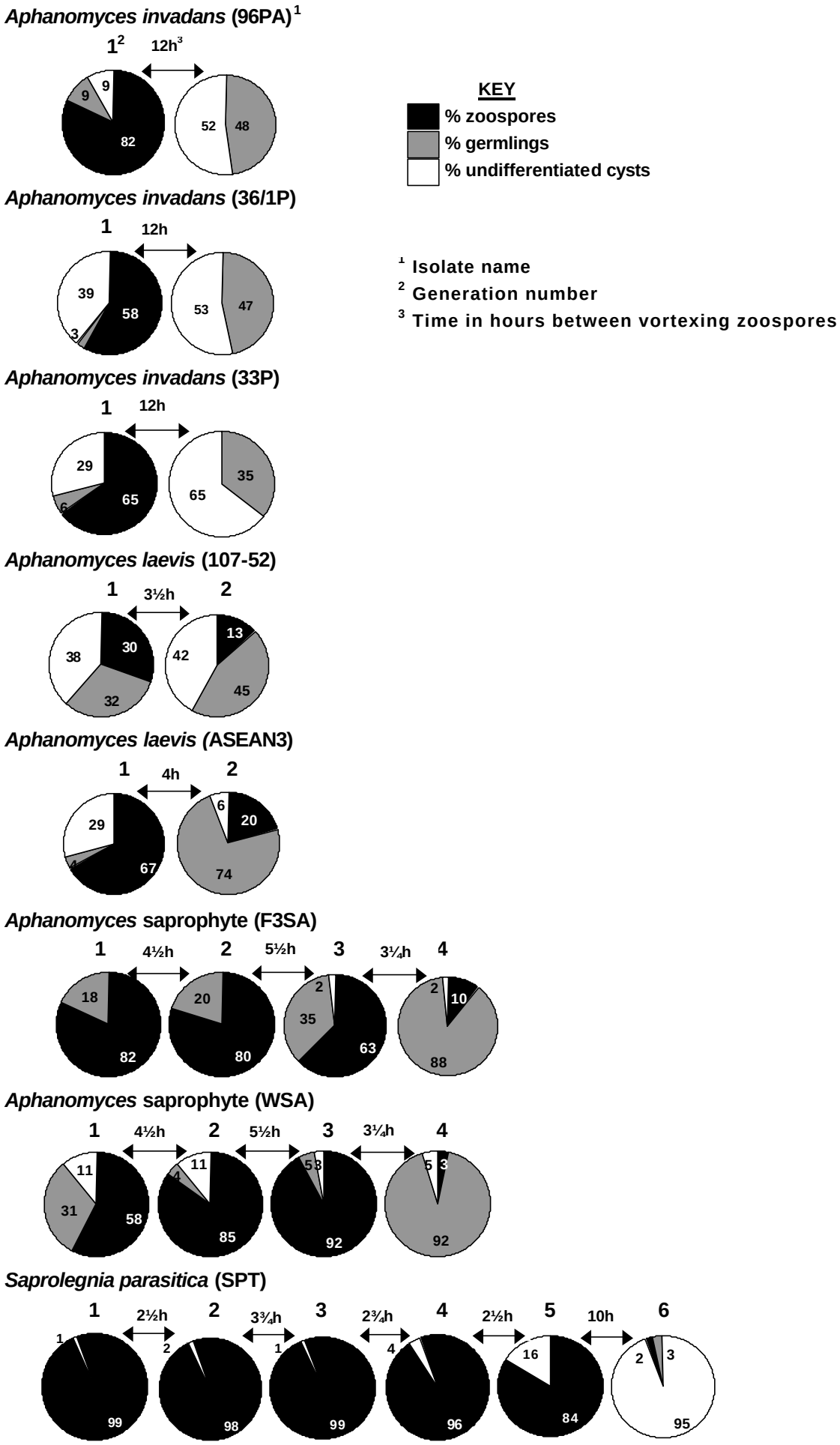


Figure 3 Percentage zoospore emergence in the presence of GPY medium (2 hours post-encystment)

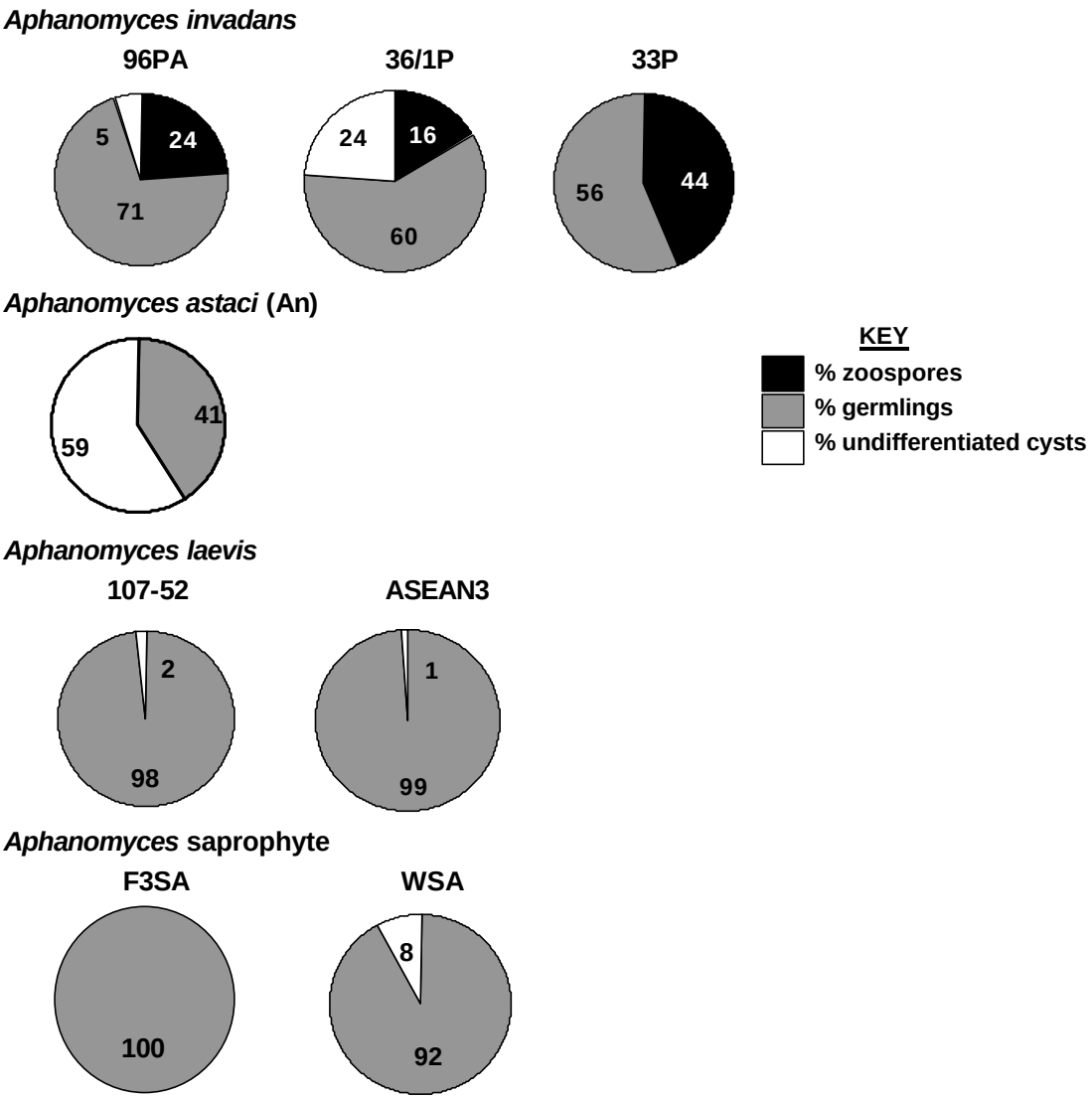
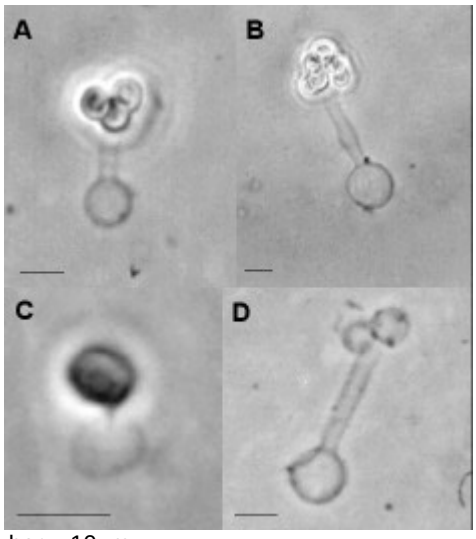


Figure 4



bar = 10µm

A *Aphanomyces invadans* mini-sporangium with a cluster of 3 cysts
B *A. invadans* mini-sporangium with a cluster of at least 6 cysts
C Tertiary zoospore stage emerging from a secondary cyst of *A. invadans*
D *A. invadans* mini-sporangium with a cluster of 2 empty cysts coats from which tertiary zoospore stages have been released

APPENDIX TWELVE

Article two - Lilley, J., and Panyawachira, W. (2000) Zoospore physiology of *Aphanomyces invadans* 2. Geotaxis. The AAHRI Newsletter, 9(1), Pp. 1-2 Aquatic Animal Health Research Institute, Bangkok (hard copy available from ARP Manager)

Zoospore physiology of *Aphanomyces invadans* 2. Geotaxis

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Motile secondary zoospores[1] of particular Oomycete fungi show negative geotaxis so that they accumulate towards the surface of a water body (Cameron and Carlile, 1977). In a survey of water samples from the Aswan High Dam Lake down to 20 metres, El-Hissy *et al* (1999) showed that surface waters yielded the highest number and diversity of aquatic fungi. However, there was some variation in the vertical distribution of different genera, with *Achlya* and *Pythium* accumulating in surface waters and other genera, including *Aphanomyces* and *Saprolegnia*, not showing any definite distribution pattern.

Microscope observations of suspensions of motile secondary zoospores of *Aphanomyces invadans* have indicated that they are less inclined to accumulate at the surface, and may actually be positively geotactic (Callinan, pers. comm.; Lilley, 1997), but no quantitative data are available. The present study was conducted to determine whether *A. invadans* zoospores show geotactic behaviour compared to other Oomycete genera.

Materials and methods

Zoospore suspensions of three fungal isolates listed in Fig. 1 were prepared as described by Willoughby and Roberts (1994). Briefly, fungi were grown in glucose-peptone yeast (GPY) broth for three days at 20^{o}C and the resulting mycelium washed by sequential transfer through five changes of filtered, autoclaved pond water, pH 6.4 (APW). Each isolate was left to sporulate overnight at 20^{o}C in duplicate Petri dishes containing 30mls of APW. The APW reached a vertical height of 6mm within the Petri dishes.

Motile zoospores in each set of duplicate Petri dishes were counted simultaneously using two improved Neubauer haemocytometers. Zoospores were sampled and counted in sequence, starting at the top, then middle, then bottom of the suspension of isolate T99G2. The same sequence was followed for ACHLYA99, and then TF20S. Counting was then restarted at the top of T99G2. This was continued until eight counts were made at each level of each Petri dish. The counting procedure lasted 5 hours. A count was made by pipetting 10µl of suspension onto one side of haemocytometer and determining the number of motile secondary zoospores in the 9-square grid under a microscope. (The average number of spores per square x 1000 = spores per ml).

Results

The total number of motile zoospores counted for each isolate is given in Fig. 1. The proportion of zoospores at each level of the Petri dish for each group of counts was calculated, averaged and plotted in Fig. 2(a) – (d). The ratio of zoospores at the top level of the Petri dish to that at the bottom level was 2.4 for *A. invadans* isolate T99G2, 2.6 for the *Saprolegnia* isolate TF20S, and 2.7 for the *Achlya* isolate ACHLYA99.

[1] See Lilley *et al*. 1999 for a diagram showing the asexual lifecycle of *Aphanomyces*

Proportions of zoospores at each level were not statistically different between the two duplicate dishes for isolates T99G2 and TF20S. Therefore data from both Petri dishes of these two isolates could be pooled for Fig. 2(a) and (b). The confidence limits in these Figures are therefore based on 16 counts per level per isolate. Isolate ACHLYA99 did show a difference between duplicate dishes (χ^2=59.06, P=0.00), and the data is presented separately in Fig. 2(c) – (d). Nonetheless, both dishes of ACHLYA99 also showed a strong accumulation of zoospores in the top level, the main difference between the dishes being a variation in the proportions in the two lower levels.

The data were also analysed to check whether there was a significant variation in the proportion of zoospores at each level over time. *A. invadans* (T99G2) showed an increasing proportion of zoospores accumulating at the top of the Petri dishes over the 5 hour experiment (Pearson's correlation, r=0.68, P=0.01 (2-tailed)), and a corresponding reduction in zoospores in the middle and bottom of the dishes. The *Saprolegnia* isolate showed a decrease in the proportion of zoospores in the middle (r=-0.64, P=0.01) and a corresponding slight increase in zoospores at both the top and bottom of the dishes over the experimental period. The *Achlya* isolate showed no significant variation in zoospore proportions over time.

Discussion

The present study shows that *A. invadans* zoospores do accumulate at the surface of suspensions, probably due to negative geotaxis, and that this tendency is comparable with isolates of *Saprolegnia* and *Achlya*. Earlier work may not have detected this tendency as *A. invadans* cultures are often poor sporulators and it is difficult to discern any trend when zoospore levels are low.

Cameron and Carlile (1977) reported that three species of *Phytophthora* showed 7 - 21 times more zoospores at the top 1mm of a 40mm capillary tube than at the bottom 1mm. The present study showed 2.4 - 2.7 times as many zoospores at the top level of Petri dish suspensions than at the bottom level, for the three species of fungi tested. However direct comparisons of top : bottom count ratios can not be made between these studies as different techniques are used, and previous unpublished studies indicated that zoospore proportions vary widely depending on the technique adopted.

Oomycete propagules have a higher density than water, and there remains a possibility that non-motile cysts of *A. invadans* could accumulate in the bottom waters, or pond sediment as postulated by Willoughby (1999), but this study shows that this would not be the case for actively swimming zoospores.

Conclusion

Aphanomyces invadans secondary zoospores are negatively geotactic, as with other Oomycete fungi that have been investigated.

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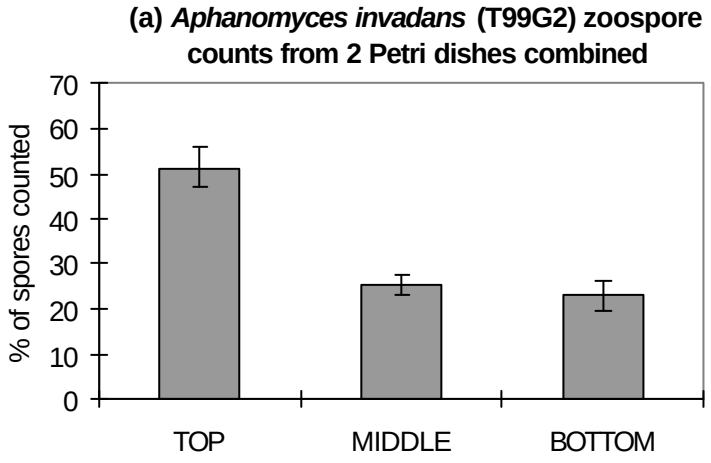
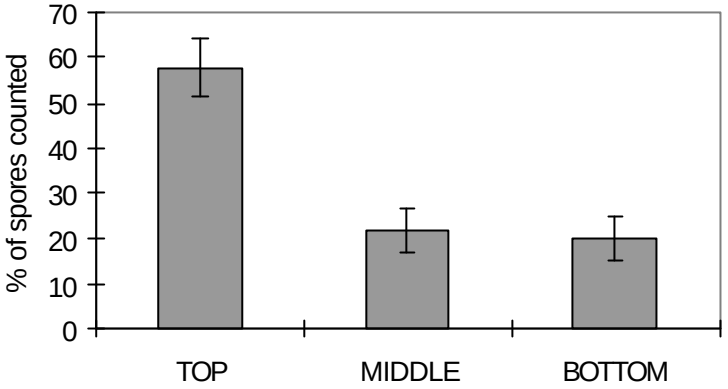
Figure 1 **Fungal isolates tested in the present study**

Isolate name	Species	Origin	Total number of zoospores counted
T99G2	*Aphanomyces invadans*	Muscle of EUS-affected giant gourami (*Osphronemus goramy*), November 1999, Thailand	3075
ACHLYA99	Saprophytic *Achlya* sp.	Surface of lesion on climbing perch (*Anabas testudineus*), December 1999, AAHRI wetlab, Thailand	3962
TF20S	Saprophytic *Saprolegnia* sp.	Surface of EUS-lesion on striped snakehead (*Channa striata*), December 1991, Udon Thani, Thailand	395

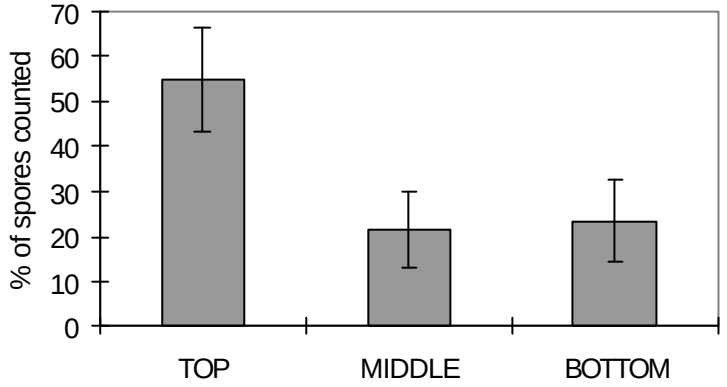
Figure 2 Proportion of motile zoospores counted at three different levels of six Petri dishes containing 30ml zoospore suspensions (Error bars show 95% confidence limits)



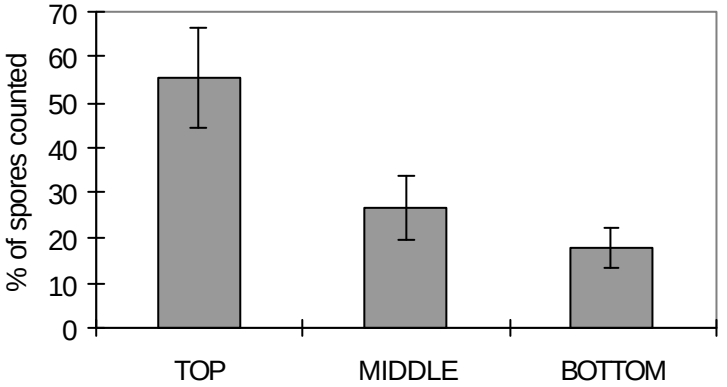
(b) *Saprolegnia* sp (TF20S) zoospore counts from 2 Petri dishes combined



(c) *Achlya* sp (ACHLYA99) zoospore counts from Petri dish 1



(d) *Achlya* sp (ACHLYA99) zoospore counts from Petri dish 2



APPENDIX THIRTEEN

Article three - Lilley, J., Petchinda, T., and Panyawachira, W. (2001) Zoospore physiology of *Aphanomyces invadans* 3. Techniques for inducing sporulation. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok. (in press)

Zoospore physiology of *Aphanomyces invadans*
3. Techniques for inducing sporulation

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Introduction

In order to identify the genus of an oomycete fungus, it is necessary to induce production of asexual fruiting bodies called zoosporangia. These structures release motile zoospores, which provide the fungi with a means of dispersal and host/substrate location. Good yields of motile zoospores are required for a large variety of experimental studies on oomycete fungi.

In order to sporulate *Aphanomyces invadans* (the fungal pathogen associated with epizootic ulcerative syndrome (EUS) outbreaks), cultures are normally grown for several days in nutrient broth media, the resulting wads of mycelium are washed to remove the media, and then left overnight in sporulating medium. At AAHRI, the most commonly used sporulating medium is autoclaved pond water (APW: Table 1).

The number of zoospores produced by a culture often decreases after repeated subculture of laboratory stocks. Therefore, procedures for triggering sporulation need to be optimized so that sufficient yields of zoospores can be obtained.

The experiment described here was initiated in order to find an acceptable alternative to APW. In studies at AAHRI to test a molecular probe for *A. invadans* in water, a sporulating medium was required that was not derived from pond water, to ensure that no external sources of *A. invadans* DNA would be added to sporulating cultures. Therefore, three sporulating media were compared against APW to identify the best alternative.

Methods

Aphanomyces invadans isolate B99C, obtained from an EUS-affected *Cirrhinus reba* in Bangladesh in March 1999, was grown on GPY agar (Willoughby and Roberts, 1994). For each experimental replicate, a 4mm agar plug of fungus was taken using a cork borer and incubated in a static Petri dish of GPY broth at 20^{o}C for three days. The resulting wad of mycelium was then sequentially transferred through four Petri dishes containing sterile distilled water, to wash out the nutrient growth medium. The wad was then transferred to a further Petri dish containing 25ml of one of the sporulating media listed in Table 1, and left for 20-24 hours at 20^{o}C. Four replicate Petri dishes were used for each of the four sporulating media. After incubation, each Petri dish was examined under a microscope for the presence of zoosporangia, and counts were made of the number of motile zoospores. For each Petri dish, four counts of nine squares on an improved Neubauer haemocytometer were made using 10µl samples taken from the middle of each Petri dish. The number of motile zoospores per millilitre was calculated by multiplying the average number of spores per square by 1000.

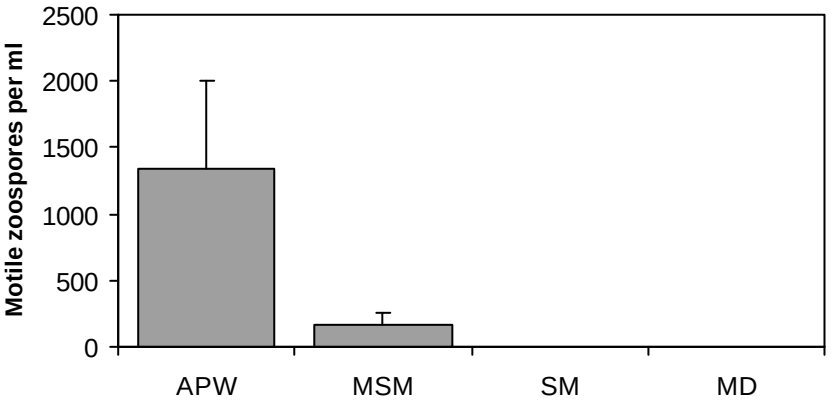
Table 1. Sporulating media tested

Sporulating medium	Medium contents
APW (adapted from Willoughby and Roberts, 1994)	Water was taken from a fish pond in Kasetsart University, filtered through Whatman 541 filter paper, mixed with two parts distilled water, adjusted to pH 7.0 and autoclaved. (water quality of the pond water was: alkalinity 161 mg/l, hardness 413 mg/l, DO 3.8 mg/l, pH 7.55, NO2 0.58 mg/l, NO3 1.30 mg/l, ammonia 0.06 mg/l, ortho-PO4 1.00 mg/l, total PO4 1.24 mg/l)
MSM (Kurata *et al,* 2000)	0.25mM KCl, 0.25mM $CaCl_2$, 20mM Hepes, adjusted to pH 7.2
SM (Griffin, 1978)	0.25mM KCl, 0.25 mM $CaCl_2$
MD (micronutrients used for GPY excluding glucose, peptone and yeast)	0.128g/l $MgSO_4.7H_2O$, 0.014g/l KH_2PO_4, 0.029g/l $CaCl_2.2H_2O$, 2.4mg/l $FeCl_3.6H_2O$, 1.8mg/l $MnCl_2.4H_2O$, 3.9mg/l $CuSO_4.5H_2O$, 0.4mg/l $ZnSO_4.7H_2O$

Results

The four cultures incubated in APW produced the highest counts of zoospores (mean=1340 spores/ml, Fig.1) and abundant zoosporangia. Cultures incubated in MSM produced intermediate numbers of zoospores (mean=163 spores/ml) and zoosporangia. Cultures incubated in SM and MD yielded no motile zoospores, although a few encysted zoospores were observed in the MD dishes. A few zoosporangia could be located in both SM and MD dishes.

Fig 1. Zoospore production using different sporulating media (mean ± 95% confidence limits)



Discussion

APW using Kasetsart pond water was shown to be the most effective sporulating medium for *A. invadans,* out of those tested here. The efficacy of the APW would, however, vary depending on the constituent micronutrients within the pond water used. MSM was the best alternative to APW in the

present study, and as it uses defined ingredients, its effect would be less variable. MSM would also be free of *A. invadans* DNA, and is therefore a good alternative for use in molecular studies.

Relatively low spore counts were achieved in all Petri dishes during the present study. Sihalath (1999) compared sporulation of a different isolate of *A. invadans* at a varying APW pH and incubating temperatures, and achieved maximum spore counts (12,150 spores/ml) using APW at pH 7.0 and at 22^{o}C. This was similar to conditions used for the APW sporulations here, but the counts here averaged only 1340 spores/ml. This may be due to repeated subculture of the B99C isolate used in the present study.

In separate studies, it has been found that spore counts can be further improved by using different substrates and growth media for the fungus, prior to sporulation. The highest *A. invadans* zoospore yields have been achieved by using a combination of hemp seeds (Willoughby and Pickering, 1977) and V8 media (Bimpong and Clerk, 1970). Sterile hemp seeds are placed on GPY agar cultures for several days to allow the fungus to attach to the seeds. The seeds are then transferred to V8 medium (5% Campbell's V8 Vegetable juice, 0.2% CaCO3, pH 6.4) for a further 2 days at 20^{o}C before using the APW washing technique. This method was shown to yield the highest number of zoospores (210,000 spores/ml) out of several methods tested by Marshall (1998), and was adopted by Khan *et al* (1998) for fish challenge experiments using *A. invadans* zoospores.

Acknowledgements

This work was supported by a grant from the Department for International Development of the UK.

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APPENDIX FOURTEEN

Article four - Lilley, J., Petchinda, T., and Panyawachira, W. (2001) Zoospore physiology of *Aphanomyces invadans* 4. *In vitro* viability of cysts. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok (in press)

Aphanomyces invadans zoospore physiology: 4. *In vitro* viability of cysts

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Introduction

To date, *Aphanomyces invadans* has only been isolated from internal tissues of fish affected with epizootic ulcerative syndrome (EUS), and there is no experimental evidence to show that it can survive for long in water or on non-fish substrates. Attempts to isolate the fungus from pond water and soil have not been successful (Willoughby *et al*, 1999). G.C. Fraser (unpublished data) has observed *A. invadans*-like colonies in water assays, but only during EUS outbreaks. Observations of the fungus have revealed no resistant spore stages that would indicate an ability to survive long periods away from a host fish.

Preliminary *in vitro* studies indicated that the asexual zoospore stages of *A. invadans* did not survive longer than nine days in sterile pond water. On this basis, Lilley *et al* (1998) suggested that one option for preventing the entry of infective propagules to fish farms at risk of EUS is to store inlet water in a fish-free reservoir for at least ten days before use.

Eradication of a fungal pathogen by excluding susceptible and carrier hosts was also recommended as a means of controlling crayfish plague (Unestam, 1969; Söderhäll *et al,* 1977). Mathews and Reynolds (1990) showed that crayfish did not get infected after spores of the pathogen *Aphanomyces astaci* were left for nine days at 10^{o}C. However, at other temperatures this time period may be much longer, as Unestam (1966) reported that a spore suspension stored for two months at 2^{o}C still contained viable spores.

The present experiment was conducted to further investigate how long encysted zoospores of *A. invadans* kept in sterile pond water would retain the ability to germinate and grow in nutrient media, and whether the resulting cultures would be able to infect snakehead fish.

Methods

Aphanomyces invadans isolate B99C, obtained from an EUS-affected *Cirrhinus reba* in Bangladesh in March 1999, was grown on GPY agar (Lilley *et al*, 1998). Four 4mm plugs of the culture were each transferred to a Petri dish containing GPY broth and grown for three days at 20^{o}C. The resulting wads were brought through 4 washes of sterile distilled water and left overnight in autoclaved pond water (APW: Lilley *et al*, 1998) at 20^{o}C to sporulate. After 24 hours the number of motile zoospores was counted using an improved Neubauer haemocytometer. Three of the sporulating cultures (A, B & C) were then kept at 20^{o}C and one (D) at room temperature (25-30^{o}C).

Each day for 23 days, 1 ml of APW was pipetted from each of the Petri dishes and added to sterile Petri dishes containing 25 ml GPPO broth (GP broth containing 100 units/ml penicillin-K and 10 µg/ml oxolinic acid). APW was taken from the bottom of the water column several centimetres from the mycelium wad, to ensure that the aliquot contained encysted secondary zoospores. On a few occasions, It is thought that fragments of hyphae were also picked up during pipetting, as the resulting colonies in GPPO were not of regular size. However, if these sample days are eliminated from the

experiment, the overall results given in Table 1 are not affected. The GPPO re-isolation cultures were incubated at 20°C, and when visible colonies were observed (usually after 5-6 days), these were transferred into Petri dishes containing APW and left overnight at 20°C to induce sporulation and confirm that the colonies were indeed *Aphanomyces*.

In order to determine whether the re-isolated cultures were *A. invadans* and remained pathogenic, 0.1ml of three of the sporulating cultures (taken from dish A on days 7 and 17, and dish B on day 7) were injected into snakeheads. For each culture, two fish were injected intramuscularly on the left flank below the dorsal fin, and kept in static 20-litre tanks at 22°C. No sporulation was achieved in cultures derived from dish B on day 17, but in order to further confirm that these were contaminant fungi, and not *A. invadans*, mycelium was inserted subcutaneously in two snakeheads as described by Roberts *et al*, (1993). All fish were sampled after ten days for histology. Sections were stained with Grocott's and counterstained with Haematoxylin and Eosin (Chinabut and Roberts, 1999).

Results

The concentrations of motile zoospores in each of the Petri dishes ranged from 500 – 3417 spores per ml (Table 1). No attempt was made to adjust the concentration to reduce the chance of contamination.

The longest period that *A. invadans* cysts remained viable when pipetted into GPPO broth was 19 days, from dish A kept at 20°C (Table 1). Cysts in the other dishes kept at 20°C could no longer be grown into colonies after 14 and 8 days. Cysts in dish D, kept at room temperature, could not be induced to grow after day 4.

Contaminant fungal colonies were observed in all dishes, but in most cases this was after the last day that *A. invadans* could be re-isolated (Table 1). Bacterial contamination was observed in two of the dishes (B and C) but only after the last day that *A. invadans* was re-isolated in those dishes (Table 1).

Table 1. Characteristics of the four zoospore suspensions

Dish (incubating temperature)	Motile zoospore count on day 1 (spores/ml)	Last day (ps) *Aphanomyces* successfully re-isolated	First day (ps) contaminant fungi observed	First day (ps) contaminant bacteria observed
A (20°C)	3417	19	21	None
B (20°C)	956	14	9	16
C (20°C)	500	8	9	10
D (rt)	583	4	5	None

rt = room temperature
ps = post-sporulation

Zoospore suspensions of re-isolated fungi, taken from dishes A and B on day 7 after sporulation, resulted in clinical lesions in all snakeheads by day 10 post-injection (Table 2). Invasive fungus and inflammatory reaction, typical of EUS lesions, were observed in histological sections of these fish. A similar result was obtained from fish injected with re-isolated fungi taken from dish A on day 17 after

sporulation, but no lesions or invasive fungus were observed in fish inoculated with mycelium derived from dish B on day 17.

Table 2. Pathogenicity of four re-isolated cultures

Dish (incubating temperature)	Day re-isolated (ps)	Motile zoospore count (spores/ml)	Clinical signs 10 days pi	Histopathology 10 days pi
A (20°C)	7	333	Lesion at injection site	Invasive fungus
A (20°C)	17	306	Lesion at injection site	Invasive fungus
B (20°C)	7	222	Lesion at injection site	Invasive fungus
B (20°C)	17	ns	No visible lesion	No invasive fungus

ps = post-sporulation
ns = no sporulation, therefore mycelium used to inoculate fish
pi = post-injection

Discussion

The present experiment shows that encysted zoospores of *A. invadans* can survive for at least 19 days *in vitro* at 20°C. It also demonstrated that cultures isolated from cysts 17 days post-sporulation are still pathogenic to snakeheads.

This has implications for control strategies against EUS. If high risk ponds can not be dried and limed before stocking, it is recommended that they are fallowed for at least 20 days. Similarly, inlet water taken from high risk areas that can not otherwise be disinfected, could be retained for at least 20 days in a fish-free reservoir prior to use. It is less than ideal to extrapolate *in vitro* trials to field situations, but as there is no reliable technique for isolating *A. invadans* from pond water, there is presently no other indicator of how long cysts remain viable outside of fish hosts.

In the present study, it is possible that growth of fungal and bacterial contaminants in some of the Petri dishes may have affected the viability of the *A. invadans* cysts, accounting for the different results between dishes A-C. Although in natural pond water *A. invadans* cysts would encounter many competing microbes, the experimental cultures were kept axenic for as long as possible, as unnaturally high concentrations of contaminants can develop quickly within Petri dishes. Temperature also has an important impact on viability, and in the present experiment, *A. invadans* cysts in dish D kept at higher temperatures (25-30°C) remained viable for only four days. However, these developed contaminant fungi only one day later, which could have reduced the viability of the cysts.

Acknowledgements

This work was supported by a grant from the Department for International Development of the UK.

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APPENDIX FIFTEEN

Paper ten - Khan, M.H., Marshall, L., Thompson, K.D., Campbell, R.E., and Lilley, J.H. (1998) Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the Oomycete fish pathogen, *Aphanomyces invadans*. Bulletin of the European Association of Fish Pathologists 18(6), 192-197. (hard copy available from ARP Manager)

Bull. Eur. Ass. Fish Pathol. 18(6),192, 1998.

SUSCEPTIBILITY OF FIVE FISH SPECIES (NILE TILAPIA, ROSY BARB, RAINBOW TROUT, STICKLEBACK AND ROACH) TO INTRAMUSCULAR INJECTION WITH THE OOMYCETE FISH PATHOGEN, *APHANOMYCES INVADANS*

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Abstract

Tilapia (Oreochromis niloticus), rosy barb (Puntius schwanenfeldi), rainbow trout (Oncorhynchus mykiss), roach (Rutilus rutilus) and stickleback (Gasterosteus aculeatus) were challenged with Aphanomyces invadans, the Oomycete fungus considered to be the causative agent of epizootic ulcerative syndrome (EUS). At least 16 fish of each species were injected intramuscularly with 1-15 x 10^3 zoospores of A. invadans, kept at 11-24°C depending on species, and periodically sampled for histology and immunohistochemistry using anti-A. invadans polyclonal antiserum. Most tilapia remained healthy throughout the experiment with no significant histopathological changes, except for two tilapia, which died on days 20 and 21 post-injection (p.i.) showing invasive fungal growth and typical EUS-type lesions. All rosy barbs rapidly developed clinical signs of EUS. Fungus was seen in histological sections by day 7 p.i. In addition, all fish died by day 22 p.i. Rainbow trout also demonstrated signs of fungal growth and the progressive development of lesions, but these occurred at a slower rate than in the rosy barbs. The sticklebacks remained clinically and histologically normal, except for two fish, which died on day 13 p.i. with fungal infection of the integument and peritoneal cavity. However, this is believed to have been a secondary non-invasive fungus. Most roach displayed some scale loss and mild histological changes, but two roach sampled on days 25 and 35 p.i., showed typical EUS histopathology with fungal hyphae associated with muscle necrosis and a granulomatous inflammatory response.

Introduction

Aphanomyces invadans is an Oomycete fungus that is considered to be the necessary biological cause of epizootic ulcerative syndrome (EUS), a disease affecting a large number of wild and cultured fish species in Asia (Roberts *et al*, 1994). EUS is now recognised as the same disease as mycotic granulomatosis (MG) in Japan (Hatai, 1994) and red spot disease (RSD) in Australia (Callinan *et al.*, 1995). Willoughby *et al.* (1995) described the causative fungus as *Aphanomyces invaderis*, and it has since been listed in the Index of Fungi as *A. invadans* (David and Kirk, 1997).

Aphanomyces invadans zoospores have been shown to be unlike those of other Oomycete fungi in that they are able to germinate and grow invasively in EUS-susceptible fish (Lilley and Roberts, 1997), resulting in the development of typical EUS lesions (Chinabut *et al*, 1995). The susceptibility of five fish species to *A. invadans* infection is tested here. Four of these have not previously been experimentally challenged. Of these, tilapia are considered to be resistant to EUS, whereas *Puntius* spp (including rosy barbs) are among the most acutely affected (Roberts *et al.*, 1994). Two native UK species previously not exposed to EUS (stickleback and roach) are also tested. The fifth species, rainbow trout, has previously been shown to succumb to *A. invadans* infection under particular conditions (Thompson, unpublished).

Materials and methods

Fish

Twenty tilapia (*Oreochromis niloticus*), rosy barbs (*Puntius schwanenfeldi*), and rainbow trout (*Oncorhynchus mykiss*), and 16 roach (*Rutilus rutilus*) and stickleback (*Gasterosteus aculeatus*) were tested for their susceptibility to *A. invadans*. The tilapia averaged 100g in weight and were bred at the Institute of Aquaculture, Stirling University. Rosy barbs, weighing 4g, were obtained from Aquatic Habitat, UK. Rainbow trout, approximately 200g in weight, were obtained from a local fish farm. Sticklebacks and

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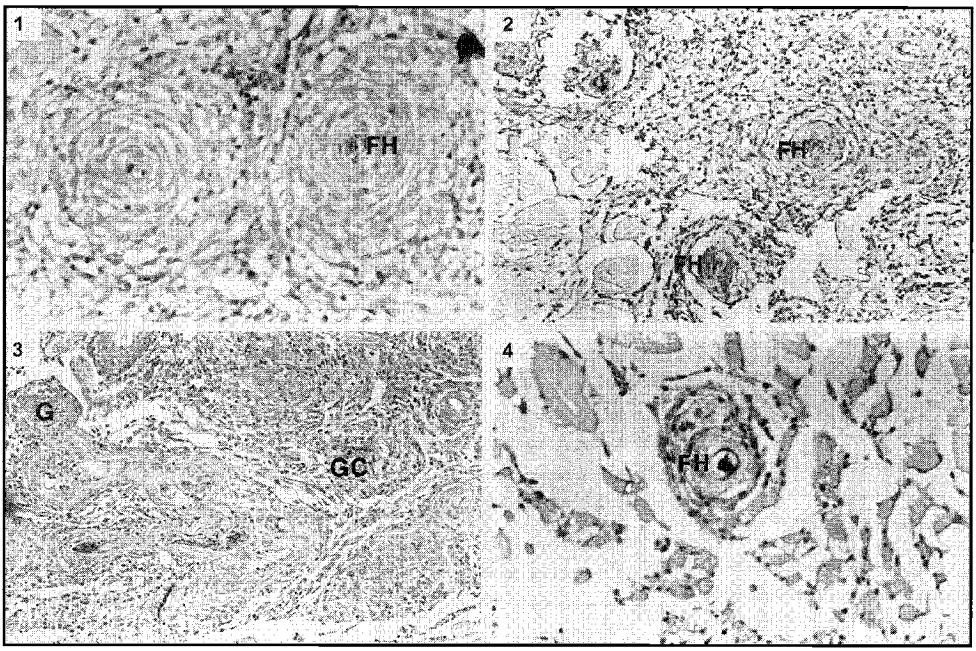


Legend

Cross-sections of fish muscle tissue after intramuscular injection with *Aphanomyces invadans* zoospores (stained by either H&E or immunohistochemistry IHC)

Fig 1 Tilapia, day 20 p.i., one of two fish sampled post-mortem, showing fungal hyphae (FH) enclosed in granulomas (H&E x40 objective). **Fig 2** Tilapia, day 20 p.i., one of 2 fish sampled post-mortem, showing EUS-like granulomas surrounding fungal hyphae (FH) (IHC x20 objective). **Fig 3** Rosy barb, day 7 p.i., showing rapid development of mycotic granuloma (G) and giant cell (GC) (H&E x20 objective). **Fig 4** Rosy barb, day 7 p.i., showing early EUS mycotic granuloma forming around fungal hypha (FH) (IHC x40 objective).

University. The average weight of the sticklebacks was 1.2g, and of the roach was 46g. The tilapia and rainbow trout were placed in covered plastic tanks holding 75L of flow-through water, heated to 18°C. The rosy barbs were maintained in 25L plastic tanks with static water heated to 24°C and partial water changes were made daily throughout the trial. Sticklebacks and roach were kept in 300L flow-through tanks, at temperatures ranging between 11-16°C. The water was dechlorinated and aerated through airstones. The fish were provided with commercial feed to satiation. All five groups of fish were acclimatized to their given water temperature for at least 1 week prior to commencing the challenge.

Preparation of fungal spores:

Aphanomyces invadans isolate PA7 was used to challenge the fish. This was isolated from snakehead fish *(Channa striata)* during an EUS outbreak in Thailand, as described by Lilley and Roberts (1997). The isolates were cultured at 22°C on Petri dishes of glucose-peptone agar (GP). Suspensions of motile secondary zoospores for the challenge of the tilapia, rosy barbs and trout, were produced following the method of Willoughby and Roberts (1994) using 4mm agar plugs of fungus incubated in GP broth at 22°C for 48

hours, then washed in autoclaved pond water (APW), and left overnight. An improved spore production method was adopted for the challenge of sticklebacks and roach. This involved growing *A. invadans* mycelium over sterile hemp seeds, and placing the "bearded" seeds in V8 broth (5% Campbells V8 Juice, 0.2% $CaCO_3$, pH 6.1) for 48 hours before washing in APW and leaving overnight as before.

Challenge with *A. invadans* spores: All fish were injected intramuscularly, just below the anterior part of the dorsal fin with a suspension of motile secondary zoospores. The volume injected was 0.1ml in all cases except for the sticklebacks, which, because of their small size, received 0.05ml. Spore counts indicated that tilapia, rosy barbs and trout were injected with 1×10^3 spores, sticklebacks received 7.5×10^3 spores and roach received 15×10^3 spores. Control fish were injected with APW alone. The trials ran for 22-40 days, depending on EUS-associated mortalities. Two fish of each species were sampled periodically for histological examination.

Histology & Immunohistochemistry (IHC): The sampled tissue blocks were fixed, processed for histology and stained with haematoxylin and eosin (H&E) (Drury & Wallington, 1980). Fungal hyphae were visualised using IHC. This was performed using anti-*A. invadans* polyclonal antiserum as described in Lilley *et al* (1997). Fungus in IHC-stained tissue sections appeared brown in colour.

Results

Tilapia (Figs 1 & 2)

Most tilapia inoculated with *A. invadans* spores showed no signs of pathological changes. As with the other species, including control fish, mild inflammatory infiltration in the region of traumatic damage caused by the insertion of the needle, was observed immediately after the inoculation of the spores. By day 5 p.i., the inoculated area consisted of focal haemorrhaging with localised macrophage infiltration and a few degenerated muscle bundles.

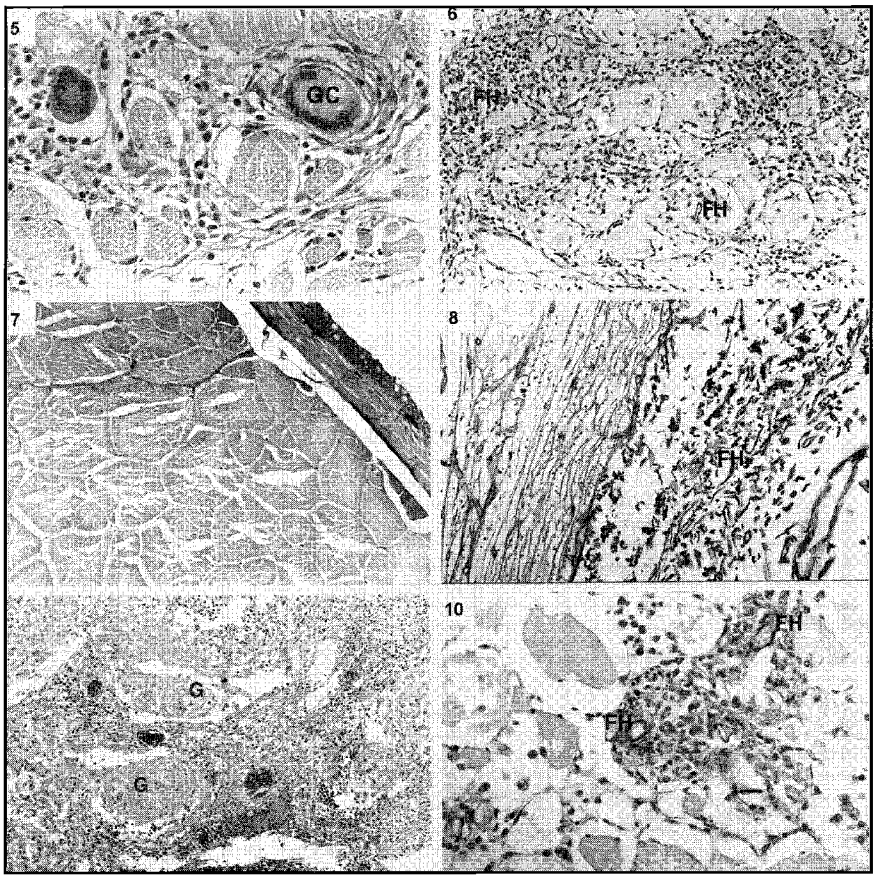
Three tilapia did, however, show minor swelling at the site of injection 13 days p.i., and two died at days 20 and 21 p.i. These were sampled within an hour of death and histology sections revealed a dramatic granulomatic response with fibrous tissue layer formation (Fig 1). A few lymphocyte aggregations were also observed in the affected areas. Fungal hyphae were detected inside the granulomas by IHC and were surrounded by epithelioid cells (Fig 2). Widespread myodegeneration and myophagia was observed. A few foreign body type giant cells were also apparent.

Rosy barbs (Figs 3 & 4)

Rosy barbs started to develop EUS-like lesions only 5 days after inoculation with *A. invadans* spores. This coincided with the start of disease-related mortalities. All fish in this group died by day 22 p.i. Two days after inoculation, macrophage infiltration was observed in the muscle around the injection site and around the adjacent peritoneal fat tissue. Some necrosis was also observed. The degenerative changes became more advanced between days 7 and 14 p.i. Epithelioid tissue surrounded the fungus and there was a tendency for mycotic granulomas to form, with a layer of fibrous tissue (Fig 3). Fungal hyphal growth was first detected in sections sampled on day 7 p.i. (Fig 4). Foreign body giant cells, with and without fungus inside, were also observed in samples on day 7 p.i.. By day 20 p.i. there was some evidence of muscle regeneration and melanisation in internal muscle, suggesting the remaining fish had the potential to recover. No lesions developed in control fish and no mortalities were recorded.

Rainbow trout (Figs 5 & 6)

In rainbow trout, only mild pathological changes were seen until day 10 p.i., with some myotic degeneration and macrophage accumulation at the injection site. After day 10 p.i., a massive inflammatory response accompanied muscle degeneration in areas



Legend

Cross-sections of fish muscle tissue after intramuscular injection with *Aphanomyces invadans* zoospores (stained by either H&E or immunohistochemistry IHC)

Fig 5 Rainbow trout, day 15 p.i., showing Langhans multinucleate giant cell (GC) (H&E x40 objective). **Fig 6** Rainbow trout day 14 p.i., showing invasive growth of fungal hyphae (FH) (IHC x40 objective). **Fig 7** Stickleback, day 5 p.i., with mild scale damage and no *A. invadans*–related pathology (H&E x20 objective). **Fig 8** Stickleback, day 13 p.i., with secondary external fungal (FH) infection (IHC x20 objective). **Fig 9** Roach, day 35 p.i., one of 2 fish showing EUS-like granuloma formation (H&E x20 objective). **Fig 10** Roach, day 25 p.i., one of 2 fish in which fungal hyphae (FH) can be visualised with an associated chronic granulomatous response (IHC x20 objective)

further away from the site of injection. Many fungal hyphae were seen, surrounded by epithelioid cells (Fig 6). Fish mortalities started to occur by day 14 p.i. In histological samples many granulomatic structures were found after day 14 p.i., with fungal hyphae in the centre, and fibrous tissue surrounding the granulomas. Huge areas of myonecrosis and myophagia were observed around the granulomas. In addition to typical EUS-like granulomas, two types of giant cell were observed in the infected regions of muscles: Langhans and foreign body type (Fig 5).

Control fish were healthy and survived for the whole period of the experiment.

Sticklebacks (Figs 7 & 8)
From day 5-40 pi., all except two sticklebacks were consistent with the controls and appeared normal with no dermatitis or development of external lesions (Fig 7). Muscle blocks displayed areas of mild tissue damage and a low-level presence of melanophores. No fungal invasion was visualised using IHC. Two sticklebacks died on day 13 p.i. with a large amount of fungus growing on the external cutaneous integument. Histological examination of these fish showed scale erosion and extensive myodegradation but no granulomatous response could be seen. IHC revealed an extensive fungal infection with hyphae growing both on the skin (Fig 8) and within the peritoneal cavity.

Roach (Figs 9 & 10)
Varying degrees of scale loss were shown by all fish during the trial. By day 5 p.i. dark patches, which resembled necrosis or haemorrhaging, had developed under the skin. Histologically, small, patchy areas of muscle damage were observed around the site of injection. The fish sampled on day 10, 15 and 20 days p.i. had similar external signs along with scale loss. Inflammation cell infiltration and further cloudy degeneration was observed in these samples around the site of injection. All tissues also contained some melanophores near the surface of the skin. IHC on these sections failed to show any signs of fungal invasion. On day 25 p.i., the fish displayed only minor scale loss, but the muscle block from one fish appeared very soft and discolored. Histologically, large areas of cloudy degeneration were seen, along with clumps of inflammatory cells which had, in some areas, formed small granuloma. IHC of this section visualised the presence of fungal hyphae (Fig 10). The second fish sampled on this day, and two fish sampled on day 30 p.i., displayed a lesser amount of degradation and inflammation, and IHC failed to visualise any fungal hyphae. On day 35 p.i., one of the fish sampled had a large swollen area on the right flank with a small red lesion near the site of injection. The muscle removed from under this area was very soft and necrotic with intense histological changes. Myodegradation was severe, with a high presence of inflammatory cells (Fig.9). IHC visualised fungal hyphae with granuloma and fibrous tissue formation.

Discussion
The results from this experiment provide confirmation that zoospores of *A. invadans* can germinate and reproduce typical EUS lesions in the muscle tissue of rosy barbs and rainbow trout. Evidence is also given that *A. invadans* can grow within muscle tissue of a few, probably immunosupressed, individuals of tilapia and roach. The small stickleback fish tested here appeared remarkably resistant to *A. invadans* infection.
The defence mechanism of the rosy barbs were generally insufficient to halt the spread of hyphae to neighbouring tissues, although by day 20 p.i., some fish were attempting to repair damaged tissue. In the rosy barbs, only a few foreign body type giant cells were found irregularly distributed in infected areas on days 10 and 14 p.i. Rainbow trout showed a more advanced inflammatory response, with signs of vascularisation and a large number of both Langhans and foreign body type giant cells engulfing the fungal hyphae. This corresponds with artificial infections described by Wada *et al.* (1996), using an MG isolate of *A. invadans*, who showed a greater giant cell proliferation in EUS-resistant carp than in EUS-susceptible ayu, and considered this to be an important aspect of the fishes cellular defence against fungal invasion. However, as the disease continued to progress in rainbow trout described here, it is apparent that such a response, involving the formation of many giant cells, does not necessarily indicate an ability to control the penetrating hyphae. In tilapia, giant cells were observed as part of a significant inflammatory response only in sections sampled from two infected dead fish, and were therefore only stimulated by active fungal growth. The two roach that became infected by *A. invadans* also mounted a massive in-

flammatory response, but giant cell formation was not a visible component of this.

The fungal infection of two of the sticklebacks was mainly concentrated on the skin and had not stimulated a granulomatous inflammatory response, suggesting that this was a secondary fungal invasion by a saprolegniacean fungal opportunist contained in the natural flora of the tank water, and not *A. invadans*. It is not unusual that such a fungus was visualised by IHC using anti-*A. invadans* polyclonal antiserum, as the antiserum is known to be cross-reactive with other saprolegniacean fungi (Lilley *et al.*, 1997).

In the natural environment, *A. invadans* spores probably can not penetrate the fish skin barrier unless it has been subjected to mechanical injury, or some form of dermatitis (Chinabut *et al.* 1995). Fish are also protected to some degree by non-specific defence mechanisms present in the mucus (Ellis, 1989). Tilapia are considered to be EUS-resistant, so it is possible that in the case of the two tilapia that became infected in the present study, the injection of spores into the muscle circumvented the normal means of protection.

Chinabut *et al* (1995) has previously shown temperature to be a crucial factor governing the ability of EUS-susceptible fish to combat infection by *A. invadans*, and it should also be considered in the interpretation of the present results. Rosy barbs are clearly susceptible at 24°C, although as some fish showed signs of recovery after 20 days p.i., it is possible that rosy barbs may be significantly more resistant at even slightly higher temperatures. In contrast to some tropical fish, which appear to be less susceptible at temperatures of 26°C or over (Chinabut *et al.*, 1995), Thompson (unpublished) has shown that rainbow trout are less susceptible to *A. invadans* infection at temperatures below 18°C. Therefore, it is possible that roach could be more susceptible to infection at higher temperatures than the 11-16°C tested here.

The potential for transmission of EUS to some temperate fish species (such as rainbow trout and roach) via susceptible ornamental species (like rosy barbs) is clearly demonstrated. However, whether EUS would be a significant problem in temperate areas is uncertain given that the UK fish tested here are not as easily infected as typical EUS-susceptible fish such as *Puntius* spp and snakeheads, even when the pathogen is introduced by intramuscular injection.

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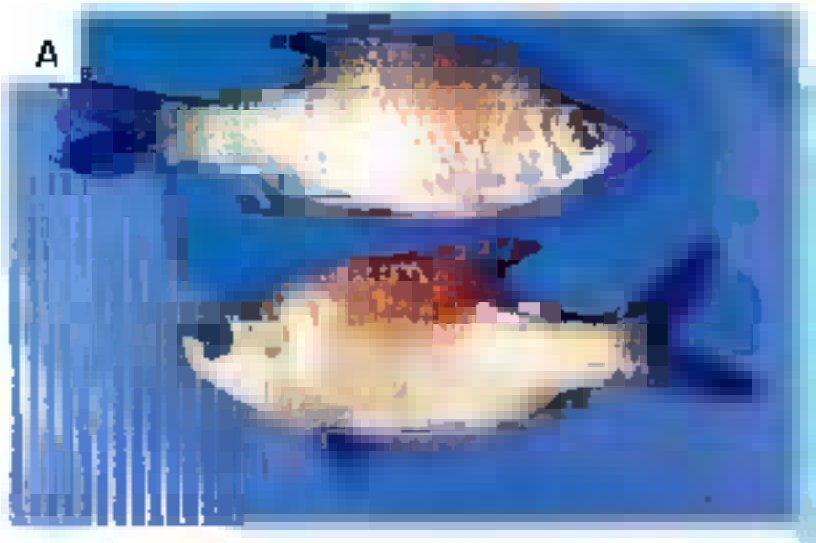
APPENDIX SIXTEEN

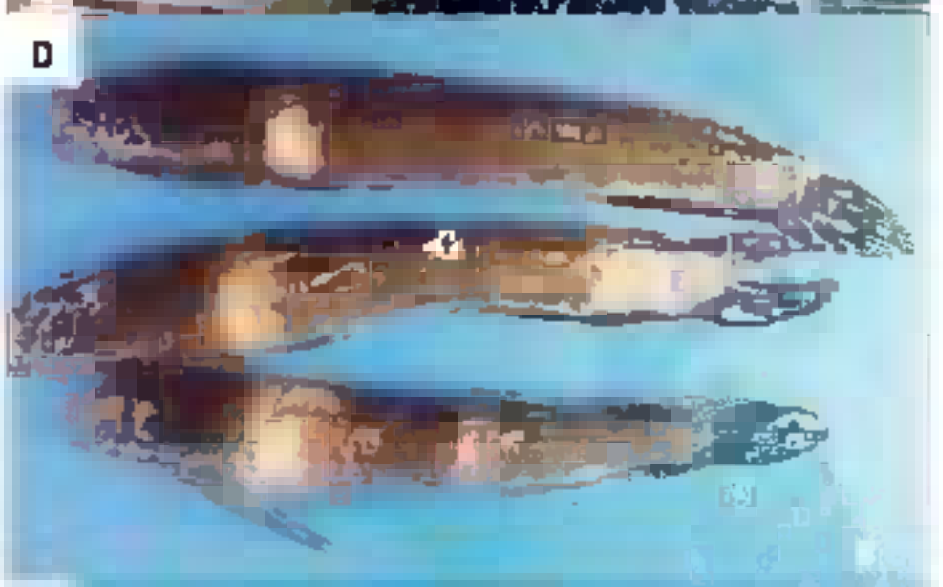
Development of an immersion challenge system (hard copy of figures available from ARP Manager)

DEVELOPMENT OF AN IMMERSION CHALLENGE SYSTEM



Injection trial
A Puntius with EUS lesions day 14 post injection
B Snakeheads with EUS lesions day 20 post-injection

Immersion trials
C. Snakeheads exposed to sporulating *Aphanomyces invadans* culture in tube



D Snakeheads abraded on left flank before bath challenge with *A. invadans* zoospores Day 21 post-challenge

E Snakeheads exposed to pH 5 for 30 min, before bath challenge with *A. invadans* zoospores Day 22 post-challenge

APPENDIX SEVENTEEN

Paper eleven - Campbell, R.E., Lilley, J.H., Taukhid, Panyawachirab, V., and Kanchanakhan, S. (2001) *In vitro* screening of novel treatments for *Aphanomyces invadans*. Aquaculture Research (in press) (hard copy available from ARP Manager)

In vitro screening of novel treatments for *Aphanomyces invadans*

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Running title:

Novel treatments for *Aphanomyces invadans*

Abstract

Forty-nine compounds were tested *in vitro* for fungicidal activity against hyphae of the fish-pathogenic Oomycete fungus, *Aphanomyces invadans*. These comprised: (a) chemicals with previous reported activity against Oomycete fungi; (b) chemicals in use in Asia to treat ulcerative disease outbreaks; (c) commercial biocides and fungicides; (d) natural products with potential anti-microbial activity; and (e) surfactants tested separately, and in combination, with some of the above treatments. No compounds tested proved as effective as malachite green, but some low toxicity natural compounds and particular surfactants showed potential for further studies. Some compounds that are currently in use in Asian aquaculture were shown to have no effect on *A. invadans* hyphae at recommended treatment rates. A range of compounds which demonstrated activity against the mycelium were selected for further testing in a zoospore motility assay. Lower treatment concentrations were required to inhibit zoospore motility than were required to inhibit hyphal growth. Zoospore activity ceased within 1 hour exposure to 2.5 parts per million (ppm) coconut diethanolamide; 1.25ppm propolis + 0.5ppm 13/6.5; 5ppm neem *(Azadirachta siamensis)* seed extract + 0.01ppm OP10; 20ppm tea tree *(Melaleuca alternifloria)* oil; and 25ppm D-limonene + 0.05ppm E-Z-Mulse™. The treated spores were shown to be non-viable in culture medium. Selected compounds were further tested for ability to inhibit zoospore production by *A. invadans* mycelium over a 72-hour period. In toxicity trials, silver barb (*Barbodes gonionotus*) exposed to 2.5ppm coconut diethanolamide; 2.5ppm OP10; 0.5ppm E-Z-Mulse™; 20ppm neem seed extract + 0.5ppm OP10; and 5ppm D-limonene + 0.5ppm E-Z-Mulse™ suffered no mortalities and no obvious behavioural changes. Similarly, rainbow trout (*Oncorhynchus mykiss*) exposed to 25ppm propolis + 1ppm OP10; 10ppm neem seed extract + 0.01ppm 13/6.5; and 10ppm D-limonene + 0.01ppm OP10 suffered no mortalities and no obvious behavioural changes.

Introduction

Aphanomyces invadans (Willoughby, Roberts & Chinabut 1995) is an invasive Oomycete fungal pathogen of Asian and Australian freshwater and estuarine fishes. The distinctive mycotic granulomas caused by *A. invadans* infection of internal fish tissues are considered the main diagnostic feature of epizootic ulcerative syndrome (EUS) (Roberts, Willoughby & Chinabut 1993; Vishwanath, Mohan & Shankar 1997). The earliest recorded outbreaks of

EUS occurred in Japan, where the disease is known as mycotic granulomatosis (MG) and *A. invadans* is referred to as *Aphanomyces piscicida* (Kurata, Kanai & Hatai 2000). EUS in Australia is also known as red spot disease (Callinan, Paclibare, Bondad-Reantaso, Chin, & Gogolewski, 1995).

There is no accepted treatment for farmed fish infected with *A. invadans,* though several useful preventative measures, such as exclusion of particular fish species and liming of fish ponds have been identified. Ahmed & Rab (1995) calculated that post-stocking applications of 9 kg ha^{-1} (9ppm) agricultural lime ($CaCO_3$) reduced the risk of EUS in Bangladesh culture ponds. Claims have also been made for the curative properties of certain indigenous plants and natural products, but none has so far been investigated for fungicidal activity against *A. invadans.*

In this study, five groups of products were assessed for activity against *A. invadans*: (a) chemicals with previous reported activity against Oomycete fungi; (b) chemicals in use in Asia to treat EUS outbreaks; (c) commercial biocides and fungicides; (d) natural products with potential anti-microbial activity; and (e) surfactants tested separately, and in combination, with some of the above treatments. The chemicals were first screened against *A. invadans* mycelium, and some products were selected for further testing in a zoospore motility assay. By combining these two assay methods and screening a wide range of chemicals, it was hoped that effective treatments would be identified which would be inexpensive, readily available, and appropriate for use in Asian aquaculture systems.

Materials and methods

Fungus

Aphanomyces invadans strain PA8 was used in all experiments. Lilley & Inglis (1997) had previously shown little variation in susceptibility to three fungicides between 26 isolates of *A. invadans*. PA8 was isolated from an EUS-affected snakehead, *Channa striata* (Bloch), in Thailand in 1995. Further isolation, growth and pathogenicity details are given by Lilley & Roberts (1997). The fungus was maintained at 20°C on Petri dishes of glucose peptone (GP) medium (Roberts *et al*. 1993) with 1.2% w/v agar. Agar plugs (4mm) taken from the edge of colonies less than seven days old were used in all experiments.

Agar plug transfer assay

The method of screening fungicide treatments used by Lilley & Inglis (1997), adapted from Bailey (1983), was used here. In this agar plug transfer method, growth of fungal mycelium is examined after exposure to test chemicals at a range of dilutions to determine the minimum inhibitory concentration (MIC).

Tenfold dilutions of each test substance were pipetted into a sterile multi-compartment 'Replidish' (Bibby-Sterilin Ltd., Stone, Staffs, U.K.) to give a total volume of 4ml in each well, with distilled water as a control. Triplicate agar plugs were placed in each of the compartments and left at room temperature (19-23°C) for 1 hour. The plugs were washed three times over a period of 1 hour in separate Replidish compartments containing distilled water. They were then blotted on sterile filter paper and placed upside down on GP agar. Growth was monitored daily for at least seven days, and the minimum concentration of each substance at which growth of the fungus was totally inhibited was recorded as the MIC for that compound.

Induction of sporulation

To induce production of motile zoospores for assays, agar plugs with mycelium were transferred into universal tubes containing GP broth and incubated at 19-23°C for 48 hours. The resulting mycelial wads were washed four times in sterile distilled water and then transferred into Petri dishes containing 20ml filtered, autoclaved pond water (APW) for 24 hours. APW contained one part filtered water from Airthrey Loch, Stirling University, combined with two parts distilled water (APW: pH 8.85, total hardness 69.0 mg $CaCO_3$ l^{-1}, alkalinity 1.63 meq l^{-1}, conductivity 155.0 µSm l^{-1}, nitrite 6.3 µg N l^{-1}, nitrate 75.7 µg N l^{-1}). This yielded approximately $5x10^4$ zoospores per ml.

Zoospore motility assay

A modification of the method described by Tomlinson & Faithfull (1979) was used to assess the effect of selected compounds on zoospore motility. Chemicals shown to have some anti-fungal activity using the agar plug assay were tested at a range of concentrations. A motile zoospore suspension was produced as described above. Five PAP pen circles, approximately 1cm in diameter, were drawn on a glass microscope slide. Twenty-five microlitres of test solution and 25µl zoospore suspension was added to each circle. The drops were observed at 20-100x magnification after 1 hour. Drops were examined quickly to avoid any effect of strong illumination on zoospore motility. If no motile zoospores were observed, the suspension was pipetted into a Petri dish containing GP broth with added penicillin-K (100units/ml) and oxolinic acid (10µg/ml). Petri dishes were observed for five days to

check whether spores remained viable. Compounds were recorded as inhibitory where zoospore motility was lost within one hour and these spores could not germinate and produce mycelia in the growth media.

Sporulation assay

Selected compounds were also tested for their ability to prevent zoospore production in *A. invadans*. Mycelial wads were produced and washed as described above, but were then left overnight in Replidish compartments containing 4mls test chemicals diluted in APW. Assays were performed in duplicate and APW were used as controls. Cultures were incubated at 19-23^{o}C and examined at 20-100x magnification for zoospore production after 24, 48 and 72 hours. Compounds were recorded as inhibitory only where no zoospores were produced over the experimental period.

Chemicals tested

Five groups of products were assessed for activity against *A. invadans.* These are listed in Tables 1(a) – 1(e). The surfactants (group e) were initially used in combination with the plant oils to overcome problems of solubility. Originally thought to be inert, surfactants have subsequently been shown to have biocidal activity of their own (Stanghellini 1997), and were therefore tested here separately, and in combination, with other treatments.

[Table 1a]

[Table 1b]

[Table 1c]

[Table 1d]

[Table 1e]

In vivo toxicity trials

Selected treatments were tested for toxic effects against silver barb, *Barbodes gonionotus* (Bleeker), (a species commonly affected by EUS, formerly known as *Puntius gonionotus*) at the Aquatic Animal Health Research Institute (AAHRI), Thailand. Eighteen tanks of six fish (4-6 cm in length) from AAHRI stock tanks were maintained in static tanks containing 10-litre tap water at 25-30^{O}C. Fish were fed pelleted feed at 3% body weight per day. After a 3-day acclimation period, chemicals were added to the tank. The fish were observed each day for seven days and presence of any uneaten food was noted. The following treatments were tested: E-Z-Mulse™ (at 0.1, 0.5 and 2.5 ppm); coconut diethanolamide (CD) (at 0.1, 0.5 and 2.5ppm); OP10 (at 0.1, 0.5 and 2.5ppm); D-limonene (at 2, 5 and 20ppm, each emulsified with 0.5ppm E-Z-Mulse™); and commercial neem seed extract (at 2, 5 and 20ppm, each emulsified with 0.5ppm OP10).

In further trials at Stirling University, Scotland, three 20 litre static tanks of ten rainbow trout, *Oncorhynchus mykiss* (Walbaum), each 20g in weight, were maintained at 17^{O}C with aeration. After a 5-day acclimation period, chemicals were added to the tank and reintroduced daily following water changes. The following treatments were tested: D-limonene (10ppm + 0.01ppm OP10); neem seed extract (10ppm + 0.01ppm 13/6.5) and propolis (25ppm + 1ppm OP10).

Results

Agar plug transfer assay

The minimum inhibitory concentrations, as determined by the agar plug transfer assays, are given in Tables 2(a) - (e). Malachite green and formalin prevented hyphal growth after one hour exposure at concentrations of 1ppm and 250ppm respectively (Table 2a). Chitosan was inhibitory at 10ppm (Table 2a), however it needed to be solubilised in 0.01M acetic acid, which was in itself found to be inhibitory against *A. invadans* mycelium.

[Table 2a]

Slaked lime ($Ca(OH)_2$) and quicklime (CaO) showed activity against mycelium, although this was probably due to the rapid increase in pH in the small Replidish compartments (Table 2b). In contrast, agricultural lime ($CaCO_3$) stabilised the pH in the compartments and showed no anti-mycelial activity. Potassium permanganate, both fresh and dried preparations of turmeric, and CIFAX were all inhibitory, but at relatively high concentrations. Neem leaf extract was found to be ineffective against *A. invadans*, however the commercial neem seed extract was effective in inhibiting mycelial growth at a minimum concentration of 7500ppm (20ppm azadirachtin). The MIC of the neem seed extract was reduced to 1000ppm (2.7ppm azadirachtin) when combined with 10ppm of particular surfactants

(NP9 and OP10) (Table 3). Filtered extracts of macgnaow (Table 2b) used in MIC tests at first appeared to inhibit mycelial growth, but this was a temporary effect, and no lasting inhibition was observed. All four varieties of ash (sugar cane, rice husk, straw, and mixed straw + cow dung) showed no activity against mycelium.

[Table 2b]

None of the products shown in Table 2(c) were effective by themselves in completely inhibiting the growth of the mycelium, at levels tested here.

[Table 2c]

Pure propolis resin solubilised in ethanol was found to have the greatest activity out of the natural products tested in group (d), with an MIC of 1000ppm (Table 2d). An effective concentration of 25ppm propolis resin was recorded in combination with NP9 or OP10, but only at high concentrations of these surfactants (100ppm) (Table 3). The commercial tincture preparation of propolis was less effective than the pure resin form (Table 2d). Of the herbal treatments obtained from MPRI, Bangkok, *Calophyllum inophyllum, Curcuma domestica, Ficus pumila* and *Eugenia caryophyllus* were the most effective against *A. invadans* mycelium, but each with high MICs of 5000ppm. Garlic was only effective at very high concentrations (10000ppm). No inhibition was detected at any concentration of D-limonene, when tested alone. D-limonene requires addition of surfactant in order to mix in water and an MIC of 1000ppm D-limonene was recorded when combined with 100ppm of particular surfactants (CD, OP10 and 91/6G) (Table 3). Tea tree oil alone was only active at very high concentrations (15000ppm) against *A. invadans* (Table 2d), and this was only slightly lower in combination with 100ppm of surfactants (CD, E-Z-Mulse™, and OP10) (Table 3). Witchhazel extract was only effective at 100% concentration.

[Table 2d]

When tested alone, the surfactants tested were found to have an inhibitory effect on *A. invadans* hyphae, although activity varied widely between compounds (Table 2e). NP9 was most effective, with a MIC of 10ppm. Coconut diethanolamide (CD), E-Z-Mulse™ and the alcohol or alkyl phenol ethoxylates (13/6.5, 91/6G and OP10) all inhibited *A. invadans* mycelial growth after one hour exposure at 25-100ppm. T-O, PE and PM demonstrated very little anti-fungal activity and were not used in further trials.

[Table 2e]

[Table 3]

Zoospore motility assay

Several products were further tested for activity against the secondary zoospore stage of *A. invadans.*

The minimum concentration at which zoospores ceased to be motile after 1 hours exposure is given in Table 4. Zoospore motility was generally more susceptible to chemical treatment than mycelial viability. Malachite green was highly effective, inhibiting motility at 0.05ppm. Several surfactants formalin, neem seed extract and propolis all prevented motility at 10ppm or less. Certain combinations of surfactant with plant extract were particularly effective, such as 1.25ppm propolis and 0.5ppm 13/6.5, and 5ppm neem seed extract and 0.01ppm OP10.

[Table 4]

Sporulation assay

Selected products were also tested for ability to prevent zoospore production during a three-day incubation period. Malachite green and formalin were inhibitory at doses that are commonly recommended for fish treatment (0.05ppm and 25ppm respectively). Surfactants (CD and E-Z-Mulse™) and neem seed extract were inhibitory at levels that are potentially toxic to fish (25ppm, 25ppm and 50ppm respectively). Potassium permanganate and CIFAX were only inhibitory at much higher levels than are currently used in aquaculture (75ppm and 750ppm respectively). Two treatments that are available at no cost to many Asian farmers (neem leaf extract and straw ash) were inhibitory, but only at very high concentrations.

[Table 5]

In vivo toxicity trials

Silver barbs in the two tanks with 2.5ppm E-Z-Mulse™ and 20ppm D-limonene with 0.5ppm E-Z-Mulse™ started to gasp at the surface of the water within five hours of applying treatment. These fish were removed to new tanks and subsequently recovered. No fish died, and no abnormal behaviour was observed, in any of the other

treatments of silver barb or rainbow trout. No uneaten pellets were observed before daily feeds in any of the tanks.

Discussion

Malachite green was the most effective treatment against *A. invadans* tested here. However, it is considered a human health hazard and is unsuitable for use in pond aquaculture. Of the other chemicals with previous reported activity against other Oomycete fungi (group a), formalin was effective in inhibiting zoospore production and motility within levels that are commonly recommended for fish treatments (Lilley, Phillips & Tonguthai 1992) and therefore may be a useful preventative measure. However the one-hour treatments of agar plugs containing mycelium were only inhibitory at levels that are toxic to fish (250ppm). Chitosan (10ppm) and 0.01M acetic acid were found to be totally inhibitory, but this treatment is considered prohibitively expensive for use in pond systems. Acidified chitosan has also recently been shown to be highly toxic to rainbow trout at concentrations down to 0.038ppm (Bullock, Blazer, Tsukuda & Summerfelt 2000). Cycloheximide showed slight inhibition at 100ppm, but this is already higher than the oral median lethal concentration (LC_{50}) for rats (2ppm, Sigma material safety data sheet).

A variety of chemicals are in use in Asia to treat EUS outbreaks (group b). The rationale behind their use may or may not be to combat biological pathogens. Lime is commonly used at 10-60ppm (Table 1b), but often no distinction is made between the different types of lime. Differences in activity are demonstrated here, with slaked lime and quicklime resulting in a rapid increase in pH within the small Replidish wells. Agricultural lime had no effect on *A. invadans* mycelium *in vitro*, but in pond conditions, it is widely reported to reduce the severity of EUS outbreaks by stabilising water pH (Lilley *et al.* 1992). Similarly, ash is considered to stabilise water pH, and Boyd (1990) states that wood ash has 30-40% value of agricultural lime in this regard. In the present studies, rice straw ash did also have slightly more affect in preventing zoospore production than the other ash materials (Table 5). It has been reported that rotting barley straw inhibits Oomycete growth (Cooper, Pillinger & Ridge 1997), and it would be of interest to investigate other straw grown in Asia.

Turmeric was only effective at high concentrations (5000ppm), but it is normally used in conjunction with lime or neem, and therefore further studies would be required to determine any synergistic effects.

Potassium permanganate and CIFAX did not demonstrate activity against *A. invadans* hyphal growth or sporulation at the concentration commonly recommended for use in aquaculture ponds (1-10ppm and 0.1ppm respectively). It should be noted that the recommended doses of potassium permanganate should also take into account the potassium permanganate demand (PPD) of the water, which is commonly 6-8ppm in Asian culture ponds.

Toxicity studies on azadirachtin, the active ingredient of neem seed extract, have shown that the 96-hour LC_{50} for silver barb is 0.185ppm, and on this basis, an estimated safe concentration was given as 0.0093ppm (Rungratchatchawal 1999). This is substantially lower than the 20ppm azadirachtin found to be effective against *A. invadans* hyphae, or the 2.7ppm level used in combination with 10ppm OP10. However, the concentration that caused loss of zoospore motility was much reduced at 0.0135ppm azadirachtin with 0.01ppm OP10. In toxicity trials described here, silver barb and rainbow trout appeared unaffected at azadirachtin levels of 0.054ppm and 0.027ppm respectively (in combination with 0.5ppm and 0.01ppm OP10 surfactant). Neem was considered a particularly suitable candidate treatment as neem trees are common in most EUS affected areas in south and southeast Asia. However, farmers normally use the leaves as pond treatments, which have much lower levels of azadirachtin. According to present studies, at least 1kg leaves m^{-3} (1000ppm) would be required to inhibit *A. invadans* sporulation. Neem seeds, which contain much higher levels of azadirachtin, do not usually become available until after the cool season when fish are most at risk of EUS. However, simple procedures for drying and storing seeds mean that it would be practicable for farmers to gather and prepare seeds for the following winter. Further pond trials on neem treatments are currently underway.

Only low concentrations of the commercial biocides and fungicides that comprised group (c) were tested, as material safety data sheets (MSDS) indicate that most of these compounds have low LC_{50}s against mammals and aquatic animals. None of the agents tested were effective in controlling the growth of the mycelium at levels that are practical for use or environmentally acceptable. There was slight inhibition of mycelial growth by Vantocil at 100ppm, however Vantocil is classified as very toxic to fish, having an LC_{50} to rainbow trout of 8ppm. (Ellis & Everard MSDS).

Some of the natural products with potential anti-microbial activity (group d) demonstrated inhibitory activity against *A. invadans* hyphae and zoospores, particularly in combination with surfactants. D-limonene, propolis and tea tree

oil all have limited availability in EUS-affected countries, but of these, D-limonene, an extract of orange rind, probably has the most potential due to its low cost. Other authors have previously reported that orange oils have anti-fungal properties (Singh, Upadhyay, Narayanan, Padmkumari, & Rao 1993). Work on the acute dermal and oral toxicity of D-limonene for rats has demonstrated an LC_{50} of 5000ppm, but insufficient studies have been carried out on aquatic life (Florida Chemical Co. Inc. MSDS). The toxicity trials described here show that 20ppm D-limonene (with 0.5ppm E-Z-Mulse™) is toxic to silver barbs.

Some factory-prepared microemulsions of natural products and various surfactants showed greater activity than our own preparations described here (unpublished data). This may demonstrate the importance of adequate mixing of the compounds, which would present particular difficulties in the treatment of aquaculture ponds.

When tested alone, surfactants (group e) were found to have an inhibitory effect on *A. invadans* hyphae and zoospores. NP9 was the most effective surfactant but it has been recently shown that the breakdown products of NP9 may mimic oestrogen, and its use may be restricted (Jobling, Sheahan, Osborne, Matthiessen & Sumpter 1996) The effect of E-Z-Mulse™ on silver barbs at 2.5ppm indicates that only very low levels of this surfactant could be used with fish. Therefore, further work will concentrate on use of the low-toxicity coconut diethanolamide and alcohol ethoxylate surfactants. Although silver barbs appeared to show no adverse effects due to exposure to 2.5ppm coconut diethanolamide or OP10, higher concentrations would probably not be tolerated, as LC_{50}s of between 2-26ppm have been demonstrated for *Daphnia* and some fish species exposed to coconut diethanolamide, OP10 or 13/6.5 (Ellis & Everard MSDS).

The agar plug method used here was shown by Bailey (1983) to give a useful, rapid indication of the *in vivo* activity of aquatic fungicides against mycelium of saprophytic fungi growing on the skin of fish. However, as *A. invadans* penetrates the muscle of infected fish, it would be protected from exposure to the chemical, and therefore the aim of this study was primarily to identify fungicides that could be used as preventative treatments. It was found that treatments were generally effective at inhibiting zoospore production and motility at lower concentrations than were inhibitory against mycelium. These lower concentration treatments of the spore stages may be sufficient to prevent infection. Strategic use of such treatments during periods when fish are at highest risk, for example during the winter season in most affected countries, would help to reduce the number of applications of treatments required. Tank and pond trials, and further fish toxicity trials, will be required for an *in vivo* evaluation of some of the treatments identified here. It should be noted, however, that water quality variables such as temperature, pH, water hardness and organic loading often have a strong effect both on the anti-fungal activity of a compound and on the toxicity of the compound to fish. Therefore, it is often impossible to give recommendations that will be effective in all circumstances. Testing should also focus on crude preparations (e.g. crushed neem seeds and orange rind, rather than azadirachtin and D-limonene) which would provide information on potential treatments that are accessible to small-scale Asian freshwater fish farmers.

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Table 1(a): Chemicals with previous reported activity against Oomycete fungi

Chemical	Reported activity
Amphotericin B	0.125µg Amphotericin B disk inhibited *Saprolegnia* growth (Bly, Quiniou, Lawson & Clem 1996).
Chitosan	Mycelial growth and zoospore production of *Saprolegnia parasitica* Coker prevented at 500ppm (Min, Hatai & Bai 1994).
Cycloheximide	Infection of rainbow trout by *S. parasitica* prevented at 6.3ppm (Yuasa & Hatai 1996).
3,5 – Dinitroaniline	Herbicide effective in suppressing plant disease due to *Aphanomyces* spp. (Bruin & Edgington 1983).
Formalin	Viability of *A. invadans (=A. piscicida)* hyphae and zoospores prevented at 31.3ppm (Yuasa & Hatai 1995).
Malachite green	Mycelial growth of *A. invadans* prevented at 0.5ppm and germination of cysts prevented at 0.08ppm (Lilley & Inglis 1997).
$MgCl_2$	Infection of *Astacus astacus* L. by *Aphanomyces astaci* Schikora zoospores prevented at 9500ppm $MgCl_2$ (Rantamäki, Cerenius & Söderhäll 1992).

Table 1(b): Chemicals in use in Asia to treat EUS outbreaks

Chemical	Reported use
Ash	Tamuli, Dutta & Dutta (1995) reported that ash of the banana plant helped to reduce mortality of ulcerated catfish *Clarias batrachus* (L.) at 400ppm. In the present study, samples of ash from sugar cane, rice husk, straw, and mixed straw + cow dung were tested (supplied by Dr M.C. Nandeesha, CARE-Bangladesh).
CaO	Quicklime, reportedly alleviates signs disease at 100-600 kg ha^{-1} (10-60ppm) (Das & Das 1993).
$Ca(OH)_2$	Slaked lime, used prophylactically and therapeutically at 40-600 kg ha^{-1} (4-60ppm) (Lilley, Phillips & Tonguthai 1992).
$CaCO_3$	Agricultural lime, used to stabilise pH in ponds (Lilley *et al.* 1992).
CIFAX	Indian propriety product used for EUS treatments at 0.1ppm (S.C. Mukherjee, pers. comm.).
Macgnaow (*Jatropha curcas* L.)	Plant of the order Euphorbiaceae. Fresh stems are put in ponds in Laos to treat EUS-affected fish (G. Haylor, pers. comm.) Tested here by macerating stems, seeds and leaves and leaving overnight in water.
Neem leaves (*Azadirachta indica* A. Juss.) and neem seed extract (from *Azadirachta siamensis* Valeton)	Tree belonging to the order Meliaceae. Used in Indian medicine for centuries, and has some reported activity against the Oomycete fungus *Phytophthora infestans* (Mont.) (Volf & Steinhauer 1997). Ground neem leaves have been used in EUS treatments (Anon. 1994). Tested here as a macerated leaf preparation left overnight in water, and as a commercial seed extract containing 0.27% active ingredient (azadirachtin). Neem seed extract was provided by Ms Attanon Paneeka, Office of Research and Development of Botanical Pesticides, Department of Agriculture, Bangkok.
Potassium permanganate	Used at preventative and therapeutic treatment of EUS at 1-10ppm (Das & Das 1993).
Turmeric (*Curcuma longa* L.)	Used in EUS treatments in India (Anon. 1994). Tested here as both dried rhizome and fresh preparations.

Table 1(c): Commercial biocides and fungicides

Chemical	Active ingredient and current use
Dimethyl formamide	Addition necessary to solubilise Griseofulvin and dinitroaniline herbicides.
Emulcid (Thor Chemicals)	Tris-(2-hydroxyethyl) hexahydratriazine: used as a biocide/ preservative in paint etc.
Griseofulvin (Sigma)	Systemic fungicide.
Radiaquat 6411 (Fina Chemicals Ltd)	Quaternary ammonium chloride + didecyl dimethyl: used in disinfectant formulations in the food industry.
Sandoteric ABD (Sandoz Chemicals Ltd)	2-methylpentane-2,4-diol: an aliphatic carboxylic acid derivative used as a biocidal detergent in the food industry.
Vantocil (Ellis & Everard)	Polymeric biguanide hydrochloride: biocide used in food industry

Table 1(d): Natural products with potential anti-microbial activity

Extracts tested	Current use and previous reported activity
Acalypha indica L. *Acalypha lanceolata* Willd. *Calophyllum inophyllum* L. (Guttiferae) *Curcuma domestica* Val. *Eugenia caryophyllus* Spreng. *Ficus pumila* L. *Psidium guajava* L. *Phyllanthus urinaria* L. *Phytolaca* americana L. *Pisidium guajava* L.	Herbal treatments supplied in powder form by Dr Ang-kana Herunsaleethe, Medicinal Plant Research Institute, Ministry of Public Health, Bangkok. Herbal treatments have been investigated for activity against bacterial and viral pathogens of aquatic animals. *Psidium guajava* has been shown to have an MIC of 625ppm[1] against *Aeromonas hydrophila* (Direkbusarakom, Ezura, Yoshimizu & Herunsalee 1998), and a number of herbal treatments showed virucidal activity against infectious pancreatic necrosis (IHN) virus and *Oncorhynchus masou* virus (OMV) at 500ppm (Direkbusarakom, Herunsalee, Yoshimizu & Ezura 1996).
D-limonene (Adrian Essential Oils Ltd)	Limonene-dextro 1,8 (9) p- Menthadienev: A low toxicity extract of orange peel
Garlic *(Allium sativum* L.*)*	Widely used in Chinese herbal medicine. Recommended for treatment of enteritis in carp at a rate of 1-2kg garlic $100kg^{-1}$ fish (NACA 1989)
Propolis (Bee Health, York)	Natural product produced by bees to keep the hive sterile. Used in human medicine due to its biocidal properties (Ghisalberti 1979). Used here solubilised in propylene glycol in the form of a tincture; and in pure resin form, macerated and solubilised in 70% ethanol.
Tea tree *(Melaleuca alternifolia* Cheel*)* oil (Health Imports Ltd., Bradford)	Used as a natural remedy for treating cuts, sprains and infection. Gustafson, Liew, Chew, Markham, Bell, Wyllie & Warmington (1998) reported an MIC of 2500ppm Tea tree oil + 80ppm Tween 80 against *Escherichia coli*.
Witchhazel (*Hamamelis mollis* Oliv.) (Power health Products, York)	An astringent and biocidal plant extract, used here as a commercial, undiluted extract.

[1]parts per million (=mg $litre^{-1}$)

Table 1(e): Surfactants tested separately, and in combination, with some of the other treatments (all surfactants from Ellis & Everard, Middlesbrough, UK except E-Z-Mulse™)

Abbreviation in text	Description
NP9	alkyl phenol ethoxylate with 9 moles of ethylene oxide based on a nonyl phenol
13/6.5	alcohol ethoxylate with 6 moles of ethylene oxide based on C13 alcohol
91/6G	alcohol ethoxylate with 6 moles of ethylene oxide based on C9-C11 alcohol
OP10	alkyl phenol ethoxylate with 10 moles of ethylene oxide based on octyl phenol
T-O	t-octylphenoxypolyethoxyethanol
PE	Phenol ethoxylate
PM	Polyethylenesorbitan monolaureate
CD	Coconut diethanolamide
E-Z-Mulse™	Low toxicity emulsifier blend formulated by Florida Chemical Co. Inc. for use with D-limonene

Table 2(a): Minimum inhibitory concentrations of chemicals with previous reported activity against Oomycete fungi, determined by exposure of *A. invadans* mycelium for one hour

Treatment	MIC (ppm[1])
Malachite green	1
Chitosan + acetic acid	10 + 0.01M
Cycloheximide	slight inhibition at 100
Formalin	250
Amphotericin B	no effect at ≤ 500
3,5-Dinitroaniline	no effect at ≤ 500
$MgCl_2$	no effect at ≤ 1000

[1]parts per million (=mg $litre^{-1}$ or µl $litre^{-1}$)

Table 2(b): Minimum inhibitory concentrations of chemicals in use in Asia to treat EUS outbreaks, determined by exposure of *A. invadans* mycelium for one hour

Treatment	MIC (ppm)
$Ca(OH)_2$	100
Potassium permanganate	250
CaO	500
Turmeric - dried rhizome extract	5000
Turmeric - fresh preparation	5000
CIFAX	5000
Neem seed extract (0.27% azadirachtin)	7500
Macgnaow extract	no effect at ≤ 10000
Neem leaf extract	no effect at ≤ 25000
Ash	no effect at ≤ 50000
$CaCO_3$	no effect at ≤ 100000

Table 2(c): Minimum inhibitory concentrations of commercial biocides and fungicides, determined by exposure of *A. invadans* mycelium for one hour

Treatment	MIC (ppm)
Sandoteric ABD	no effect at ≤ 10
Radiaquat 6411	no effect at ≤ 10
Dimethyl formamide	no effect at ≤ 10
Vantocil	slight inhibition at 100
Griseofulvin	no effect at ≤ 10000
Emulcid	no effect at ≤ 10000

Table 2(d): Minimum inhibitory concentrations of natural products with potential anti-microbial activity, determined by exposure of *A. invadans* mycelium for one hour

Treatment	MIC (ppm)
Propolis resin	1000
Propolis tincture	2500
Calophyllum inophyllum	5000
Curcuma domestica	5000
Ficus pumila	5000
Eugenia caryophyllus	5000
Pisidium guajava	no effect at ≤ 5000
Phyllanthus urinaria	10000
Phytolaca americana	10000
Garlic clove	10000
Acalypha lanceolata	no effect at ≤ 10000
Acalypha indica	no effect at ≤ 10000
D-limonene	no effect at ≤ 10000
Tea tree oil	15000
Witchhazel	1000000

Table 2(e): Minimum inhibitory concentrations of various surfactants, determined by exposure of *A. invadans* mycelium for one hour

Treatment	MIC (ppm)
NP9	10
CD	25
E-Z-Mulse™	30
13/6.5	50
91/6G	50
OP10	100
T-O	1000
PE	10000
PM	no effect at ≤ 10000

Table 3: Minimum inhibitory concentration of plant product/surfactant combinations, determined by exposure of *A. invadans* mycelium for one hour

Treatment	MIC (ppm)
Neem seed extract + NP9	1000 + 10
Neem seed extract + OP10	1000 + 10
Propolis resin + NP9	25 + 100
Propolis resin + OP10	25 + 100
D-limonene + CD	1000 + 100
D-limonene + OP10	1000 + 100
D-limonene + 91/6G	1000 + 100
Tea tree oil + CD	10000 + 100
Tea tree oil + E-Z-Mulse™	10000 + 100
Tea tree oil + OP10	10000 + 100

Table 4: Minimum concentration of selected compounds at which *A. invadans* zoospores lost motility after one hour incubation

Treatment	MIC (ppm)
Malachite green	0.05
CD	2.5
E-Z-Mulse™	5
13/6.5	5
Formalin	10
Propolis	10
Propolis + 13/6.5	1.25 + 0.5
Neem seed extract	10
Neem seed extract + OP10	5 + 0.01
Tea tree oil	20
D-limonene + E-Z-Mulse™	25 + 0.05

Table 5: Minimum concentration of selected compounds at which *A. invadans* mycelium failed to produce zoospores over a 72 hour period

Treatment	MIC (ppm)
Malachite green	0.05
Formalin	25
CD	25
E-Z-Mulse™	25
Neem seed extract	50
Potassium permanganate	75
CIFAX	750
Neem leaf extract	1000
Ash (straw)	25000
Ash (sugar cane)	slight inhibition at 50000
Ash (mixed straw + cow dung)	slight inhibition at 50000
Ash (rice husk)	no effect at 50000

APPENDIX EIGHTEEN

Paper twelve - Lilley, J.H., Panyawachira, V., Khan, M.H. and Chinabut, S. (2001) Pond trials to test treatments against epizootic ulcerative syndrome. Asian Fisheries Science. (in preparation, selected results shown, hard copy of figures available from ARP Manager)

TRIAL TO TEST PREVENTATIVE TREATMENTS AGAINST EUS - 1

Site: Bangsai Fisheries Station, Ayuthaya, Thailand

Objective: To examine the ability of three treatments to prevent EUS in juvenile striped snakeheads (*Channa striata*) and juvenile giant gourami *(Osphronemus gouramy)*

Methods

Ponds

Five 400m^2 earthen ponds were used for the trial (Figure 2A). The water level was fixed at a depth of 1 metre. One pond was randomly allocated to each of the three treatments or one control (listed below). Two small (1 x 1 x 1.2 m) 1m^2 hapas were fixed in each treatment pond. Each had a net lid to prevent predation and escapees. Bamboo walkways were constructed to the hapas to ensure that workers did not risk spreading fungus by wading through water. Two large (2 x 3 x 1.2m) 6m^3 hapas were fixed in the remaining pond to contain the stock fish. There was no water change during the experiment. Equipment was sterilised in 100ppm calcium hypochlorite (containing 60% available chlorine). At the end of the experiment, the pond water was sterilised in 50ppm calcium hypochlorite.

Fish

Nine days before challenge, 300 juvenile striped snakeheads and 300 juvenile giant gouramis were put in stock hapas for acclimatisation. Fish were fed pelleted feed to satiation. On the morning of the day of the challenge (day 0), fungal mats and treatments were added to the ponds (Figure 2B and 2C). On the afternoon of day 0, 20 fish were weighed, artificially abraded on the left flank, just below the start of the dorsal fin, and put in each of the 8 small hapas (Figure 2D and 2E). At the end of the first part of the experiment (day 10), all fish were examined for clinical signs, weighed, and sampled for histology. New fungal wads were added on the morning of day 10, and 20 new pre-acclimatised fish were abraded and added to the hapas in the afternoon of day 10. These were examined, weighed and sampled on day 20.

Fungus

80 "mats" of *Aphanomyces invadans* isolate T99G2 (from giant gourami, Thailand, December 1999) were prepared by growing 4mm plugs in GP (glucose-peptone) broth for 4 days at 20-25^oC prior to day 0. On day 0, the 80 mats were placed into 8 small plastic tubes with holes. The tubes, each containing 10 mats, were suspended in each hapa in the middle of the water column. On day 3, the tubes were removed from each pond, and the mats examined under a microscope for evidence of sporulation.

Treatments

The following treatment regimes were tested:

i. Control - no treatment
ii. 0.15ppm malachite green (Fig. 1A)
iii. 2.5ppt salt and 38ppm $Ca(OH)_2$ (Fig. 1B)
iv. 125ppm dried, crushed neem *(Azadirachta siamensis)* seed (Fig. 1C)

Histology

After sampling, fish were processed for Uvitex histology at AAHRI and examined for mycotic granulomas to determine efficacy of the treatments.

Water quality

Basic water quality of each pond was measured on day -3, 10 and 20. Temperature was measured daily at 09:00 am.

Results

A high proportion of the untreated control snakeheads sampled on day 10 showed EUS-like clinical lesions (Fig. 3A); none of the malachite green treated fish showed lesions (Fig. 3B), and a small proportion of the salt and lime group (Fig. 3C) and neem group (Fig. 3D) had lesions. All fish with EUS-like clinical lesions were diagnosed as EUS-positive by observation of invasive fungus. Early stage granulomas were also observed in most circumstances. Table 1.1 shows the difference in

numbers of fish infected with *A. invadans* in each of the treatment groups. Although none of the malachite green treated fish showed clinical lesions, four did show some evidence of fungal invasion histologically.

Table 1.1 Percentage of snakeheads histologically confirmed with EUS after 10 days

Treatment	Number fish with EUS	Number sampled	Percentage of fish with EUS
Control	18	20	90%
Malachite green	4	19	21%
Salt and $Ca(OH)_2$	4	20	20%
Neem seeds	6	20	30%

Only 8 out of 71 gouramis (11%) sampled from all groups were confirmed as infected, and therefore no comparison between treatments could be made from the gourami challenge. The percentage difference body weight of sampled snakeheads and gourami are given in Table 1.2.

Table 1.2 Weight of fish sampled on day 10

Treament		Day 0	Day 10	% difference
Control	Snakeheads	41.3	35.5	-14%
	Gourami	5.9	5.6	-5%
Malachite green	Snakeheads	40.0	33.5	-16%
	Gourami	6.3	5.9	-6%
Salt and $Ca(OH)_2$	Snakeheads	43.8	42.0	-4%
	Gourami	5.5	6.8	+24%
Neem seeds	Snakeheads	38.0	38.0	0%
	Gourami	5.3	7.9	+49%

Only 4 out of 75 snakeheads (5%) sampled, and 1 out of 78 gouramis (1%) sampled were confirmed as infected from the fish challenged on day 10 after pond treatment, and sampled on day 20. This is probably due to a rise in water temperature from 23-26^{o}C on days -9 to 10, to 26-28^{o}C on days 10-20. Other water temperature parameters recorded during the first half of the experiment are shown in Table 1.3.

Table 1.3 Water quality of experimental ponds

Treatment		Day -3	Day 10
Control	pH	7.81	8.00
	alkalinity (mg/l)	141	161
	hardness (mg/l)	155	172
	NH_3 (mg/l)	0.019	0.289
	salinity (ppt)	0	0
Malachite green	pH	7.73	8.41
	alkalinity (mg/l)	143	145
	hardness (mg/l)	197	220
	NH_3 (mg/l)	0.005	0.019
	salinity (ppt)	0	0
Salt and $Ca(OH)_2$	pH	7.33	8.41
	alkalinity (mg/l)	157	168
	hardness (mg/l)	197	311
	NH_3 (mg/l)	0.025	0.019
	salinity (ppt)	0	3
Neem seeds	pH	7.37	8.91
	alkalinity (mg/l)	125	171
	hardness (mg/l)	136	126
	NH_3 (mg/l)	0.015	0.046
	salinity (ppt)	0	0

Conclusions

- None of the treatments were totally effective in preventing infection by *Aphanomyces invadans*.
- Malachite green was the most effective of the treatments, with only four fish from this group showing low levels of fungus in skin tissue, and none showing clinical lesions. Malachite green is not, however, advised for use in ponds due to its potential health hazards. It was included in the present study as it was the most successful of the fungicides tested *in vitro*, and is a benchmark by which fungicides are tested. The malachite green treated fish showed the greatest decrease in body weight, which may be an indication that the fish were stressed.
- The snakeheads in the salt and lime and neem groups did show lower percentage infection than untreated controls. However, water quality deterioration in the neem treated pond may indicate that this treatment is unsuitable for use by farmers.
- Giant gourami is not a suitable model for EUS treatment trials, due to the low number of fish affected in all groups, including untreated controls.
- High temperatures reduce the chance of infection by *Aphanomyces invadans*. Low numbers of both snakeheads and giant gouramis were infected in the second phase of this study, when temperatures had risen above 26^{o}C.

FIGURE 1. PREVENTATIVE TREATMENT TRIAL - TREATMENTS

A

A. 0.15ppm malachite green

B

B. 2.5 ppt salt + 38ppm $Ca(OH)_2$

C

C. 125ppm dried crushed neem seeds

FIGURE 2. PREVENTATIVE TREATMENT TRIAL - METHODS

A

A. Treatment ponds

B

B. Adding fungus to hapas

C

C. Adding treatment to ponds

D

D. Abrading fish

E

E. Adding fish to hapas

FIGURE 3. PREVENTATIVE TREATMENT TRIAL - RESULTS

A. No treatment. 10 days post-challenge

B. 0.15ppm malachite green treatment. 10 days post-challenge

C. 2.5 ppt salt + 38ppm $Ca(OH)_2$. 10 days post-challenge

D. 125ppm dried crushed neem seeds. 10 days post-challenge

TRIAL TO TEST PREVENTATIVE TREATMENTS AGAINST EUS - 2

Site: Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh

Objective: To examine the ability of four low-cost treatments to prevent EUS in mrigal *(Cirrhinus mrigala)*

Methods

Ponds

Ten earthen ponds, ranging in size from 46-113m^2, were used for the trial. The water level was fixed at a depth of 1 metre. One pond was randomly allocated to each of the four treatments or one control (listed below).

Fish

100 *Cirrhinus mrigala* fingerlings (12-15 cm) were fed daily at 2-5% body weight per day (rice bran and oilcake). 10 clinically-normal fish were weighed and acclimatised in hapas for 10 days before the experiment. On day 0, they were artificially abraded on the left flank, just below the start of the dorsal fin and released into the main pond. At the end of the experiment (day 14), they were weighed and sampled for histology.

Fungus

200 "mats" of *Aphanomyces invadans* isolate B99C (from *Cirrhinus reba* sampled in BAU in March 1999) were prepared by growing 4mm plugs in GP (glucose-peptone) broth for 4 days at 20-25^{o}C prior to use for the fish challenge. On day 0, the 200 mats were placed into 50 histology processing cassettes. 5 containers were distributed in each pond in the middle of the water column. On days 1, 7, and 14, a cassette was removed from each pond and examined under the microscope for active sporulation.

Treatments

i. Control - no treatment
ii. Green water – pre-stocking treatment of cowdung (250ppm) + urea (5ppm) + TSP (5ppm) and post-stocking treatments every 3 or 4 days of cowdung (25ppm) + urea (2.5ppm) + TSP (2.5ppm).
iii. Lime - pre-stocking treatment of 25ppm. Post-stocking treatments of 6ppm added every week. Stone-like lime was used.
iv. Ash - 25ppm sieved ash was added every week.
v. Neem seed - one 50ppm treatment of finely-crushed neem seeds

Histology

10 fish from each pond were processed for H&E and Uvitex histology.

Water quality

Basic water quality (secchi disk, pH, temperature, DO, alkalinity, hardness) were measured on day -10 and day 14.

Results

Table 2.1 shows the number of fish sampled from each experimental pond confirmed infected with EUS by Uvitex and H&E histology. Infection rates were highest in untreated controls (average 94.4%), followed by ash (83.3%), lime (66.7%), neem (52.9%) and fertilised “green water” ponds (29.4%).

Table 2.1 Percentage of mrigal confirmed with EUS after 14 days (number of fish sampled is given in brackets)

Treatment	Pond 1	Pond 2
Control	88% (8)	100% (10)
Green water	14% (7)	40% (10)
Neem seeds	33% (9)	75% (8)
Lime	44% (9)	89% (9)
Ash	78% (9)	89% (9)

The fish in all treatment ponds showed higher weight gains than in untreated control ponds. Out of the treatments, limed and fertilised ("green water") ponds had the highest production levels (Table 2.2).

Table 2.2 Weight of fish sampled on day 14

Treament		Day -10	Day 14	% difference
Control	Pond 1	56.4	76.1	+34.9%
	Pond 2	59.8	79.4	+32.8%
Green water	Pond 1	60.8	85.0	+39.8%
	Pond 2	59.4	82.4	+38.7%
Neem seeds	Pond 1	58.9	80.1	+36.0%
	Pond 2	59.1	82.4	+39.4%
Lime	Pond 1	58.7	81.7	+39.2%
	Pond 2	63.8	89.3	+40.0%
Ash	Pond 1	60.7	83.3	+37.2%
	Pond 2	59.1	80.4	+36.0%

Conclusions

- None of the treatments were totally effective in preventing infection by *Aphanomyces invadans*.
- The fertilised ("green water") ponds had the lowest levels of infection. Fertilsation has the added benefit of increasing production in the ponds, and is one of the most cost-effective treatments. Previous work has shown that the are much lower levels of fungal propagules in ponds with high levels of plankton. Studies are planned at RIA1, Vietnam to further assess the effect of plankton on fungal propagules.
- Neem, lime and ash ponds also contained significantly lower numbers of infected fish than untreated controls, but the benefits achieved from these treatments are marginal when labour and cost is considered.
- Mrigal is a suitable fish for use in pond treatment trials against EUS. The untreated control groups showed high levels of infection. Mrigal were adopted as the treatment model for subsequent pond trials at CIFA, India.

APPENDIX NINETEEN

Paper thirteen - Islam, S., Karim, M., Nandeesha, M.C., Khan, M.H., Chinabut, S. and Lilley, J.H. (2001) Farmer-based investigation of treatments for ulcerative disease in polyculture carp ponds in Bangladesh. Asian Fisheries Science. (draft)

Farmer-based investigation of treatments for ulcerative disease in polyculture carp ponds in Bangladesh

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Abstract

Ulcerative disease was identified by polyculture carp farmers participating in the CARE project, Locally Intensified Farming Enterprises (LIFE), as the most significant constraint to higher production. In order to address this problem, six candidate prophylactic treatments were identified through consultation with researchers and farmers. These comprised of: ash, lime, salt, salt&lime, neem branches (*Azadirachta indica*), and fertilizer. Two hundred and seventy-eight farmers (94 in Rajshahi district and 184 in Kishoregonj district) participated in the trial. Each farmer selected one of the treatments for their experimental pond. Lime and ash were the most popular selections in both districts. For each treatment except neem, fortnightly applications were advised over the study period from 1st October 1999 to 28th February 2000. However, at the end of the study, the number of applications of these treatments averaged only 3.3 times. The occurrence of ulcerative disease was reported by farmers in a structured questionnaire at the end of the study. A proportion of affected sites were visited by CARE staff during the study and 30 fish samples were taken to determine whether the disease was epizootic ulcerative syndrome (EUS), using presence of mycotic granulomas in histological section as the diagnostic feature of EUS. Seventy percent of the samples could be confirmed as EUS-positive.

Farmers from all treatment groups reported a lower incidence of ulcerative disease than farmers with control ponds. In total, 9.4% of farmers adopting a treatment reported ulcerative disease, compared to 61.9% of farmers with control ponds. No farmers applying fertilizer reported disease, but only three farmers adopted this treatment. Only two of the 60 farmers adopting lime (3.3%) reported disease, and 9.8% of ash farmers, 10% of salt farmers and 15.4% of salt&lime farmers reported disease. Neem was the least effective of the treatments, with 19% of farmers reporting disease. Fish production levels, as reported by farmers, were higher in all treatment groups than controls in Kishoregonj, but production from lime, neem and ash treatment ponds was lower than controls in Rajshahi. The percentage increase in production on the previous year, as reported by farmers, was higher in all treatment groups than controls in both districts. The cost of production compared to the previous year, as reported by farmers, was higher than controls for all treatment farmers, except for those using ash. However, in all cases, the reported increase in production was higher, or equal to, the increase in costs. Farmers adopting the salt treatment reported the highest increase in input costs. Of the farmers that adopted one of the treatments, 95% indicated they were satisfied with the results. The majority indicated that this was due to no occurrence of disease. The most popular treatments were lime and ash, with 38% and 32% of farmers expressing a desire to adopt these respective treatments in the next season. Other pond variables were recorded to check whether they were associated with the occurrence of ulcerative disease in the experimental ponds. The entry of wild fish in ponds, and the occurrence of ulcerative disease in nearby ponds, were significant risk factors for ulcerative disease in Kishoregonj and Rajshahi respectively. The problems of obtaining reliable quantitative data from farm-level studies are discussed. However, it is suggested that farmer participatory research is an important means of identifying treatments that are acceptable to farmers.

Introduction

The Locally Intensified Farming Enterprises (LIFE) project run by CARE – Bangladesh is working at Rajshahi and Kishoregonj districts located in the northern part of the country to enhance food security through improving farmer's knowledge and skills with regard to major agricultural activities. Farmers working with the LIFE project identified EUS (epizootic ulcerative syndrome) as the main problem they encounter in fish cultivation. The LIFE project undertook an initiative to address the EUS problem. Four possible EUS treatments (ash, lime, salt, and salt&lime) were identified through farmer participation and during the 1998-1999 season were found to reduce the incidence and intensity of the disease (Nandeesha *et al*, 2001). To investigate the treatments further, the project continued the study during the 1999-2000 season, in collaboration with the Department for International Development (DFID)-funded regional EUS project. In addition to the previous treatments, leaves and stems of the neem tree (*Azadirachta indica)* and fertilizer treatments were also studied. The study aimed to determine whether the treatments reduced the incidence of EUS as reported by farmers, and to assess whether the farmers were satisfied with the treatments and were prepared to continue using the treatment in subsequent years.

Materials and Methods

Formation of Participatory Action Research Groups (PARGs)

Participatory action research groups (PARGs) were formed at the village level. PARGs were formed by organising a general meeting of village farmers, informing them of project objectives and principles, and inviting interested farmers to become members. PARG members also had to satisfy certain selection criteria. They had to be defined as "food insecure" and show interest in solving problems using available resources. The LIFE project does not provide credit or any other incentives other than enhancing farmer knowledge and skills. Each PARG consisted of 25-30 farmers with either male-only or female-only members.

Participatory Needs Assessment (PNA)

After PARG formation, farmers' needs, or problems associated with agricultural activities, were identified through PNA (participatory needs assessment) sessions. In the majority of the PARGs, EUS was identified as the major problem in the fisheries sector.

Trial design and Implementation

A learning session was conducted for the farmers of those PARGs where EUS was recognized as a major problem. The session included the history of EUS, known causes, and possible control measures using locally available resources. The results of the previous year's study were also shared. Based on these discussions, six possible preventative treatments were identified for further study. Farmers were urged to adopt one of the following treatments, and to make the first treatment application early in the cold season:

Ash treatment

Ash was applied at an initial dose of 3 kg/decimal (741 kg/ha), followed by 1.5 kg/decimal (371 kg/ha) at fortnightly intervals. The application procedure involved sieving the required quantity of ash, dissolving it in water, and then spreading it over the pond.

Lime treatment

This treatment comprised of an initial dose of 1 kg/decimal (247 kg/ha) lime followed by 0.5 kg/decimal (124 kg/ha) lime at fortnightly intervals. Farmers were instructed that the lime should be dissolved in water, cooled, and the resulting solution further diluted and then dispersed throughout the pond.

Salt treatment

This treatment comprised of an initial dose of 1 kg/decimal (247 kg/ha) table salt (NaCl) followed by 0.5 kg/decimal (124 kg/ha) at fortnightly intervals. The required amount of salt was dissolved in water before dispersal in the pond.

Salt&lime treatment

Ponds under this treatment were given an initial dose of 0.5 kg/decimal (124 kg/ha) of both salt and lime. Farmers were instructed that subsequent doses of 0.25 kg/decimal (62 kg/ha) of both salt and lime should be applied at fortnightly intervals. The salt and lime were dissolved separately, the lime solution was cooled and mixed with salt solution, and the mixture was spread over the pond.

Neem treatment

Farmers adopting this treatment inserted neem (*Azadirachta indica*) stems with leaves into the pond. Farmers applied a variable number of neem stems (between 2-188 stems per hectare).

Fertilizer treatment

This treatment comprised of 2 kg/decimal (494 kg/ha) decomposed cow dung, 200g/decimal (49 kg/ha) urea, 200g/decimal (49 kg/ha) TSP and 250g/decimal (62 kg/ha) mustard oil cake at fortnightly intervals. The number of applications varied depending on the level of phytoplankton in the pond water.

Control

Participating farmers who did not want to follow a treatment were designated as control farmers.

In order to satisfy the criteria for selection in the study, all study ponds contained at least one of the species that has been listed as being susceptible to EUS by Lilley *et al* (1998). The susceptible species may have been stocked and/or wild species. The trial was conducted in the cold season from 1st October 1999 to 28th February 2000, when EUS is more prevalent. Farmers normally looked for clinical signs on fish on a daily basis, but in addition, they agreed to check fish at least once every fifteen days using a cast net. Farmers identified EUS-affected fish by the presence of clinical lesions. A proportion of the farmers that reported EUS outbreaks to LIFE field trainers were visited by the authors to sample affected fish for later histological confirmation at Bangladesh Agricultural University (BAU).

Questionnaire and Focus Group Discussions

At the end of the study, a series of workshops was arranged in Rajshahi and Kishoregonj to bring together farmers to discuss the treatments they had adopted. A structured questionnaire was used to gather results from each of the farmers. This was followed by group discussions, where the experiences and lessons learned by the farmers were recorded.

Statistical analysis

Differences between treatments and controls were analysed using one-way ANOVA followed by pairwise multiple comparisons using the least significant difference method. Significantly different groups are shown at an alpha level of 0.05.

Results

A total of 94 ponds in 32 PARGs of Rajshahi and 184 ponds in 50 PARGs of Kishoregonj were used to study the impact of EUS treatment strategies for the prevention of EUS during the 1999-2000 season. The average size of the farmer's family was slightly larger in Kishoregonj (6.1 people) than in Rajshahi (5.9 people), while the average land holding and average pond area were smaller in Kishoregonj than in Rajshahi (Table 1). The majority of ponds were under single ownership in both project districts. Interestingly, the districts contrasted in respect of the number of leased ponds. The larger ponds found in Rajshahi were more likely to be leased out to commercial fish producers. In Rajshahi, there is an established organised trade in fish from these ponds.

Effect of treatment on the prevention of ulcerative disease

The majority of farmers (83.8%) commenced treatment as recommended, early in the cold season, before any signs of fish disease were observed. The treatment selected by these farmers, and the number of applications of each treatment, are listed in Table 2. Uptake in both districts was similar in that lime and ash were the most popular treatments, and fertilizer the least popular treatment. The main difference between districts was that 16 (10.5%) Kishoregonj farmers adopted salt, making it one of the more popular treatments, whereas in Rajshahi only four farmers (4.9%) tried the salt treatment (Table 2).

Very few farmers applied the recommended number of treatments over the study period. The majority of the neem treatment group made one application of 2-188 stems/ha at the beginning of the study period (Tables 2-3). Excluding neem, the number of applications averaged 3.3 times, with 76.5% of farmers making three or more applications during the study period.

According to data on disease occurrence obtained from farmers, all six treatments had a significant effect in the prevention of ulcerative disease, with the exception of the neem treatment in Rajshahi (Table 4). Overall, only a small proportion of the treatment ponds was affected by ulcerative disease (9.4%), compared to 61.9% of control ponds. These figures were remarkably similar for both Rajshahi and Kishoregonj districts. No farmers applying fertilizer reported disease, but only three farmers adopted this treatment. Only two of the 60 farmers adopting lime (3.3%) reported disease; and 9.8% of ash farmers, 10% of salt farmers and 15.4% of salt&lime farmers reported disease. Neem was the least effective of the treatments, with 19.0% of farmers reporting disease (Table 4).

All groups, including control ponds, had a lower incidence of ulcerative disease than the previous year (Table 4).

The data was re-analysed using only the ponds that had encountered ulcerative disease the previous year (i.e. ponds with a high risk of getting disease). In this case, the incidence of ulcerative disease was slightly higher in all affected pond groups. Overall, 12.4% of treatment ponds (17.1% in Rajshahi and 10.8% in Kishoregonj) got ulcerative disease, compared to 73.3% of control ponds (75% in Rajshahi and 72.7% in Kishoregonj).

Species affected

There was a high variability in species combinations and stocking densities in the study ponds as farmers added fish to the ponds according to their resources and the species available. In some cases, farmers took measures to exclude wild fish from their pond, but in most cases, wild species could be observed in the ponds.

Table 5 shows the species that farmers reported to be affected by ulcerative disease. Most of the species listed are considered to be EUS-susceptible. However in 13 cases, farmers identified fish that are considered to be EUS-resistant (Lilley *et al*, 1998) as "EUS-positive", i.e. tilapia, common carp, grass carp and silver carp. A common carp sample taken for histology was indeed found to be negative. However, in all cases where farmers identified affected resistant fish, they also recorded affected susceptible fish.

Of 30 samples taken for histological confirmation of EUS, 70% were found to be EUS-positive by demonstration of invasive hyphae and associated granulomatous response (Table 5). This indicates that most, although not all, of the diseased fish that the farmers had diagnosed as "EUS-positive", were positive according to the pathological definition. The samples comprised of both stocked and wild fish, and were taken from a variety of treatment ponds.

Effect of treatments on production

Production levels, calculated from farmers reports of the Taka value of fish sold, were higher in all treatment groups than controls in Kishoregonj, but only production from salt and salt&lime treatment groups was higher than controls in Rajshahi (Table 6). The percentage increase in production on the previous year, as reported by farmers, was higher in all treatment groups than controls in both districts. However, it should be noted that there was wide variation in both production and input costs, both within and between the treatments (Table 6).

In Rajshahi, neem and salt treatment groups showed the highest increase in production over the previous year (88% and 63% respectively). In Kishoregonj, all treatment groups showed high increases in production over the previous year, with fertilizer, salt&lime and neem treatment groups showing the highest increases (208%, 140% and 110% respectively) (Table 6).

The reported increase in production in the salt treatment group was associated with a similar increase in input costs in both Rajshahi and Kishoregonj. In all the other treatment groups, the reported increase in production was much higher than the increase in input costs (Table 6).

Further analysis was carried out to show the proportion of farmers that reported high and low changes in production. Table 7 shows that production increase had taken place with all groups of farmers, including that of controls. Overall, 24% of treatment farmers recorded an increase in production over the previous year of over 200%, compared to 7% of control farmers.

Attitude of farmers to treatment results

Of the farmers that adopted one of the treatments, 95% indicated they were satisfied with the results (Table 8). The majority indicated that this was due to "no occurrence of ulcerative disease", although "better fish growth" was another important reason for satisfaction with the treatment. Some farmers indicated that the treatment helped the fish recover from disease, and a few mentioned that fish could be sold at a higher price because of the lower incidence of disease (Table 8).

Of the farmers that reported increased production, the highest ranked reason for this was given as "treatment effect", but "better management" and "more feed and fertilizer", as advised by LIFE staff, also contributed to higher production. (Table 9).

In ponds where ulcerative disease occurred, the majority of farmers (64% overall) continued treatment (Table 10). However, in Rajshahi the highest proportion of farmers (41%) stopped treatment and did not harvest the fish. In both districts, a minority of farmers undertook an emergency harvest and sold the fish after noticing disease (Table 10).

The most popular treatments were lime and ash, with 38% and 32% of farmers in both districts expressing a desire to adopt these respective treatments in the next season (Table 11).

Other factors influencing occurrence of disease

Other pond variables were recorded to check whether they were associated with the occurrence of ulcerative disease in the experimental ponds. The entry of wild fish in ponds, and the occurrence of ulcerative disease in nearby ponds, were significant risk factors for ulcerative disease in Kishoregonj and Rajshahi respectively (Table 12). If the data is recalculated for relative risk (RR), presence of wild fish in the experimental pond almost doubled the likelihood of disease in Kishoregonj ponds (RR=1.74), and presence of ulcerated fish in nearby ponds increased the risk of disease in Rajshahi ponds by over four times (RR=4.29).

Phased-out farmers

Continued uptake of treatments by farmers that had participated in the 1998-9 LIFE supervised study was high. [put further details here].

Discussion

The study showed that EUS remains a common problem, and is an issue of concern to farmers in particular areas of Bangladesh. Khan & Lilley (2001) reported that 50% of 64 ponds sampled from each district of Bangladesh had ulcerative disease, and of these ponds, 94% contained fish diagnosed as EUS-positive. Of the control ponds in the present study, occurrence of ulcerative disease was slightly higher with 64% of ponds in Rajshahi and 61% of ponds in Kishoregonj being reported as affected. However, of the 30 samples taken for histological diagnosis, a slightly lower proportion (70%) were found to be EUS-positive.

In this study, farmers that adopted any of the six suggested preventative treatments reported a lower incidence of disease in their ponds than control farmers. The difference between treatments and controls was significant in all groups with the exception of the neem treatment in Rajshahi. Combining the results of the two study districts, the reports of ulcerative disease were lowest in fertilizer ponds (0%) (but only three farmers adopted this treatment), followed by lime (3.3%), ash (9.8%), salt (10%), salt&lime (15.4%), and neem (19%). In comparison, ulcerative disease was reported in 61.9% of control ponds.

The lime treated ponds had the lowest reported incidence of disease (excluding the three fertilizer ponds). However, farmers adopting lime reported the lowest increase in production over the previous year compared to the other treatments. Despite this, Table 7 shows that a proportion of lime farmers did report very high (>200%) increases in production levels. After discussions, lime was voted the most popular treatment in both districts combined. Liming is commonly used to stabilise water quality, particularly in areas susceptible to low pH. Khan & Lilley (2001) reported that lime applications, both before and after stocking, reduce the risk of EUS, and Ahmed and Rab (1995) showed that post-stocking applications reduce the severity of EUS. The lime that farmers are able to purchase is variable in terms of chemical composition. Both studies described above noted that farmers used lime that was predominantly calcium carbonate ($CaCO_3$). This has been shown to have little or no anti-fungal activity (Campbell *et al*, 2001) so its effect is probably in reducing the susceptibility of fish to infection by stabilising water quality.

The ash treatment ponds had the second lowest reported incidence of disease. Farmers adopting this treatment reported an increase in production levels over the previous year, with no increase in input costs. Ash was a more popular treatment among farmers in Kishoregonj than Rajshahi owing to its easy availability in that district. There are many small-scale paddy processing plants in Kishoregonj which utilise rice husk as fuel, therefore farmers can procure the resulting ash, usually at no cost. Following focus group discussions, 32% of farmers indicated that they would use ash in the next season. Several farmers also commented that use of ash reduces turbidity in the pond. Experimental studies have shown that ash has very little *in vitro* fungicidal activity on the EUS fungal pathogen *Aphanomyces invadans* (Campbell *et al*, 2001). However, it is possible that it may be acting to improve water quality and prevent fish becoming predisposed to infection. Boyd (1990) reported that wood ash has 30-40% value of agricultural lime in terms of stabilising water pH.

The salt treatment ponds had a low (10%) reported incidence of disease, but it is the most expensive of the treatments tested, and the high cost was reflected in the data given in Table 6. Salt at 2 ppt has been shown to inhibit sporulation of the EUS pathogen, *A. invadans* (Fraser *et al*, 1992), however it is unlikely that salinity levels in experimental ponds reached these levels. It is probable, therefore, that the salt treatment may have had a greater effect on improving water conditions for the fish, than on treating the fungus, or any opportunistic pathogens.

The salt&lime group had a low (15.4%) reported incidence of disease and a high increase in production levels was reported from Kishoregonj farmers and was associated with a small increase in input costs. Combined salt and lime treatments are commonly used as preventative and therapeutic treatments for EUS, but there are no controlled studies that have positively demonstrated a treatment effect.

Neem was one of the least efficacious treatments, but reports of disease in these ponds were still substantially lower than controls. High increases in production were reported from both Rajshahi and Kishoregonj farmers, along with little or no increase in input costs. Neem was identified by farmers in India and Pakistan as a treatment for lesions on fish, but it is also used as a piscicide, so care should be taken in its application. In pond trials, fish challenged with *A. invadans* and treated with 50-125 ppm dried, ground neem seeds demonstrated a lower incidence of EUS than untreated fish (unpublished data). In addition, Campbell *et al* (2001) showed that a commercial seed extract containing 0.27% active ingredient (azadirachtin) inhibited *A. invadans* zoospore motility after 1 hour exposure at 10ppm (100 kg/ha), but neem leaf extract had little or no effect. Neem leaves are known to have much lower azadirachtin levels than seeds (Schmutterer, 1995) but during the present study, it was apparent that farmers would not have the resources to collect and process an adequate number of neem seeds, and therefore neem leaves were used. There was a very wide range in the number of stems added to each treatment pond (Table 3) and it is very unlikely that the lower application rates used had any effect on the occurrence of disease.

Fertilizer was adopted by only three farmers overall, none of whom reported an outbreak of disease during the study period. These farmers also reported the highest increase in production levels over the previous year, but the low number of ponds in this group reduces the significance of the results. The use of fertiliser was suggested in order to induce production of high levels of algae in the ponds. Lilley (1992) has shown that there are lower fungal counts in pond water containing high levels of phytoplankton, and Khan & Lilley (2001) have shown that there is a lower incidence of EUS in ponds in Bangladesh with green (high phytoplankton) or red (high zooplankton) water. Although this treatment warrants further study, it will not be possible to prescribe a particular type and dose of fertilizer in order to achieve optimal water colour, as fertilization levels should be adjusted according to the development of the algal bloom.

The reported reduction in disease, and increase in production levels, compared to previous years (Tables 4 and 6) may be due to management advice given to farmers by project staff during PARG sessions. Table 9 shows that farmers believed that better management practices were almost as important as the treatment effect in improving

production levels over the previous year. However, this does not explain the higher increase in production reported by treatment farmers over control farmers. Control farmers were present in the same PARG groups as treatment farmers, so it is likely that improved management practices, other than treatments, were adopted equally between control and treatment farmers.

The high level of satisfaction expressed by farmers, particularly with ash and lime treatments, are indicators that uptake of these treatments would be high among the wider population of Rajshahi and Kishoregonj farmers. The high number of phased-out farmers that continued these treatments of their own accord is further evidence of this [insert further details here]. The fact that the project did not provide the farmers with any of the treatments helped to ensure that farmer's uptake of the treatment was sustainable, and motivated by a genuine concern for the health of the fish.

It is important to be aware that the high level of satisfaction that the farmers expressed may, in itself, be a cause for bias in the study. Attempts were made during the course of the study to verify information provided by individual farmers, but given the number of farmer participating in the study, it was not possible for the authors to visit all the sites. It is suggested that future studies should provide the farmers with a better means of obtaining the data themselves. For example, teaching record keeping procedures and providing weighing scales would better equip farmers with the means to estimate production. Farmers could also be provided with formalin and dissection equipment and instructed in histological sampling techniques, so that samples could be taken from each outbreak, for later diagnosis by laboratory staff.

Care should be taken in extending information about the uptake of treatments beyond the target beneficiaries, in this case, polyculture farmers in Rajshahi and Kishoregonj. There may be important regional differences in farmer conditions that account for farmer responses. For example, in this study farmers in Kishoregonj expressed a greater desire to adopt the ash treatment than farmers in Rajshahi, because of the greater availability of ash in Kishoregonj.

The variability between small-scale carp polyculture ponds in Bangladesh makes it very difficult to undertake a controlled, replicated treatment study. An attempt was made here to investigate possible compounding factors that may influence the occurrence of disease to a greater extent than the adoption of a treatment. Similar to findings by Khan & Lilley (2001), presence of wild fish in the pond, and presence of ulcerated fish in nearby water bodies, increased the risk of outbreaks. It is suggested that more stringent selection criteria should be used in future studies, to ensure that experimental ponds are as similar as possible. It is particularly important that participating farmers adopting a particular treatment apply the same treatment dose and number of treatment applications over the entire study period. If enough farmers participate in the study, it may be possible to exclude data from particular ponds that do not conform to study criteria.

Treatments should be pre-tested in controlled pond trials to ensure that they are capable of yielding positive benefits for the farmer. In this case, treatments were tested in pond trials in Thailand and Bangladesh and found to reduce the number of snakeheads and mrigal affected by EUS, after challenge with *A. invadans* (unpublished data). The success of the treatments as reported by farmers in the present farm-based study was even greater than was indicated by the previous pond studies. Despite the problems concerned with accurately assessing the impacts of the treatments on ulcerative disease in field situations, it is suggested that farmer participatory research is an important means of enabling and assessing uptake of these treatments by farmers.

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Table-1: Background information

	Districts		Overall
	Rajshahi	Kishoregonj	
Average family size	*5.9 ± 3.1	6.1 ± 2.5	6.1 ± 2.7
Average land size (m^2)	9909 ± 9395	6408 ± 5874	7639 ± 7456
Average pond area (m^2)	1433 ± 1534	656 ± 461	915 ± 1032
Average pond depth (m)	2.1 ± 0.7	2.2 ± 2.5	2.2 ± 2.1
Pond ownership pattern			
-Single	42 (45%)	127 (69%)	169 (61%)
-Multiple	32 (34%)	53 (29%)	85 (31%)
-Leased	20 (21%)	4 (2%)	24 (9%)

**Denotes mean ± standard deviation*

Figures in the parentheses show data as a proportion of the total

Table-2: Application frequency of preventative treatments

Treatment	Rajshahi				Kishoregonj			
	Total number of ponds	Application frequency			Total number of ponds	Application frequency		
		Mean	Min	Max		Mean	Min	Max
Salt	4	3.5	3	4	16	3.8	1	6
Lime	30	2.6	1	6	30	3.3	1	6
Salt&lime	11	2.0	1	3	15	4.2	3	6
Neem	6	2.0	1	3	15	1.2	1	2
Ash	19	2.7	1	4	7	3.9	3	7
Fertilizer	Nil	Nil	Nil	Nil	3	4.3	3	6

Table-3: Application of neem

District	Neem application (average no. of stems/ha)		Neem application range (number of stems/ha)
	Affected pond	Not affected pond	
Rajshahi	*62.6 ± 27.7	70.5 ± 102.0	2.3 – 187.7
Kishoregonj	73.9 ± 68.7	58.2 ± 46.1	8.2 – 171.5

**Denotes mean ± standard deviation*

Table-4: Occurrence of ulcerative disease after starting treatment

Treatment	Rajshahi				Kishoregonj			
	Total number of ponds	Disease occurrence		% disease occurrence in 1999-2000	Total number of ponds	Disease occurrence		% disease occurrence in 1999-2000
		1998-99	1999-2000			1998-99	1999-2000	
Salt	4	2 (50%)	0 (0%)[a]	8.6%	16	13 (81%)	2 (13%)[a]	9.9%
Lime	30	11 (37%)	1 (3%)[a]		30	22 (73%)	1 (3%)[a]	
Salt&lime	11	4 (36%)	1 (9%)[a]		15	13 (87%)	3 (20%)[a]	
Neem	6	4 (67%)	3 (50%)		15	14 (93%)	1 (7%)[a]	
Ash	19	14 (70%)	1 (5%)[a]		42	37 (88%)	5 (12%)[a]	
Fertilizer	Nil	Nil	Nil		3	3 (100%)	0 (0%)[a]	
Control	11	8 (73%)	7 (64%)	63.6%	31	22 (71%)	19 (61%)	61.3%
Total	**81**	**43**	**13**		**152**	**124**	**31**	

Figures in the parentheses show data as a proportion of the total

Figures with superscript show significant differences with Control at 5% level of significance

Table-5: Species affected by ulcerative disease

	Rajshahi		Kishoregonj	
Treatment	**Cultured species**	**Wild species**	**Cultured species**	**Wild species**
Salt	Not affected	Not affected	Catla (*Ca. catla)* Rohu *(L. rohita)** Mrigal (*C. mrigala*) *(1-ve) Silver carp (*Hy. molitrix)** Silver barb (*B. gonionotus*) *(1+ve) Gonia (*L. gonia*) Tilapia (*O. nilotica*)	Snakehead (*Ch. punctatus*) Striped snakehead (*Ch. striatus*) Catfish (*My. tengara*) *(1+ve) Stinging catfish (*H. fossilis*) Walking catfish (*Cl. batrachus*) Puti (*P. sophore*)*
Lime	Catla (*Ca. catla)* Rohu *(L. rohita*) Mrigal (*C. mrigala*) Silver barb (*B. gonionotus*)	Mola (*A. mola*) Puti (*P. sophore*) Snakehead (*Ch. punctatus*) Flying barb (*E. danrica*) Chanda (*Cha. nama*)	Catla (*Ca. catla) *(2+ve)* Rohu *(L. rohita)** Mrigal (*C. mrigala*) *(3+ve, 3-ve) Silver carp (*Hy. molitrix*) Silver barb (*B. gonionotus*) Gonia (*L. gonia*) Common carp (*Cy. carpio*) Calbaush (*L. calbasu*) *(1-ve)	Snakehead (*Ch. punctatus*) Striped snakehead (*Ch. striatus*) Catfish (*My. tengara*) Puti *(P. sophore)* Climbing perch (*An. testudineus*) *(1+ve)
Salt&lime	Catla (*Ca. catla)* Rohu *(L. rohita*) Mrigal (*C. mrigala*) *(1+ve, 1-ve) Silver barb (*B. gonionotus)** Grass carp (*Ct. idella*)	Puti (*P. sophore)** Flying barb (*E. danrica*) *(1+ve)	Catla (*Ca. catla*) Mrigal (*C. mrigala*) Silver barb (*B. gonionotus*)	Snakehead *(Ch. punctatus)*
Neem	Catla (*Ca. catla)* Mrigal (*C. mrigala*) Tilapia (*O. nilotica*) Grass carp (*Ct. idella*)	Wallago (*W. attu*)	Catla (*Ca. catla)* Rohu *(L. rohita*) Mrigal (*C. mrigala*) Silver barb (*B. gonionotus*)	Snakehead (*Ch. punctatus*) Puti *(P. sophore)* Climbing perch (*An. testudineus*)
Ash	Catla (*Ca. catla)** Rohu *(L. rohita*) Mrigal (*C. mrigala*) Silver barb (*B. gonionotus*) *(1+ve)	Snakehead (*Ch. punctatus*) Striped snakehead (*Ch. striatus*) Puti (*P. sophore*) Catfish (*M. vittatus*) *(1+ve)	Catla (*Ca. catla*) Mrigal (*C. mrigala*) *(1-ve) Silver barb (*B. gonionotus*) Common carp (*Cy. carpio*) *(1-ve)	Snakehead (*Ch. punctatus*) Armed spiny eel (*M. armatus*)*
Fertilizer	Not applicable	Not applicable	Not affected	Not affected
Control	Catla (*Ca. catla)* Rohu *(L. rohita*) Mrigal (*C. mrigala*) Silver carp (*Hy. molitrix*) Silver barb (*B. gonionotus*)	Snakehead (*Ch. punctatus*) Striped snakehead (*Ch. striatus*) Puti (*P. sophore*)	Catla (*Ca. catla)* Rohu *(L. rohita*) *(1+ve) Mrigal (*C. mrigala*) *(4+ve, 1-ve) Silver carp (*Hy. molitrix*) Silver barb (*B. gonionotus*) Gonia *(L. gonia*)	Snakehead (*Ch. punctatus*) *(1+ve) Puti *(P. sophore)* Climbing perch (*A. testudineus*) *(2+ve) Catfish (*M. tengara*) *(1+ve) Kholsa (*Co. fasciata*)*

All fish listed had lesions and were recorded as diseased for study analyses. Samples of fish shown here with asterices were taken for histological diagnosis. Only a proportion of samples were processed.
-ve Fish sample found to be EUS-negative by histology, the number of samples examined is indicated
+ve Fish sample found to be EUS-positive by histology, the number of samples examined is indicated

Key
A.= Amblypharyngodon, An.= Anabas, B.= Barbodes, C.= Cirrhina, Ca.= Catla, Ch.= Channa, Cha.= Chanda, Cl.= Clarias, Co.= Colisa, Ct.= Ctenopharygodon, Cy.= Cyprinus, E.= Esomus, H.= Heteropneustes, Hy.= Hypophthalmichthys, L.= Labeo, M.= Mastacembelus, My.= Mystus, O.= Oreochromis, P.= Puntius, W.= Wallago

Table-6: Total input cost and fish production compared to the previous year

Treatment	Rajshahi						Kishoregonj					
	Input cost (Tk/ha)		% increase in input cost	Production (kg/ha)		% increase in production	Input cost (Tk/ha)		% increase in input cost	Production (kg/ha)		% increase in production
	1998-99	1999-2000		1998-99	1999-2000		1998-99	1999-2000		1998-99	1999-2000	
Salt	*15892 ±14696	23899 ±6741	50%	1503 ±1044	2445[a] ±1028	63%	16076 ±11511	28025 ±56791	74%	1387 ±1289	2418 ±872	74%
Lime	20662 ±13137	21513 ±12071	4%	1815 ±1398	1910[a] ±822	5%	18005 ±23766	23171 ±28730	29%	1683 ±3173	2855[a] ±4117	70%
Salt&lime	26802 ±14508	26616 ±10340	-1%	2010 ±776	2395[a] ±1150	19%	14687 ±6220	17504 ±3579	19%	930 ±687	2234 ±833	140%
Neem	10306 ±8176	9820 ±5481	-5%	1033 ±1028	1938[a] ±1185	88%	16471 ±9045	17622 ±8019	7	1017 ±685	2132 ±873	110%
Ash	12841 ±12502	12463 ±7816	-3%	1263[a] ±892	1875 ±921	48%	17482 ±9887	15202 ±7913	-13%	1314 ±774	2390[a] ±842	82%
Fertilizer	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	11703 ±5777	15748 ±3457	35%	818 ±228	2525 ±299	208%
Control	21063 ±27001	18814 ±23414	-11%	2954 ±5153	2238a ±2445	-24%	12661 ±6329	12690 ±6131	0%	1386 ±717	1560 ±643	13%
Total	**19125 ±15767**	**19357 ±13239**	**1%**	**1791 ±2050**	**2194 ±1255**	**23%**	**16191 ±14013**	**18790 ±25592**	**16%**	**1356 ±1718**	**2329 ±2152**	**72%**

**Denotes mean ± standard deviation*

Figures with superscript show significant difference with Control at the 10% level of significance

Production calculated from Taka value of fish harvested, as given by farmers

Table-7: Number of ponds with increased and decreased production in 1999-2000 compared to 1998-1999

Treatment	High production increase (above 200%)	Low production increase (below 200 %)	Decrease in production
Salt	10 (36%)	15 (54%)	3 (11%)
Lime	16 (21%)	53 (68%)	9 (12%)
Salt&lime	7 (22%)	19 (59%)	6 (19%)
Neem	7 (29%)	15 (63%)	2 (8%)
Ash	16 (23%)	49 (69%)	6 (8%)
Fertilizer	1 (33%)	2 (67%)	0 (0%)
Control	3 (7%)	21 (50%)	18 (43%)
Total	60	174	44

Figures in the parentheses show data as a proportion of the total for each group

Table-8: Satisfaction with treatment results

Reason for satisfaction	Rajshahi	Kishoregonj
No occurrence of ulcerative disease	51%	53%
Better fish growth	38%	37%
Fish recovered from ulcerative disease	9%	9%
Fish sold at a higher price	2%	1%
Total indicating satisfaction	**79 (94%)**	**147 (96%)**

Note: more than one reason for satisfaction was given by some farmers

Table-9: Reason for increased production in 1999-2000 compared to 1998-1999

Reason for increased production	Rajshahi	Kishoregonj
Treatment effect	46%	58%
Better management	35%	24%
More feed and fertilizer	16%	17%
Suitable weather	2%	0%
Good quality seed	1%	0%
Total reporting increased production	**79 (83%)**	**163 (89%)**

Note: more than one reason for satisfaction was given by some farmers

Table-10: Action taken by farmers after noticing ulcerative disease

Action taken	Rajshahi	Kishoregonj	Both districts
Continued treatment	6 (35%)	30 (75%)	36 (64%)
Stopped treatment and not sold	7 (41%)	6 (15%)	13 (23%)
Distress sale	3 (18%)	4 (10%)	7 (13%)

All treatments except neem are included

Figures in the parentheses show data as a proportion of the total

Table-11: Desire of farmers (including control farmers) to adopt treatment in the next (2000-2001) season

Treatment	Rajshahi	Kishoregonj	Both districts
Lime	44 (47%)	63 (34%)	107 (38%)
Salt	5 (5%)	19 (10%)	24 (9%)
Salt&lime	17 (18%)	13 (7%)	30 (11%)
Ash	20 (21%)	68 (37%)	88 (32%)
Neem	5 (5%)	18 (10%)	23 (8%)
Fertilizer	1 (1%)	3 (2%)	4 (1%)
Not decided	1 (1%)	0 (0%)	1 (0%)
Not continue	1 (1%)	0 (0%)	1 (0%)
Total	**94**	**184**	**278**

Note: includes farmers that started treatment after ulcerative disease occurred
Figures in the parentheses show data as a proportion of the total

Table-12: Possible confounding factors influencing ulcerative disease occurrence in ponds

Variable	Rajshahi		Kishoregonj	
	Chi square	P value	Chi square	P value
Pond was dried prior to stocking	0.01	0.934	0.02	0.879
Lime was applied prior to stocking	0.46	0.500	1.40	0.237
Pond is connected to external water source	0.06	0.771	1.20	0.273
Wild fishes were removed prior to stocking	0.07	0.797	0.20	0.656
Source of seed (Patilwala, Hatchery or Nursery)	2.92	0.232	0.69	0.709
Fertilization after stocking (Regular, Irregular or Never)	0.33	0.848	2.33	0.312
Supplementary feeding (Regular, Irregular or Never applied)	2.30	0.317	1.85	0.397
Wild fish entered the pond during culture period	0.98	0.321	4.44	0.035*
Net used in fish catching/harvesting (Own net or Borrowed net)	0.04	0.833	0.51	0.477
Pond was flooded in 1998-99	0.03	0.860	0.53	0.466
Cirrhinus mrigala present in the pond	0.03	0.863	1.24	0.265
Barbodes gonionotus present in the pond	0.09	0.769	1.28	0.257
Ulcerative disease present in nearby ponds	5.15	0.023*	1.26	0.261

**Significant difference (P<0.05)*

APPENDIX TWENTY

Article five - Miles, D.J.C. (1999) Lessons learned in macrophage culture. The AAHRI Newsletter, Aquatic Animal Health Research Institute, Bangkok. 8(2), 9-12. (hard copy available from ARP Manager)

Lessons Learned in Macrophage Culture

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Miles, D. J. C. (1999) Lessons learned in macrophage culture. The AAHRI Newsletter, 8(2), Pp. 9-12. Aquatic Animal Health Research Institute, Bangkok.

However remarkable the results of a study, the most incredible part of any paper is surely the materials and methods. There is invariably a clear and concise account of how the authors carried out the work without needing to spend so much as ten minutes optimising their assays, dealing with contamination or dropping their samples. Of course, as soon as one tries to repeat the study, one immediately finds such irritations in abundance, and has to find one's own solutions.

I have recently completed a study involving the successful isolation and culture of head-kidney macrophages from four fish species (striped snakehead *Channa striata*, silver barb *Puntius gonionotus*, Nile tilapia *Oreochromis niloticus* and giant gourami *Osphronemus gourami*) and the complete failure to isolate anything from the head-kidney of a fifth (swamp eel *Fluta alba*). Macrophages were used for fungicidal assays, which were cultured for 24h in the presence of germinating *Aphanomyces invadans* propagules. While basic protocols are available in abundance, this article is intended cover what I wish someone had told me before I started, but will definitely not be appearing in my materials and methods section.

All suggestions are based on my own experience and that of others doing similar work. They are inevitably biased by the fact that the work was done at AAHRI and the Institute of Aquaculture, University of Stirling, UK. I do not claim to have produced a all-encompassing text, but hope it may be useful to anyone else planning to work with fish macrophages *in vitro*.

The techniques were adapted from Secombes (1990), which remains the quintessential text on macrophage isolation, and details of the solutions used is given in the appendix.

1 Kill the fish

Handling stress during this stage can severely affect the ability of the macrophages to respond to the presence of a pathogen**.** Although it is acceptable to stun the fish with a blow to the head and then sever the spinal cord, far better results are attained by the gradual introduction of anaesthetics such as benzocaine or quinaldine. A few drops should be used initially to immobilise the fish, then more added.

2 Bleed the fish

If the fish is not bled, blood cells will be isolated with the macrophages and may have a detrimental effect on culture. The simplest method for bleeding is to slash the caudal vessel near the tail, though blood may be collected with a syringe. The caudal vein lies along the vertebral column and may be accessed laterally, usually just below the lateral line in the anterior third of the fish, or ventrally.

Dissection should be carried out immediately as macrophages die without their blood supply.

3 Dissect out the head-kidneys and place in isolation medium

Many protocols recommend spraying the surface of the fish before dissection and the peritoneum before removal of the head-kidney with 70% ethanol solution. While this is probably unnecessary, dissection instruments should be kept in absolute alcohol and flamed and the head-kidney should be placed straight in isolation medium without touching any other part of the fish.

The position of the head-kidney varies between species. For example, snakehead head-kidneys lie along the upper surface of the peritoneum well behind the head, while tilapia head-kidneys are much less prominent and

recessed into the top of the skull. As much of the head-kidney should be taken as possible as it is better to have too many macrophages than too few.

The required volume of isolation medium depends on the size of the head-kidney, but 5ml is sufficient for most species up to approximately 500g.

4 Macerate the head-kidneys by pushing through a 100µl nylon mesh

The autoclaved mesh should be stretched over a petri dish and the medium containing the head-kidneys poured on to it. The kidney is usually pushed through the mesh with the plunger of a 3ml or 5ml syringe.

Pouring a little isolation medium into the petri dish beforehand facilitates the procedure, but if too little isolation medium was used to collect the head-kidneys, migration of cells will be inhibited.

Meshes can be cleaned and re-used, though they tend to deteriorate and produce poor quality macrophages after several uses.

5 Layer the suspension on to a percoll gradient

This stage causes more broken friendships than the rest of the protocol put together, especially as gradients must be prepared immediately before use. Sadly, it can only be mastered by experience but the following protocol may be used as a starting point:

I/ Pipette 10 ml of 34% percoll solution into a centrifuge tube.
II/ Carefully pipette 7.5ml of 51% percoll solution under it to form a gradient. A soft bulb is essential. The tip of the pipette should be placed on the slope just above the apex of the tube and the bulb squeezed steadily. Trying to add the suspension too slowly is likely to lead to jerkiness and a poor gradient. The last of the fluid should not be expelled to avoid producing bubbles that will break up the gradient.
III/ If there is no visible gradient, throw it away and start again.
IV/ If there is a visible gradient, carefully pipette the head-kidney suspension on to the surface. When it is collected, the surface of the petri dish should be washed several times to ensure the collection of macrophages that have adhered to the petri dish surface.

Excessive agitation of the gradient at any time will break up the gradients and homogenise the solution. If this happens accidentally, for example if the sample is dropped, the homogenised head-kidney can be recovered by completely homogenising the suspension, centrifuging at 900g for 15min at 4ºC, pouring off the supernatant, resuspending the pellet in 5ml isolation medium and repeating this step. However, a large loss of macrophages can be expected.

6 Centrifuge gradients at 400g, 25min, 4°C

After centrifuagation, a white band of macrophages should be clearly visible at the gradient.

7 Collect macrophages and add 5ml washing medium

Macrophages should be collected in a second centrifuge tube without removing any of the 51% layer, which may contain other leukocytes. If very few macrophages are present, the band may not be visible, but some may still be collected from the same area.

8 Centrifuge at 1000g, 15min, 4°C

9 Discharge the supernatant and resuspend the pellet in 3ml washing medium

10 Centrifuge at 900g, 15min, 4°C

The purpose of these stages is to wash off any residual percoll and to ensure that the macrophages are thoroughly exposed to antibiotics.

11 Resuspend pellet in washing medium and count macrophages with a haemocytometer

1ml of washing medium is usually sufficient for resuspension. For counting, the macrophage suspension should be mixed 1:1 with 0.1% trypan blue and diluted for ease of counting. Suggested volumes are:

100µl	0.1% trypan blue
90µl	washing medium
10µl	macrophage suspension

The trypan blue solution should be placed on a haemocytometer (ideally improved Neubauer pattern) and counted at 100x magnification using a phase contrast microscope. Macrophages are relatively large and circular, although they rapidly loose their shape under a microscope light. They should not be confused with elliptical red blood cells or the many smaller cells that may be present. Any macrophage that appears blue is non-viable and should not be included within the count.

The concentration can be calculated by the following formula with most haemocytometers:

$$^{cells}/_{ml} = {}^{cells}/_{large\ square} \text{ x dilution factor x } 10^4$$

12 Adjust the cell suspension to the required concentration with washing medium and seed culture vessel

The culture vessel and macrophage concentration used require consideration based on the study. Most fish of more than 50g will yield more than 10^7 viable macrophages in total, although the exact quantities vary between species. For respiratory burst assays, a concentration of 10^7 cells ml^{-1} is usually required, while for bactericidal or fungicidal assays, 10^6 cells ml^{-1} may be adequate.

The culture vessel of choice is usually a sterile, flat-bottomed polystyrene 96-well microplate with a lid. However, if macrophages are to be collected and stained for microscopic examination, various types of slide are available. The best, but most expensive design is the chamber slide with a removable plastic gasket. However, macrophages may be cultured on a ordinary slide in wells made with a pap-pen.

13 Incubate for 4h at 22-24°C

During the incubation, viable macrophages adhere to the surface of the culture vessel to form a monolayer.

Protocols based on trout usually require 2h at 15-18°C. However, experience has shown the above to be optimal for most species of tropical fish. While some flexibility may be allowed over the time, higher or lower temperatures frequently prevent macrophages from adhering to the culture vessel.

14 Wash off non-adherent macrophages with L-15 medium

A large proportion of macrophages do not adhere and must be removed by three washes of L-15 warmed to the incubation temperature. Although media are usually stored at 4°C, washing with media at this temperature may cold shock macrophages and reduce viability.

15 Add maintenance medium

Maintenance medium should be warmed to the incubation temperature before it is added to the cultures. The choice of maintenance medium depends on the study. However, it must be based on L-15 medium containing 5% v/v foetal calf serum. The latter may require heat inactivation (a 55°C water bath for 30min) before use and should ideally be tissue culture grade.

If pathogen suspensions are to be added for bactericidal or fungicidal assays, the L-15 may be prepared at double strength to allow for the dilutions. If no pathogens are to be added, antibiotic solutions may be added to the maintenance medium to reduce the risk of contamination.

16 Culture

Although any macrophage culture deteriorates over time, the period a culture can be kept for varies widely between species. For example, rainbow trout macrophages can be kept alive and activated for three months while snakehead macrophages are unlikely to remain viable for more than 24h. Any technique should be performed as soon as possible.

A further consideration is that there is considerable variation in the proportion of cells that adhere both between and within species. For example, in the aforementioned fungicidal study, 1-5% of the snakehead macrophages adhered while 2-69% of the silver barb macrophages adhered.

17 Assessment of macrophage number

Due to the inconsistencies of the number of macrophages present, it is necessary to enumerate them for any quantitative assay. Macrophages are lysed so that the nuclei are suspended and may be counted. Usually, two wells per fish are counted and the results averaged. The counting technique is as follows:

I/ Prepare cultures identical to those used for the assay.
II/ Add lysis buffer at 4°C. The volume varies with the expected number of macrophages but suggested volumes are 200µl for cultures prepared at 10^7 cells ml^{-1} and 100µl for cultures prepared at 10^6cells/ml.
III/ Incubate for 10-30min.
IV/ Mix the suspension well without producing bubbles.
V/ Count nuclei in a haemocytometer at 200x magnification. See section 11 for interpretation of result.

The main difficulty with this procedure is recognising nuclei from other debris. Counting cells from cultures prepared at different dilutions is a good way to start, as nuclei vary in concentration while debris does not.

General Procedures: Sterile Technique

Although the presence of contaminants is inevitable during the early stages of the procedure due to the large number of microbial symbionts present in fish, they must be completely removed by the end of the procedure. The antibiotics in the solutions should achieve this provided no more are introduced by poor handling.

Once the kidney is removed, all steps must be carried out in a sterile hood of some type. All spillages of media must be cleaned immediately as they provide a viable environment for bacteria. The operator should wear latex gloves and wash them regularly with 70% ethanol solution, especially after removing the hands from the hood. Above all, the operator must not touch anything which will come into direct contact with the macrophages.

All materials and solutions used must be sterile, and not opened outside a sterile hood.

General Procedures: Use of Ice

Many protocols recommend carrying out all steps on ice to prevent macrophages adhering to surfaces during the isolation and washing stages. This sometimes works but usually complicates the technique unnecessarily.

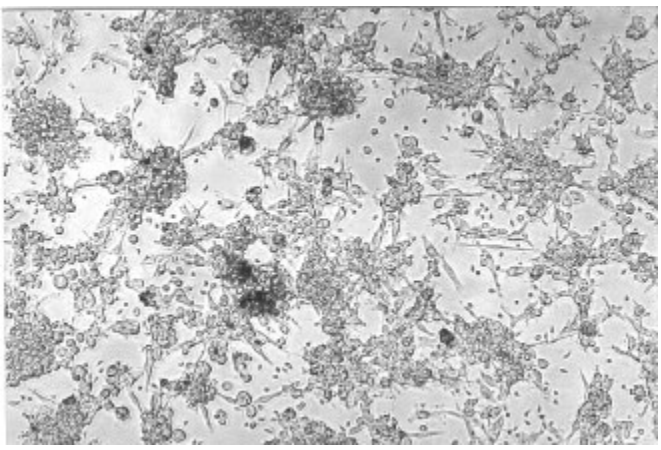

Figure 1. Rainbow trout Oncorhyncus mykiss macrophages innoculated with Aphanomyces invadans cysts. Note the clusters forming around fungi, and the pseudopodia of the unclustered macrophages. Both are characteristic of healthy cells.

Acknowledgements

The reader has probably surmised that the acquisition of the above experience was not without a degree of angst. I would like to thank everyone whose senses of humour continued to function after mine had failed, particularly Miss Phuttachat Seetubutim who not only bore the brunt of my temperament but produced many of the above suggestions.

Other solutions came from the painfully acquired knowledge of Dr. Natalio Garcia Garbi and Dr. Mags Crumlish of the Institute of Aquaculture, University of Stirling.

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Secombes CJ (1990). Isolation of Salmonid Macrophages and Analysis of their Killing Activity in Stolen JS, Fletcher TC, Anderson DP, Roberson BS & van Muiswinkel WB (eds) *Techniques in Fish Immunology*, SOS Publications, 43 DeNormandie Ave, Fair Haven, NJ 07704-3303, USA.

Appendix: Solutions used

It is recommended to prepare the following quantities and store at 4°C.

Isolation medium:	100ml L-15 medium 1ml penicillin-streptomycin solution (stock: 10,000U ml^{-1}) 400µl heparine (stock: 2,500U ml^{-1} in L-15) 2ml Foetal Calf Serum
Washing medium:	100ml L-15 medium 1ml penicillin-streptomycin solution (stock: 10,000U ml^{-1}) 100µl Foetal Calf Serum
34% percoll solution:	34ml percoll 10ml 10x Minimum Essential Medium (MEM) 56ml sterile distilled water
51% percoll solution:	51ml percoll 10ml 10x Minimum Essential Medium (MEM) 39ml sterile distilled water
Lysis buffer:	100ml 0.1M citric acid 1ml tween 20 100µl crystal violet Sterile filter at 0.45 nm

APPENDIX TWENTY ONE

Paper fourteen - Miles, D.J.C., Kanchanakhan, S., Lilley, J.H., Thompson, K.D., Chinabut, S., and Adams, A. (2001) Effect of macrophages and serum of fish susceptible or resistant to epizootic ulcerative syndrome (EUS) on the EUS pathogen, *Aphanomyces invadans*. Fish and Shellfish Immunology (in press)

Effect of macrophages and serum of fish susceptible or resistant to Epizootic Ulcerative Syndrome (EUS) on the EUS pathogen, *Aphanomyces invadans*

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Key words: Epizootic ulcerative syndrome, *Aphanomyces invadans*, macrophage, serum, respiratory burst, antibody.

Abstract

Epizootic ulcerative syndrome (EUS) is one of the most destructive diseases of fresh and brackish water farmed and wild fish in the Asia-Pacific region. The *in vitro* germination and growth of the propagules of the EUS pathogen, *Aphanomyces invadans* (=*A. piscicida*), were assessed in the presence of the head-kidney macrophages, serum, and serum heated to inactivate complement proteins, of three EUS-susceptible and one resistant fish species. The susceptible species were striped snakehead *Channa striata*, giant gourami *Osphronemus gouramy* and silver barb *Barbodes* (= *Puntius*) *gonionotus*, and the resistant species was Nile tilapia *Oreochromis niloticus*. Fish of all species were acclimatised to either low temperature (20°C ± 1.6) at which EUS is known to occur, or to high temperature (32 °C ± 5.0) at which EUS does not occur, except for giant gouramis which were only studied at low temperature. The respiratory burst of the macrophages was assessed in the presence of *A. invadans* or the stimulant phorbol myristate acetate (PMA), and compared to that of controls. Anti-*A. invadans* antibody concentrations were assessed in all species except silver barbs. All assays were carried out at the same temperature, regardless of the temperature that the fish were kept at.

Macrophages of all species other than snakeheads inhibited fungal germination at both temperatures, though only silver barb and gourami macrophages could inhibit germling growth. PMA increased the respiratory burst in nearly all cases. Respiratory burst in the presence of *A. invadans* was consistently lower than that of controls, though the difference was only significant in the case of snakeheads. Respiratory burst of all macrophage treatments was higher at low temperature. Except in the case of PMA-stimulated macrophages, regressions between respiratory burst and inhibitory action were only found in susceptible species, suggesting that the respiratory burst is important in those species, but is unable to prevent the proliferation of *A. invadans*.

Serum inhibited fungal germination in all cases other than low temperature tilapia, indicating that the EUS-resistance of tilapia is not due to the serum. Inhibition of germling growth by serum only occurred in silver barbs and gourami. Heated serum did not inhibit germination in any case except that of high temperature snakehead, and stimulated it in the case of tilapia. Heating serum did not affect the growth inhibiting activity of

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silver barbs and gouramis, but it stimulated growth in some groups. Snakeheads at high temperature had high anti-*A. invadans* antibody concentrations, which may explain the inhibitory activity of their heated serum. A role for complement and antibodies in defence against *A. invadans* in susceptible species is suggested.

I. Introduction

Epizootic ulcerative syndrome (EUS) was first reported from Japan in 1971 (Egusa & Masuda, 1971) and since then it has spread across the Asia-Pacific region, causing substantial losses to fresh and brackish water aquaculture systems and large scale mortalities of wild fish populations (Chinabut, 1998).

EUS outbreaks usually occur during the colder seasons of the year when the temperature is below 25°C (Tonguthai, 1985) although optimal mycelial growth in culture is nearer 30°C (Willoughby *et al*, 1995). It is not clear whether such seasonality results from the reduced immunity of fish at low temperatures making them more prone to infection (Chinabut *et al*, 1995) or from reduced infectivity by the pathogen at higher temperatures (Willoughby, 1993), or a combination of both.

The first few outbreaks in an area are often associated with high mortality, though subsequent occurrences are usually less severe (ODA, 1994). EUS may not be reported from an area for a number of years, but outbreaks still recur (Chinabut, 1998).

EUS is diagnosed by the presence of the invasive hyphae of the oomycete, *Aphanomyces invadans* (=*A. piscicida*) (ODA, 1994). Granuloma formation is a common response of fish to invasive pathogens (Secombes & Fletcher, 1992), and is so frequently associated with EUS that it was incorporated into the case definition (ODA, 1994). The importance of granulomata in defence against *A. invadans* was illustrated by Wada *et al's* (1996) observation that EUS-resistant common carp *Cyprinus carpio* develop granulomata far more rapidly than susceptible ayu *Plecoglossus altivelis*.

The mechanism by which the granulocytes inhibit the invasive hyphae is unclear. The size of the hyphae precludes phagocytosis, although multinucleate giant cells observed to encapsulate the mycelium when non-susceptible common carp were injected with *A. invadans* (Wada *et al*, 1996). One possibility is the production of superoxide (O^-_2) by the respiratory burst reaction of granulocytes. It is employed in the immune responses of many animals and plants (Sutherland, 1991), and in responses to hyphal pathogens as diverse as that of human macrophages to the imperfect fungus, *Aspergillus fumigatus* (Diamond & Clark, 1982) and that of potato tubers to the oomycete, *Phytophthora infestans* (Doke, 1983). Superoxide production is not the only mechanism by which macrophages may interact with pathogens, but the strength of the respiratory burst of head-kidney macrophages *in vitro* is a widely used indicator of immunocompetance, especially when provoked by stimulators (Secombes, 1990).

Humoral components of the innate immune system have also been associated with resistance to fungi. The alternative complement pathway (ACP) is involved in resistance to many fungal pathogens of mammals (Lehman, 1985). Teleost complement proteins differ very little from those of mammals (Nonaka *et al*, 1984), and Ji *et al* (1997) suggested that complement proteins are conserved throughout the chordates. Although the role of fish ACP in immunity to fungi and oomycetes has not been previously investigated, such a similarity of form suggests a similarity of function.

Specific antibody responses to hyphal infection of mammals are commonplace (Casadevall, 1995), though they have only been conclusively proven to be protective in a few cases such as that of horses to

Trichophyton equinum (Pier & Zancanella, 1993). In fish, antibodies to *A. invadans* have been found in striped snakehead *Channa striata* (Thompson *et al*, 1997) and rainbow trout *Oncorhynchus mykiss* (Thompson *et al*, 1999), though whether they are protective is unclear.

The present study compared the mechanisms by which EUS-susceptible and non-susceptible fish respond to *A. invadans*, and to establish whether any of those mechanisms are attenuated at the low temperatures at which outbreaks typically occur.

II. Methods

EXPERIMENTAL FISH

All experimental fish were from central Thailand (Table 1), where EUS had regularly been reported for 17 years preceding the study (Tonguthai, 1985).

Fish from all four species were acclimatised to 20ºC (s.d. ± 1.6) for at least three weeks. Other snakeheads, silver barbs and gouramis were acclimatised to the ambient temperature of approximately 32°C (s.d. ± 5) at which EUS is rarely reported. All fish were fed twice daily on Charoen Pokphand (Bangkok, Thailand) brand no. 9910 pellets. Water quality was maintained by partial water changes twice weekly. All fish were kept in 150l glass tanks.

It was not possible to use all individuals for every analysis due to difficulties with bacterial contamination of some samples, but all analyses involved a minimum of five and a maximum of twelve individuals per group.

EXPERIMENTAL PATHOGEN

A. invadans isolate NJM9701 was isolated from an EUS-affected ayu in Shiga Prefecture, Japan, two years previously. Pathogenicity was confirmed by injection of zoospores into healthy striped snakeheads and histopathological examination.

Zoospores were collected after Khan *et al* (1998) and encysted by shaking vigorously for 20s.

REAGENTS

All solutions were prepared in distilled water unless otherwise specified.

Reagents for macrophage isolation, respiratory burst assays and fungicidal assays were prepared after Secombes (1990) and Miles *et al* (in press). Reagents for ELISA were prepared after Chen *et al* (1996) and for ELISA antigen collection after Miles *et al* (in press). All culture medium for fungicidal or respiratory burst assays was based on L-15 medium (Miles *et al*, in press).

COLLECTION OF SERUM AND MACROPHAGES

Fish were killed by overdose of 10% w/v benzocaine in absolute ethanol. As much blood as possible was immediately taken from the caudal vein. It was allowed to clot for 2h at 25°C (± 1.0), centrifuged at 10,000g for 15min and serum was pipetted off. Fungicidal assays were prepared immediately and serum for assessment of relative antibody concentration was stored at -70°C until the assay was performed.

Macrophages were isolated from the head kidney after Miles *et al* (in press) and adjusted to 10^6 cells ml^{-1} for fungicidal assays and 10^7 cells ml^{-1} for respiratory burst assays. All assays were carried out in 96-well

microplates and incubated 23ºC ±1, which preliminary studies had shown to be optimal for all species used in the study. Non-adherent macrophages were washed off and the wells prepared for fungicidal assays.

Duplicate wells were seeded for assessment of number of viable cells.

FUNGICIDAL ASSAYS

Fungicidal assays were carried out after Miles *et al* (in press).

Triplicate wells per treatment were prepared with macrophages, 10% v/v serum, 10% v/v serum heated at 55°C for 30min to inactivate complement or control wells containing medium only. *A. invadans* cysts prepared on the day the fish were sampled were added to all wells other than controls at a concentration of 10^4 cysts ml^{-1} (Thompson *et al*, 1999).

After 24h, germination in each well was assessed by counting visible germlings within one microscope field of view at 40x magnification, and extrapolating the count to the total area of the well for comparison between treatments and control. Germling growth was assessed by measuring ten randomly chosen germlings from each well using a graticule.

Viable macrophage numbers at the time of counting were assessed (Secombes, 1990)

RESPIRATORY BURST ASSAY

Three groups of triplicate wells were seeded with macrophages as described above. One group was inoculated with 10^4 fungal cysts ml^{-1}. Other triplicate wells were seeded only with fungal cysts to confirm that the germlings themselves had no effect on absorbance.

After incubation for 20h, intracellular superoxide production was evaluated by measuring the reduction of nitro-blue tetrazolium (NBT) over 30min (Secombes 1990). One group of uninoculated wells was stimulated with 4-α-phorbol 12-myristate 13-acetate (PMA) and the other used as a control for comparison with both stimulated and inoculated macrophages.

Viable macrophage number was assessed in duplicate wells per fish (Secombes, 1990).

To calibrate the results of the assays, serial doubling dilutions were made of a high but unknown concentration of superoxide, generated from the highest possible concentration of giant gourami macrophages. A standard curve of absorbance against superoxide concentration in arbitrary units was prepared, and shown to be extremely precise ($r^2 = 0.99$). All subsequent measurements were compared to the standard and results were converted into a superoxide concentration in arbitrary units per 10^5 macrophages.

ASSESSMENT OF RELATIVE ANTIBODY CONCENTRATION

Relative anti-*A. invadans* antibody concentrations were quantified by ELISA, after Chen *et al* (1996) with adaptations after Miles *et al* (in press), using duplicate serial dilutions for each individual fish.

MAb supernatants were prepared from hybridoma cell lines 7D2 raised against striped snakehead immunoglobulin, 5G11C2 against giant gourami immunoglobulin and M40 against Nile tilapia immunoglobulin. No anti-silver barb immunoglobulin MAb was available.

Antibody was considered to be present if mean test serum absorbance exceeded three times that of the negative control at any dilution. If antibodies were found, the relative antibody concentration was calculated for each fish (Miles *et al*, in press).

STATISTICAL ANALYSIS

Initial analysis on the growth and germination inhibition assays was carried out by comparing the results from treatment and control wells by 2-way ANOVA with interaction, followed by Tukey multiple comparisons, or by non-parametric equivalents where necessary.

Cases where treatments were different to controls were compared. Each treatment count was converted into an index of inhibition by dividing it by the mean control value (Miles *et al*, in press). Indices of inhibition by serum and heated serum were compared by nested ANOVA, followed by Bonferroni multiple comparisons. Indices of inhibition by macrophages were compared by nested ANCOVA, using macrophage counts as the covariate.

The respiratory burst of PMA-stimulated and *A. invadans*-inoculated macrophages was compared to controls by 2-way ANOVA with interaction and Tukey multiple comparisons within each case. Comparisons between macrophages with the same treatment were made between cases by nested ANOVA and Bonferroni multiple comparisons.

Three measures of superoxide production in the inhibition assay were calculated for each individual by dividing the superoxide production per 10^5 macrophages from each of the three treatments in the respiratory burst assay by 10^5, and multiplying by the number of macrophages in the inhibition assay. Regression analyses of the mean index of inhibition for each fish against the adjusted superoxide production for each fish were carried out. The significance of the regressions was assessed by F-test.

It was not possible to compare relative antibody concentrations between species as the MAb's used to assess them were not standardised. Comparison between individuals of the same species at different temperatures was carried out by independent samples t-tests.

The relationships between antibody concentration and inhibition of germination and growth were examined by regression of mean indices of inhibition against relative antibody concentration for each fish. The significance of the regressions was assessed by F-test.

Critical values of $p < 0.05$ were accepted as significant in all tests.

III. Results

FACTORS INHIBITING FUNGAL GERMINATION

Macrophages of all groups other than snakeheads reduced germination ($p < 0.05$). Serum of all groups other than low temperature tilapia reduced germination ($p < 0.05$). Only the heated serum of high temperature snakeheads reduced germination ($p < 0.005$), and that of tilapia at either temperature increased it ($p < 0.05$) (Table 2).

Heating reduced the activity of serum in all cases ($p < 0.05$).

FACTORS INHIBITING GERMLING GROWTH

Only silver barb and gourami macrophages, serum or heated serum reduced germling growth ($p < 0.01$). The heated serum of high temperature snakeheads and low temperature tilapia increased germling growth ($p < 0.05$) (Table 3).

Germlings were shorter when treated with low temperature silver barb heated serum than with unheated serum ($p < 0.005$), but there was no difference in the effect of any other case where both treatments reduced growth ($p > 0.05$). Heated sera of high temperature snakeheads and low temperature tilapia increased growth ($p < 0.05$).

COMPARISONS OF THE EFFECT OF MACROPHAGES FROM DIFFERENT SPECIES AND TEMPERATURE GROUPS ON GERMINATION AND GROWTH

No differences in macrophage activity on fungal germination or growth were found between groups other than those described above ($p > 0.05$).

COMPARISONS OF THE EFFECTS OF SERA AND HEATED SERA FROM DIFFERENT SPECIES AND TEMPERATURE GROUPS ON GERMINATION AND GROWTH

At low temperature, there were no significant differences between the activity of sera of different species on germination other then those described above ($p > 0.05$). At high temperature, snakehead serum was more inhibitory than that of silver barbs ($p < 0.05$), though not that of tilapia, and tilapia serum was not different to silver barb serum ($p > 0.05$).

No significant differences ($p > 0.05$) in the effects of sera or heated sera on germling growth, or the effect of heating serum, were found between groups other than those described.

EFFECT OF PMA AND *A. INVADANS* ON INTRACELLULAR RESPIRATORY BURST

The PMA-stimulated macrophages produced significantly ($p < 0.05$) more superoxide than control macrophages in all cases except that of high temperature silver barbs ($p > 0.05$). Control macrophages produced more superoxide than inoculated macrophages in all cases other than low temperature tilapia, although the difference was only significant in the case of snakeheads at either temperature ($p < 0.05$) (Fig. 1).

COMPARISONS OF INTRACELLULAR RESPIRATORY BURST BETWEEN GROUPS

Among low temperature fish, silver barb macrophages had the lowest respiratory burst in all three treatments ($p < 0.05$). There were no other significant differences.

Tilapia macrophages showed higher superoxide production than snakehead and silver barb macrophages in all treatments at high temperature ($p < 0.05$).

Respiratory burst was higher at low temperature in all groups and treatments, though the differences were not significant in any treatment of silver barb macrophages ($p > 0.05$) and in the case of tilapia, were only significant in control macrophages ($p < 0.0005$). All three treatments of snakehead macrophages showed significant difference between temperatures ($p < 0.01$) (Fig. 2).

RELATIONSHIP BETWEEN RESPIRATORY BURST AND INHIBITION OF GERMINATION BY MACROPHAGES

An assessment of respiratory burst based on control macrophages showed a positive relationship in the case of high temperature tilapia with superoxide production derived from PMA-stimulated macrophages ($r^2 = 0.78$, $p <$

0.01), and low temperature gourami with superoxide data derived from control macrophages ($r^2 = 0.56$, $p < 0.05$) (Fig. 3).

Superoxide production data from both temperature groups of silver barbs, which was did not vary between temperature groups, were pooled. Weak positive regressions were obtained from data derived from control ($r^2 = 0.33$, $p < 0.05$) and inoculated macrophages ($r^2 = 0.31$, $p < 0.05$) (Fig. 3).

RELATIONSHIP BETWEEN RESPIRATORY BURST AND INHIBITION OF GROWTH BY MACROPHAGES

Low temperature snakeheads gave positive quadratic regressions with superoxide data derived from PMA-stimulated ($r^2 = 0.95$, $p < 0.0005$), inoculated ($r^2 = 0.86$, $p < 0.01$) and control ($r^2 = 0.76$, $p < 0.05$) macrophages. The only other group to give significant regressions was that of low temperature gourami, where control macrophages gave a negative linear relationship ($r^2 = 0.80$, $p < 0.005$) and inoculated macrophages gave a negative quadratic relationship ($r^2 = 0.74$, $p < 0.05$) (Fig. 4).

RELATIVE ANTIBODY CONCENTRATION

Anti-*A. invadans* antibodies were found in all individuals examined with the exception of one low temperature tilapia.

High temperature snakeheads and tilapia had higher relative anti-*A. invadans* antibody concentrations than low temperature snakeheads and tilapia respectively ($p < 0.05$) (Fig. 5). No significant relationships between concentration and inhibitory activity were found.

IV. Discussion

The anti-*A. invadans* activity of the macrophages and serum of EUS-susceptible fish was compared to that of non-susceptible fish at EUS-permissive and non-permissive temperatures.

Snakeheads have been repeatedly described as one of the most EUS-susceptible species (e.g. Tonguthai, 1985; ODA, 1994). The lack of inhibition by macrophages indicates that the granuloma response that forms in the early stages of infection is probably ineffective at preventing the spread of infection in that species. By contrast, the inhibitory properties of serum equalled or exceeded those of the other species in all cases.

Silver barbs and gourami are both EUS-susceptible (Lilley *et al*, 1998). They were the only groups in which serum inhibited growth, and the activity was not adversely affected by heating. Kurata *et al* (2000) found that the serum of the common carp, a cyprinid like the silver barb, had similar anti-fungal properties. It is possible that a similar factor is present in silver barbs, though its chemical nature remains unknown.

Tilapia have never been reported with EUS in the wild, and rarely showed even histopathological signs after injection with high concentrations of zoospores (Khan *et al*, 1998). In the light of such findings, the poor inhibition by most of the parameters measured in the present study is remarkable. Tilapia macrophages were unable to inhibit fungal growth, unlike those of silver barbs and gouramis, and there was no inhibition parameter in which tilapia macrophages or serum exceeded that of any other species. The effects of serum were particularly ambiguous as it could only inhibit germination at high temperature, but could only inhibit growth at low temperature.

Tilapia macrophages performed at least as well as those of susceptible species in all respiratory burst treatments. Wolf & Smith (1999) found that the granuloma response of hybrid tilapia *O. niloticus* x *O.*

mossambicus x *O. aureus* was unusually effective against mycobacteriosis, suggesting that there are mechanisms available to tilapia macrophages which are not present in other species. However, such mechanisms may only be implied by the present study.

The relationships between silver barb and gourami respiratory burst and inhibition of germination indicate that the respiratory burst may be important in preventing the early stages of infection, though the weakness of the relationships suggest that it is not the only mechanism involved. Also, the efficacy of the respiratory burst in this context must be questioned, as neither species is resistant. The lack of a relationship between superoxide production and inhibition of growth in silver barbs suggests either that macrophages are diverting their resources to a different mechanism of inhibition, or that the germlings are actively interfering with the macrophages. The fact that the respiratory burst of inoculated macrophages is consistently lower than that of control macrophages similarly indicates one of these effects.

The negative regression of gourami macrophage superoxide production and inhibition of growth further suggests a change of mechanism. The macrophages of both silver barbs and gouramis are able to inhibit growth, which argues against the presence of inhibitory factors produced by the germlings. However, both species are susceptible and as the germlings in this study were only 24h old, they may not yet have produced such factors. Macrophage-inhibiting virulence factors have not previously been observed in oomycetes, though they are used by bacterial fish pathogens such as *Yersinia ruckeri* (Stave *et al*, 1987) and factors derived from the oomycete *P. infestans* inhibit superoxide production by potato tubers (Doke, 1983).

There was also a strong regression of tilapia respiratory burst with inhibition of germination. However, the relationship only occurred with superoxide production data derived from PMA-stimulated macrophages, which suggests that the relationship is a result of both assays reflecting the general state of the macrophages, rather than fungicidal activity by superoxide.

The strong relationship between respiratory burst and inhibition of growth by low temperature snakehead macrophages suggests that the respiratory burst may be a major mechanism of defence in that species. However, inhibition of growth is poor while snakeheads were the only species in which the respiratory burst was significantly inhibited in the presence of *A. invadans.* The high susceptibility of snakeheads to EUS may derive from the ability of *A. invadans* to inhibit the main mechanism of defence against it. Such strong relationships between respiratory burst and inhibition of growth are not present in other species, implying the existence of other mechanisms that may not be available to snakeheads.

The respiratory burst is not the only microbicidal mechanism available to macrophages. Nitric oxide has often been associated with fish granulocytes, and is involved in the killing of bacteria such as *Aeromonas hydrophila* (Yin *et al*, 1997). Human granulocytes secrete lysozyme to attack the hyphae of *Candida albicans* (Diamond *et al*, 1978), and lysozyme has been associated with fish granulocytes (Murray & Fletcher, 1976). The role of such mechanisms in defence against hyphal pathogens such as *A. invadans* remains to be elucidated.

The ability of serum to inhibit germination was reduced by heating in all cases. This indicates that complement was responsible for the inhibitory activity as few other serum factors are inactivated at 55°C. If this is the case, the role of complement appears to be important, as most sera completely lost their germination-inhibiting activity after heating. Complement did not appear to be involved in the inhibition of fungal growth as silver barb and gourami serum did not lose growth-inhibiting activity after heating and unheated tilapia and snakehead

serum did not inhibit growth. It is not clear whether complement was activated directly by the alternative pathway, or by the antibody-mediated classical pathway as all species tested had anti–*A. invadans* antibodies.

Only the high temperature snakehead serum inhibited germination after heating, although less effectively than unheated serum. This is probably related to the high anti-*A. invadans* antibody concentrations found in that group. The Suphanburi snakehead population suffered high mortalities when EUS first occurred in Thailand (Tonguthai, 1985), and subsequent outbreaks were less severe, which may indicate the development of resistance (Chinabut, 1998). In recent years, Suphanburi snakeheads have been widely used for experimental challenges (ODA, 1994; Chinabut *et al*, 1995), which indicates that the resistance conferred by the antibody response is far from complete. The inhibitory activity of snakehead serum in this study was restricted to germination, which suggests that, like complement, its importance is in preventing infection rather than controlling it once it has begun.

Immunosuppression at low temperatures has been suggested as a likely mechanism for the seasonality of EUS (Chinabut *et al*, 1995), though no evidence of macrophage suppression by low temperature was found in this study. *In vivo* studies have associated the resistance of otherwise susceptible species such as snakehead at high temperatures with the rapid development of granulomata (Chinabut *et al*, 1995). Bly & Clem (1992) suggested that granulomata form more slowly at low temperature due to the suppression of the T cells that control granulocyte activity by the release of cytokines. T cell suppression has been implicated in rendering channel catfish *Ictalurus punctatus* vulnerable to winter saprolegniasis (Bly *et al*, 1992), and similar effects may be responsible for the EUS-susceptibility of some species.

Superoxide production was higher at low temperature in all cases, though only significant in the case of snakeheads. Higher macrophage activity at low temperature has also been reported in tench *Tinca tinca* (Collazos *et al*, 1994) and common carp (Le Morvan *et al*, 1997), raising the possibility that the respiratory burst may be particularly important at temperatures where other mechanisms are suppressed. The apparent ability of *A. invadans* to withstand, and possibly inhibit, the respiratory burst may explain the higher prevalence of EUS at relatively low temperatures.

Snakehead and tilapia both showed improved antibody production at high temperature, although there was no strong evidence that tilapia antibodies were inhibitory. Carlson *et al* (1995) found that the antibody response of striped bass *Morone saxatilis* was enhanced at high temperatures, although several parameters of the cellular immune response were attenuated. If the antibody response is protective in snakeheads, seasonal immunosuppression may explain why successive outbreaks within the same area become more restricted to the colder months (Tonguthai, 1985; ODA, 1994).

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Table 1. Species, source, weight and EUS-susceptibility of fish.

Species	Source	Mean weight (g)	s.d. of weight	EUS-Susceptibility*
Striped snakehead *Channa striata* (Bloch) (Perciformes: Channidae)	Pond farms, Suphanburi Province	87.2	22.3	Yes
Silver barb *Barbodes* (= *Puntius*) *gonionotus* (Bleeker) (Cypriniformes: Cyprinidae)	National Aquaculture and Genetic Research Institute, Patum Thani Province	198.8	68.1	Yes
Giant gourami *Osphronemus gouramy* Lacepède (Perciformes: Osphronemidae)	Pond farms, Uthai Thani Province	609.6	219.1	Yes
Nile tilapia *Oreochromis niloticus* (Linnaeus) (Perciformes: Cichlidae)	Singburi Province Fisheries Station.	95.9	25.8	No

*Lilley *et al* (1998)

Table 2. Factors that significantly ($p < 0.05$) affected the germination of *A. invadans* cysts in comparison to control cysts. ↓ indicates less germination than the controls, x indicates no difference to the controls and ↑ indicates more germination than the controls.

Species	Temperature	Serum	Heated serum	Macrophages
Snakeheads	Low	↓	x	x
Channa striata	High	↓	↓	x
Silver barbs	Low	↓	x	↓
Barbodes gonionotus	High	↓	x	↓
Giant gourami *Osphronemus gouramy*	Low	↓	x	↓
Nile tilapia	Low	x	↑	↓
Oreochromis niloticus	High	↓	↑	↓

Table 3. Factors that significantly ($p < 0.05$) affected the growth of *A. invadans* germlings in comparison to control germlings. ↓ indicates shorter germlings than the controls, x indicates no significant difference to the controls and ↑ indicates longer germlings than the controls.

	Temperature	Serum	Heated serum	Macrophages
Snakeheads	Low	x	x	x
Channa striata	High	x	↑	x
Silver barbs	Low	↓	↓	↓
Puntius gonionotus	High	↓	↓	↓
Gourami *Osphronemus gouramy*	Low	↓	↓	↓
Tilapia	Low	x	↑	x
Oreochromis niloticus	High	x	x	x

Fig. 1. Superoxide production of I) macrophages stimulated with PMA, II) control macrophages and III) macrophages incubated with *A. invadans* for 24h from fish kept at a) low and b) high temperature. Bars indicate 95% confidence interval. * indicates groups significantly different to controls.

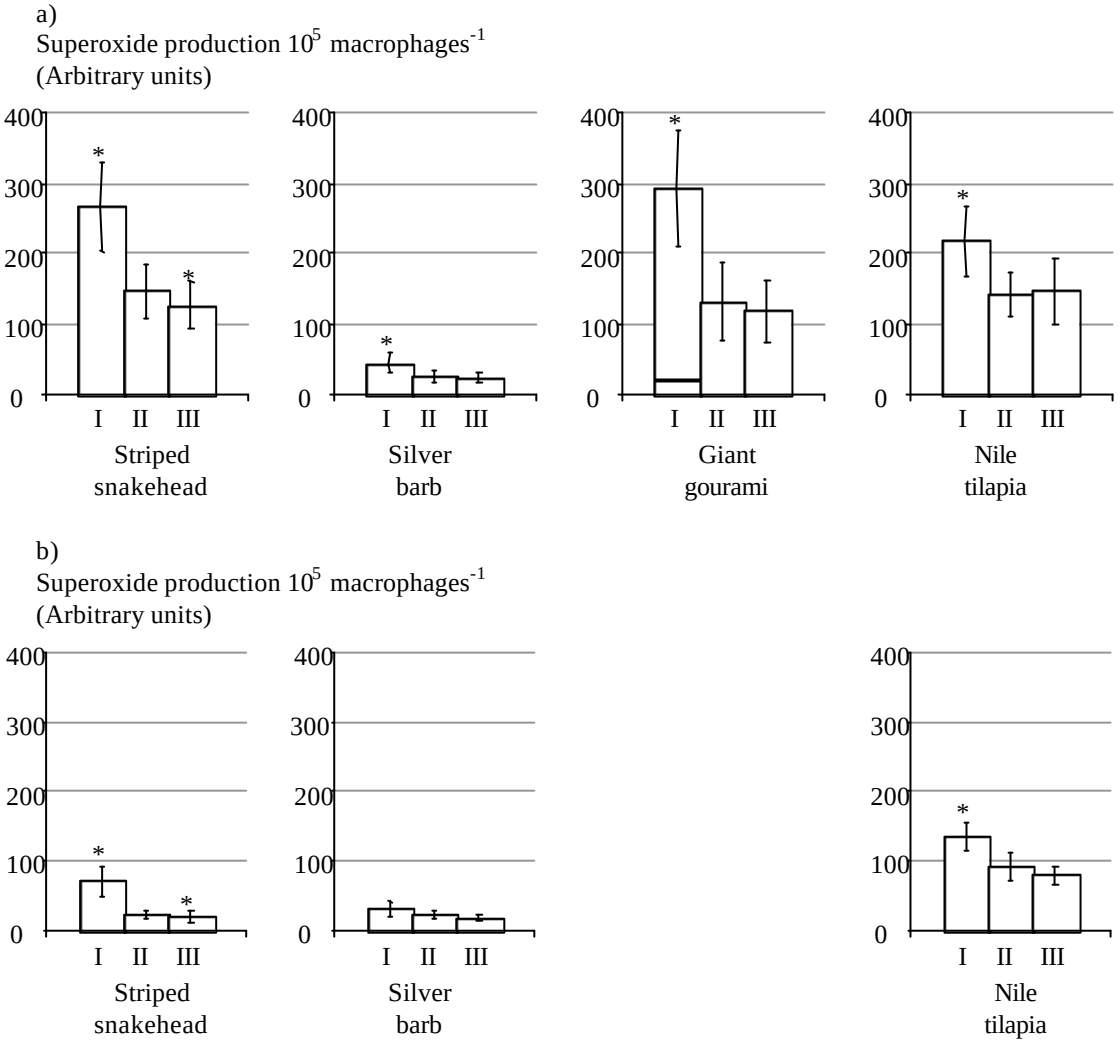
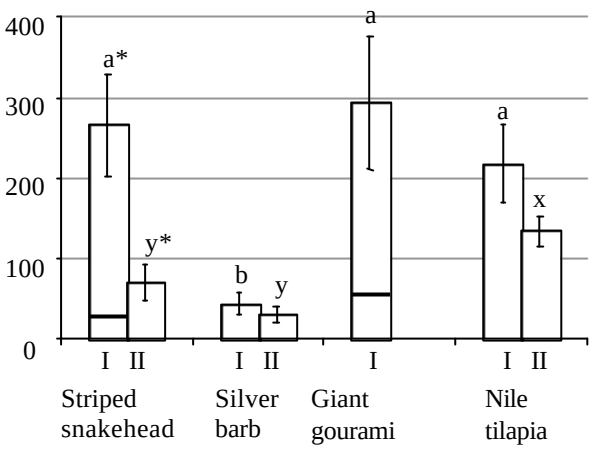
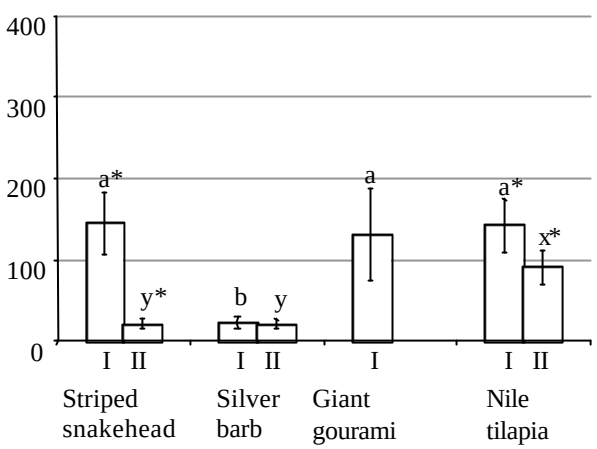


Fig.2. Superoxide production of a) macrophages stimulated with PMA, b) control macrophages and c) macrophages incubated with *A. invadans* for 24h from fish kept at I) low and II) high temperature. Bars indicate 95% confidence interval. Letters indicate significant differences between different species at the low temperature (a,b) or high (x,y) temperature. * indicates a significant difference between the macrophages of low and high temperature fish of the same species after the same treatment.

a)



b)



c/

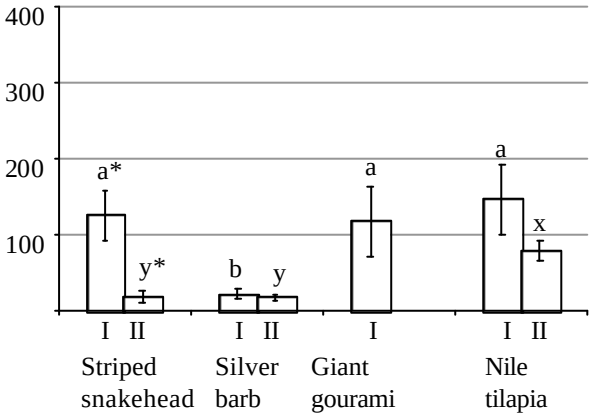
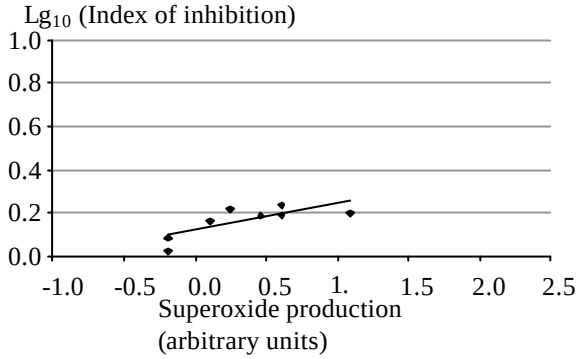


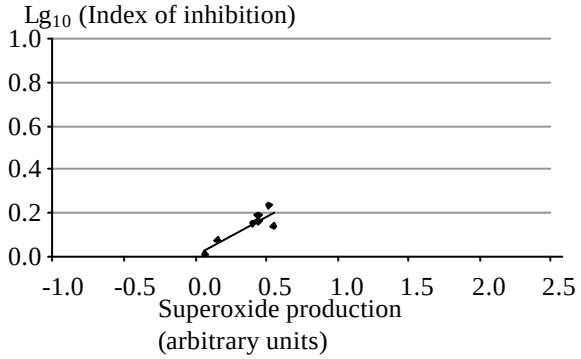
Fig. 3. Regression analysis of index of inhibition of germination plotted against total superoxide production per well in the cases of a) low temperature giant gourami with superoxide production calculated from control macrophages, b) high temperature tilapia with superoxide production calculated from PMA-stimulated macrophages, c) pooled silver barbs with superoxide production calculated from control macrophages and d) pooled silver barbs with superoxide production calculated from inoculated macrophages. Lines of best fit, Pearson regression coefficient, F-tests of significance of regression and line equations are included.

a)



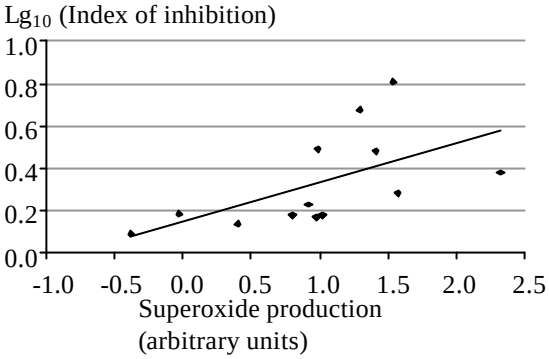
$r^2 = 0.56$, F = 7.57, n = 8, $p < 0.05$
$y = 0.12x + 0.12$

b)



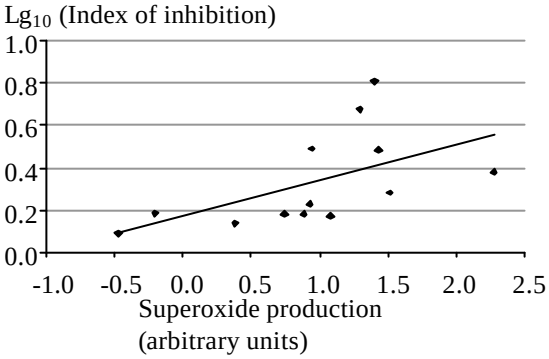
$r^2 = 0.78$, F = 18.14, n = 7, $p < 0.01$
$y = 0.36x + 0.00078$

c)



$r^2 = 0.33$, F = 5.50, n = 16, $p < 0.05$
$y = 0.18x + 0.15$

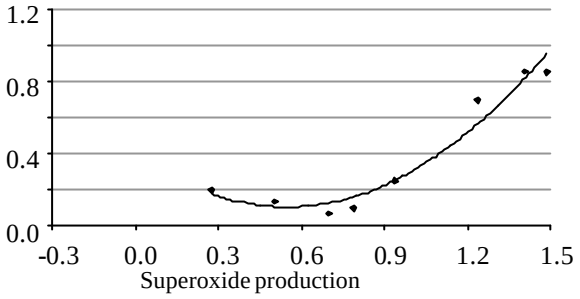
d)



$r^2 = 0.31$, F = 4.94, n = 16, $p < 0.05$
$y = 0.17x + 0.17$

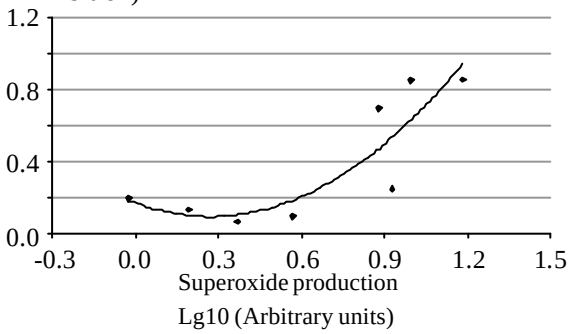
Fig. 4. Regression analysis of index of inhibition of growth and total superoxide production per well in the cases of low temperature snakeheads with superoxide production calculated from a) PMA-stimulated, b) control and c) inoculated macrophages and giant gourami with superoxide production calculated from d) control and e) inoculated macrophages. Lines of best fit, Pearson regression coefficient, F-tests of significance of regression and line equations are included.

a) Lg_{10} (Index of inhibition)



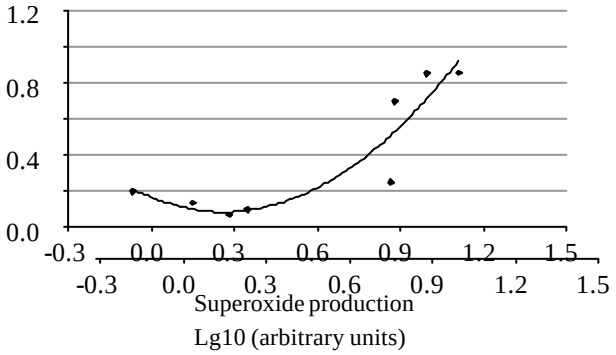
r2 = 0.95, F = 51.61, n = 8, $p < 0.0005$
$y = -1.06x + 0.97x^2 + 0.40$

b) Lg10 (Index of inhibition)



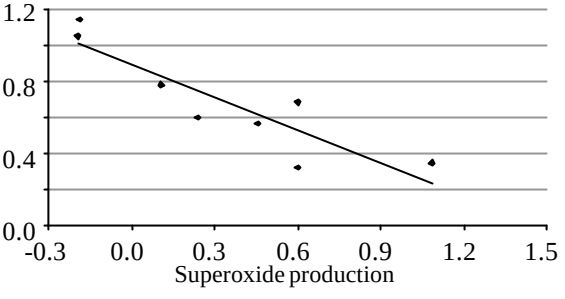
r2 = 0.76, F = 7.94, n = 8, $p < 0.05$
$y = -0.55x + 1.01x^2 + 0.17$

c)
Lg10 (Index of inhibition)



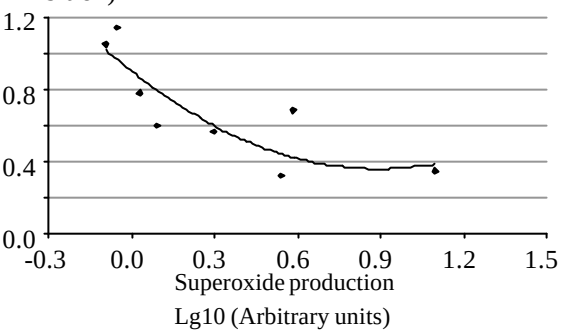
r2 = 0.86, F = 14.80, n = 8, $p < 0.01$
$y = -0.60x + 1.15x^2 + 0.16$

d) Lg10 (Index of inhibition)



r2 = 0.80, F = 23.69, n = 8, $p < 0.005$
$y = -0.61x + 0.89$

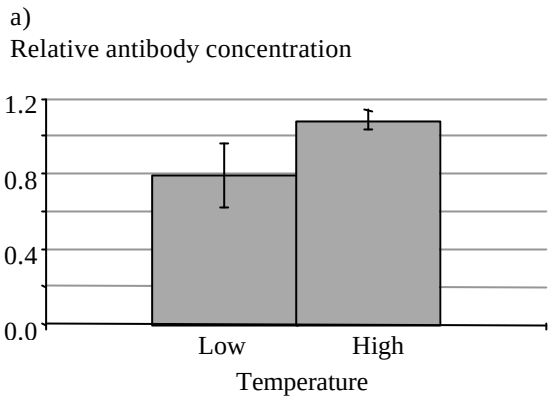
e) Lg10 (Index of inhibition)



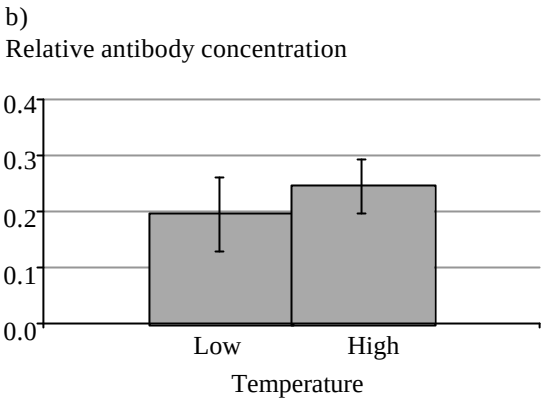
r2 = 0.74, F = 7.01, n = 8, $p < 0.05$
$y = -1.21x + 0.68x^2 + 0.90$

Fig. 5.

a)


Relative antibody concentration
1.2
0.8
0.4
0.0
Low
High
Temperature

b)


Relative antibody concentration
0.4
0.3
0.2
0.1
0.0
Low
High
Temperature

APPENDIX TWENTY TWO

Paper fifteen - Thompson, K.D., Lilley, J.H., Chen, S.-C., Adams, A., and Richards, R.H. (1999) The immune response of rainbow trout (*Oncorhynchus mykiss*) against *Aphanomyces invadans*. Fish and Shellfish Immunology 9(3), 195-210. (pdf or hard copy available from ARP Manager)

APPENDIX TWENTY THREE

Paper sixteen - Miles, D.J.C., Polchana, J., Lilley, J.H., Kanchanakhan, S., Thompson, K.D., and Adams, A. (2001) Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome (EUS). Aquaculture 195 (1-2), 1-15. (pdf or hard copy available from ARP Manager)

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Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome

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Abstract

Five immunostimulants were injected intraperitoneally into striped snakeheads (*Channa striata*). The inhibitory effects of the serum and macrophages collected from the treated fish on the germination and subsequent growth of *Aphanomyces invadans* (= *piscicida*), the causative agent of epizootic ulcerative syndrome (EUS), were then assessed. Salar-bec, a vitamin premix, and Ergosan, an alginate, both stimulated the inhibitory effects of serum on the germination and subsequent growth of *A. invadans* cysts, and the inhibitory effect of macrophages on growth. Betamak C85, a yeast extract containing β glucans and mannans, and Lysoforte, a lysophospholipid biosurfactant, induced little or no improvement in the parameters measured. Oro glo layer dry, a xanthophyll preparation, was rejected because of high mortalities among injected fish.

Salar-bec showed the greatest improvement in the inhibition of both germination and growth by serum, and of growth by macrophages. It was selected for a tank trial in which snakeheads were fed on pellets coated with 2 g kg^{-1} Salar-bec, then injected with *A. invadans*. Control fish were fed on uncoated feed. Although the incidence of infection was not affected, hyphae appeared later in treated fish and granulomata developed faster at the infection site, suggesting an enhanced ability to contain the infection. Relative percent survival of treated fish was 59.2% higher than the controls over the 40-day trial. Anti-*A. invadans* antibody concentration was higher in treated fish, which may also have contributed to the containment of the infection.

Keywords: Immunostimulant; *Channa striata*; Epizootic ulcerative syndrome; *Aphanomyces invadans*; Vitamin

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1. Introduction

Epizootic ulcerative syndrome (EUS) is one of the most destructive diseases affecting freshwater aquaculture in Asia (Lilley et al., 1998; Chinabut, 1998). The causative agent is the oomycete *Aphanomyces invadans* (= *piscicida*) (Hatai et al., 1977; Willoughby et al., 1995), which is diagnosed histologically by the presence of invasive aseptate hyphae in muscle tissue, associated with a granulomatous response (Lilley et al., 1998). Outbreaks are frequently precipitated by adverse environmental conditions (Tonguthai, 1985) and compounded by secondary infections (Callinan and Keep, 1989).

The striped snakehead *Channa striata* (Bloch) (Perciformes: Channidae) industry suffered heavy losses to EUS when the disease first reached Thailand as snakeheads are very susceptible to infection (Tonguthai, 1985). While the possibility of vaccination appears remote (Thompson et al., 1997), the observation that infection usually occurs during the winter months when the host immune system is suppressed (Chinabut et al., 1995) raises the possibility of improving resistance by stimulating the innate immune response.

Since the discovery that adjuvants used in the application of vaccines confer considerable resistance in themselves (Olivier et al., 1985), a wide range of products have been tested for immunostimulatory effects in fish (Sakai, 1999). In Thailand, striped snakehead are farmed intensively and fed on a preparation of minced trash fish mixed with rice bran, which would allow application of oral immunostimulants.

Many methods of testing immunostimulants have been reported. Substances may be injected or mixed with feed, and the results may be tested by examination of the immune parameters of the fish or challenge with an experimental pathogen (Sakai, 1999). However, while injection and examination of immune parameters is the simplest method of screening, it may not reflect the effect that the immunostimulant would have if farmed fish were exposed to the pathogen. Farm trials, on the other hand, are expensive and may lead to ambiguous results.

Galeotti (1998) suggested that in vitro screening methods should be used to elucidate the mechanisms of immunostimulation, then in vivo methods should be used to establish whether the benefits occur in live fish. This study followed such an approach with preliminary screening of a range of putative immunostimulants by injection, then examination of immune parameters in vitro. The most effective substance was selected for oral administration to fish, which were challenged with *A. invadans*.

2. Methods

2.1. In vitro studies

Five substances were injected into fish to assess their immunostimulatory properties.

2.1.1. Experimental fish

Striped snakeheads weighing 66.7 g (s.d. $\pm$ 17.7) were acquired from pond farms in Suphanburi province, Thailand and placed in 50-l opaque plastic tanks with a through-

flow system at a density of five fish per tank. They were acclimatised at 21 ± 1°C for 14 days before the trial to simulate the temperature at which EUS may be expected to occur (Tonguthai, 1985; Chinabut et al., 1995). Fish were fed twice daily to satiation with Charoen Pokphand (Bangkok, Thailand) brand no. 9910 pellets containing at least 30% protein.

2.1.2. Experimental pathogen

A. invadans isolate NJM9701 was isolated from an ayu *Plecoglossus altivelis* in Shiga Prefecture, Japan, by Prof. K. Hatai 2 years previously. Pathogenicity was confirmed by injection of zoospores into healthy striped snakeheads and histological confirmation of infection.

A. invadans cysts were prepared by growing the mycelium on GPY-agar (16.7 mM glucose, 1 g l^{-1} mycological peptone, 0.5 g l^{-1} yeast extract, 0.52 mM magnesium sulphate heptahydrate, 0.102 mM monopotassium phosphate, 0.20 mM calcium chloride dihydrate, 8.88 μM ferric chloride hexahydrate, 9.10 μM manganese chloride tetrahydrate, 0.016 mM cupric sulphate pentahydrate, 0.014 mM zinc sulphate heptahydrate) at 20°C. Mycelium was collected on autoclaved hemp seeds and grown out in V8 broth (5% v/v V8 vegetable juice, 20.0 mM calcium carbonate) for 2 days. Sporulation was induced by passing the seeds through six washes of autoclaved distilled water and incubating in filtered, autoclaved pond water (APW) at 22°C for 24 h. Zoospores were collected and encysted by shaking for 20 s.

2.1.3. Application of immunostimulants

Table 1 lists the immunostimulants used, all of which were in the form of insoluble powders. All were provided by Aquaculture Vaccines, Saffron Walden, UK.

For application, the immunostimulants were suspended at 5 mg ml^{-1} in sterile phosphate buffered saline (PBS) (7.3 mM monosodium phosphate, 18.0 mM disodium phosphate, 0.15 M sodium chloride, pH 7.2). Fish were lightly anaesthetised with benzocaine solution (10% w/v benzocaine in ethanol) until movement ceased. Each fish was injected intraperitoneally with 100 μl of the immunostimulant suspension with a 21-G hypodermic needle.

Table 1
Active ingredients of putative immunostimulants used in experiment 1

Product	Active ingredient[a]
Salar-bec	300 g kg^{-1} vitamin C, 150 g kg^{-1} vitamin E, trace quantities of vitamins B_1, B_2, B_6, B_{12}
Ergosan	0.002% unspecified plant extract, 1% alginic acid from *L. digitata*, 98.998% algal based carrier
Betamak C85	Brewers' yeast containing 32% β 1,3 and 1,6 Glucans, 30% uspecified mannan
Lysoforte	Lysophospholipids
Oro glo layer dry	Yellow xanthophylls derived from marigold *Tagetes erecta*, principally lutein with significant amounts of zeaxanthin

[a]Information courtesy of Aquaculture Vaccines, Saffron Walden, UK and Elorisan-Biostimulatoren, Deggendorf, Germany.

One group of five fish was used per treatment. In addition to the treatments in Table 1, a control group was injected with sterile PBS.

2.1.4. Sampling protocol

Fish were killed by overdose with benzocaine solution 14 days after injection with immunostimulants. They were weighed and blood samples were collected from the caudal vein, allowed to clot for 2 h at room temperature (24°C) and centrifuged at $10{,}000 \times g$ for 15 min. Serum was then collected.

Head kidneys were dissected out and placed in isolation medium (L-15 medium containing 1000 U ml^{-1} penicillin, 1000 μg ml^{-1} streptomycin, 100 U ml^{-1} heparin, 2% v/v foetal bovine serum (FBS) heat inactivated at 55°C for 30 min) for macrophage collection (Secombes, 1990). The head kidneys were macerated through a 100 μm mesh into 10 ml isolation medium. A Percoll density gradient was prepared in a centrifuge tube by layering 51% Percoll solution (51% Percoll, 10% $10 \times$ minimum essential medium (MEM), 39% distilled water) below 34% Percoll solution (34% Percoll, 10% $10 \times$ MEM, 56% distilled water). The macerated head kidney was layered on top of the gradient and centrifuged at $400 \times g$ for 25 min. Macrophages were collected from the gradient interface and washed by centrifuging twice in washing medium (L-15 medium containing 1000 U ml^{-1} penicillin, 1000 μg ml^{-1} streptomycin, 0.1% v/v FBS) at $900 \times g$ for 15 min. Viable macrophages were counted by mixing an aliquot of macrophage suspension with an equal volume of 0.1% trypan blue and counting on an improved Neubauer haemocytometer. Macrophages were adjusted to 10^6 cells ml^{-1} and 100 μl aliquots were seeded into 96-well microtitre plates. These were incubated for 2 h at 23 ± 1°C to allow attachment of macrophages, which previous studies had shown to be an optimal period of time (Dr. M. Crumlish, University of Stirling, personal communication). Non-adherent macrophages were removed with three washes of pre-warmed L-15 medium and the culture wells were loaded with 100 μl maintenance medium ($2 \times$ L-15 prepared from powder, containing 5% v/v FBS).

2.1.5. Fungicidal assays

Fungicidal assays were adapted from Diamond et al. (1978). Cysts were prepared on the day the fish were sampled. Cysts were counted with an improved Neubauer haemocytometer and diluted to a concentration of 4.0×10^4 ml^{-1} in APW. Aliquots of 50 μl were added to each well. Triplicate wells containing macrophages or 20 μl serum from each fish, or control wells containing medium only, were prepared and the volume of each made up to 200 μl with APW so that the final cyst concentration was 10^4 cysts ml^{-1} (Thompson et al., 1999).

After 24 h, inhibition of germination was assessed by counting visible germlings within one randomly selected microscope field of view at $40 \times$ magnification and extrapolating the count to the total area of the well. Inhibition of growth was assessed by measuring 10 randomly chosen germlings from each well using a graticule.

2.2. In vivo challenge

Fish were fed on Salar-bec and challenged with *A. invadans*.

2.2.1. Experimental fish

Striped snakeheads were acquired from the same source as in experiment 1. Fish were placed in 150-l glass tanks at a density of 40 fish $tank^{-1}$. Two tanks were allocated as treatments and two as controls. Water quality was maintained by frequent water changes. All fish were fed twice daily to satiation on Charoen Pokphand brand no. 9910 pellets.

Fish were acclimatised at $21 \pm 1°C$ for 14 days prior to the experiment.

2.2.2. Application of immunostimulant

The most successful immunostimulant from the in vitro study, Salar-bec, was selected for the in vivo challenge. Immunostimulant (2 g) was mixed with 1 kg of feed. Vegetable oil (10 ml) was sprayed into the mixture to attach the immunostimulant to the pellets. Control feed was prepared by adding oil only. Fish were fed to satiation twice daily.

After acclimatisation, fish were placed on experimental diets for 14 days prior to challenging them with *A. invadans* spores.

2.2.3. Challenge

A. invadans isolate B99C was isolated from an EUS-infected reba carp *Cirrhinus reba* caught in Mymensingh, Bangladesh 2 months previous to the experiment. The most recent isolate was used rather than the same isolate as used in the in vitro trial in order to use a pathogen as similar as possible to that found in natural outbreaks. Culture of the fungus was as described for the in vitro study, though zoospores were not encysted before application.

A zoospore suspension of 100 zoospores ml^{-1}, 67% of which were motile, was prepared and assessed with a modified Neubauer haemocytometer. A 100-μl dose of the suspension was injected intramuscularly into the right of the leading edge of the dorsal fin of each fish.

2.2.4. Sampling protocol

Three fish were sampled from each tank at 5-day intervals. Each fish was weighed and the injection site excised and fixed in 10% neutral buffered formalin for histological examination. Blood samples were taken from the caudal blood vessels and serum collected for assessment of antibody concentration.

Incidental mortalities were also recorded.

2.2.5. Histology

All samples were stained using Grocott's (1955) methenamine silver stain for muco-polysaccharides. They were then counter-stained with haemotoxylin and eosin.

The presence or absence of invasive fungal hyphae was recorded. The cellular immune response was classified either as acute inflammation, with infiltration of granulocytes into the region of infection but little or no organisation, or chronic inflammation where distinct granulomata could be observed enclosing hyphae. Fig. 1 illustrates the different stages of the response. The area of each type of response in each slide was measured with a grid graticule.

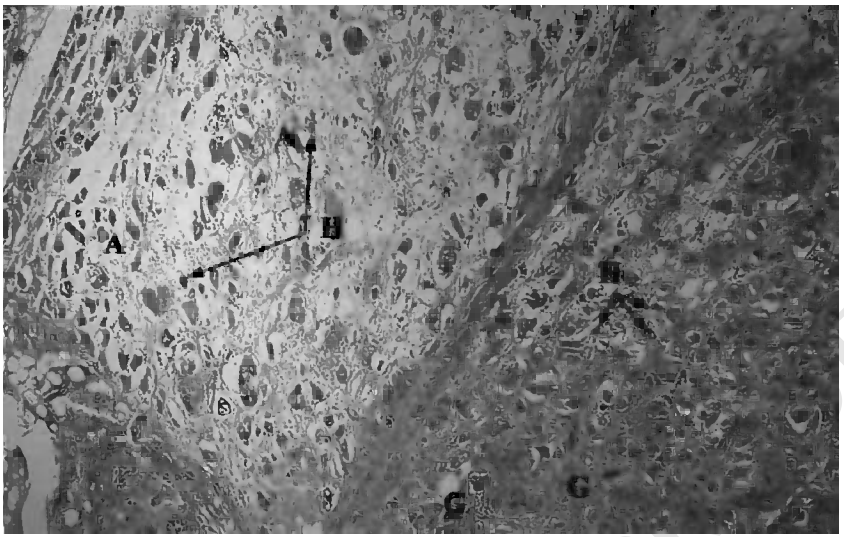

Fig. 1. Muscle section of striped snakehead 20 days after injection with *A. invadans* zoospores, 40×. H = hyphae; A = acute inflammation; G = granulomata.

2.2.6. Assessment of relative antibody concentration

Relative antibody concentration was quantified by Enzyme Linked Immunosorbant Assay (ELISA).

Antigen was prepared by grinding 3-day germlings in liquid nitrogen, thawing and collecting in Wood's (1988) extraction buffer (85 mM Tris, 1 mM magnesium chloride, 1 mM EDTA, 10 mM potassium chloride, 1.12 mM ascorbic acid, 10.9 mM glycerol, pH 7.5) with the addition of 5 μm phenylmethylsulphoxylflouride. Protein concentration was assayed with a Bio-Rad protein assay (Bio-Rad, Hercules, USA).

Monoclonal antibody supernatants were prepared from hybridoma cell line 7D2 raised against striped snakehead immunoglobulin (courtesy of Dr. Suppalak Puttinaowarat, AAHRI, Thailand).

The ELISA was carried out after Chen et al. (1996) with one modification. When fish serum was added, it was prepared by diluting 1/8 in antibody buffer (PBS containing 1% w/v bovine serum albumin). Doubling dilutions were carried out to 1:4096. Positive controls were prepared by adding 100 μl snakehead serum previously shown to be positive diluted 1/10 in antibody buffer to duplicate wells, and negative controls were prepared by adding 100 μl antibody buffer only to duplicate wells.

All absorbance values for each fish were plotted and a curve fitted. A relative antibody concentration was calculated for each fish by reading the absorbance at the midpoint of the serum dilutions (Harlow and Lane, 1988).

2.3. Statistical analysis

All data were subjected to parametric analyses other than the incidence and mortality data in experiment 2, which was treated as frequency data. For all tests, a significance level of $p < 0.05$ was regarded as significant.

3. Results

3.1. In vitro studies

Five substances were injected into fish to assess their immunostimulatory properties.

3.1.1. Mortality

Of the five fish injected in each treatment group, one fish died before sampling in every group including the control group, except for the case of fish injected with Oro glo layer dry where three of the five fish died. No further studies were carried out on this treatment, as there were insufficient survivors to make up a sample group.

3.1.2. Comparison of inhibition within treatment groups

The effect of serum and macrophages on growth and germination of *A. invadans* germlings were compared to control germlings for each treatment.

Macrophages significantly inhibited germination within all treatment groups compared with the control germlings (one-sample *t*-test, $p < 0.05$), with the exception of Salar-bec where a lower level of significance was obtained (one-sample *t*-test, $t = 2.03$, $df = 11$, $p = 0.07$). Serum inhibited germination in the case of fish injected with Salar-bec (one-sample *t*-test, $t = 3.08$, $df = 11$, $p < 0.01$) and Ergosan (one-sample *t*-test $t = 2.93$, $df = 7$, $p < 0.05$), but not in the case of the controls, Betamak C85 or Lysoforte (one-sample *t*-test, $p > 0.05$).

The number of cases in which germling growth was inhibited by serum or macrophages isolated from treated or control fish, is shown in Table 2. Macrophages or serum from control fish showed no inhibitory activity. Fish injected with Betamak C85 were also unable to inhibit germling growth, and serum actually stimulated growth in one case. Only one fish showed any inhibitory activity in the case of Lysoforte, but serum and macrophages were inhibitory in the case of all or most fish injected with Salar-bec or Ergosan.

3.1.3. Comparison of inhibition between treatments

The effect of serum and macrophages on growth and germination of *A. invadans* germlings in each treatment were compared.

Table 2
The number of striped snakeheads injected with putative immunostimulants or phosphate buffered saline (PBS) for which serum or macrophages (MØ) were significantly ($p < 0.05$) inhibitory, stimulatory or had no effect on the growth of *A. invadans* germlings in vitro

	PBS		Salar-bec		Ergosan		Betamak C85		Lysoforte	
	MØ	Serum	MØ	Serum	MØ	Serum	MØ	Serum	MØ	Serum
Inhibitory	0	0	3	4	3	3	0	0	1	1
No effect	4	4	1	0	1	0	3	2	3	3
Stimulatory	0	0	0	0	0	0	0	1	0	0

The inhibitory effects of macrophages and serum on germination derived from the different treatments is shown in Fig. 2. There was no difference between the inhibition of germination from any of the treatments in terms of the effect of macrophages on inhibition (one-way ANOVA, $F = 2.28$, $df = 4$, $p = 0.07$, no differences revealed by Tukey multiple comparisons).

The two cases in which serum was inhibitory, the Salar-bec and Ergosan groups, were compared and Salar-bec was the more inhibitory of the two (independent samples t-test, $t = -3.12$, $df = 18$, $p < 0.01$).

The effects of macrophages and serum from the different treatments on germling growth were examined (Fig. 3). Macrophages from fish injected with Salar-bec were more inhibitory than the control or any other treatment, and those from fish injected with Ergosan were more inhibitory than those of control fish, though not compared with those of fish injected with Betamak C85 or Lysoforte (ANOVA, $F = 14.44$, $df = 4$, $p < 0.001$, Tukey multiple comparisons).

Serum from fish injected with Salar-bec, Ergosan or Lysoforte were all more inhibitory than the control (ANOVA, $F = 19.06$, $df = 4$, $p < 0.001$, Tukey multiple comparisons). Serum from fish injected with Salar-bec was more inhibitory than any treatment except for Ergosan, which was more inhibitory than Betamak C85 but not significantly different to Lysoforte.

3.2. *In vivo* challenge

Fish were fed on Salar-bec and challenged with *A. invadans*.

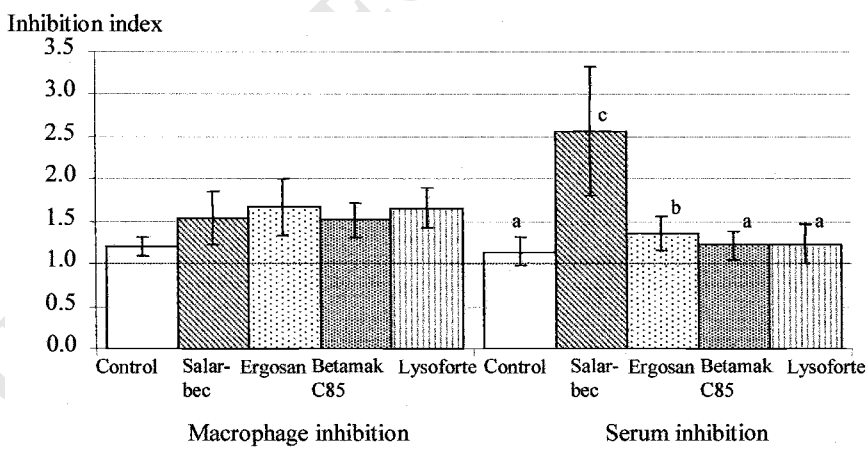

Fig. 2. The inhibitory effect of macrophages and serum from striped snakeheads injected with putative immunostimulants on the germination of *A. invadans* in vitro. Inhibition index was calculated by dividing control by treatment germling counts. An index above 1.0 indicates inhibition. Letters indicate significance groups in the case of serum, but are absent from macrophages as there are no significant differences. Bars indicate 95% confidence interval.

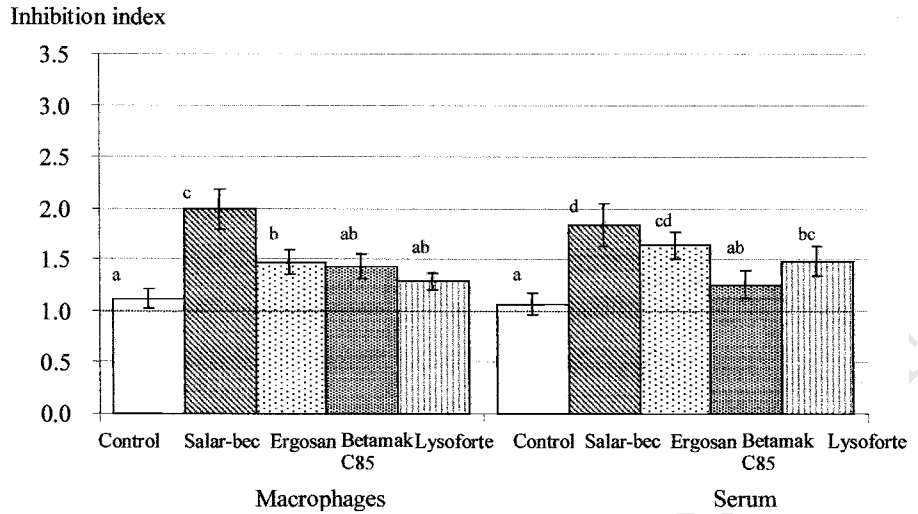

Fig. 3. The inhibitory effect of macrophages and serum from striped snakeheads injected with putative immunostimulants on the growth of *A. invadans* germlings in vitro. Inhibition index was calculated by dividing control by treatment germling lengths. An index greater than 1.0 indicates inhibition. Letters indicate significance groups. Bars indicate 95% confidence intervals.

3.2.1. Weights

The average weight of sampled fish was 46.1 g (s.d. $\pm$ 17.1). Weights of fish in all tanks in each trial were compared. In no case was there any difference (one-way ANOVA, $df = 3$, $p > 0.05$).

3.2.2. Incidence and survival

Incidence of EUS, as determined by the presence of fungal hyphae, among the 24 fish sampled, and mortality in unsampled fish were recorded.

The first recorded incidence of EUS from sampled control fish was on day 15, and from treated fish on day 20. There was no significant difference in the incidence of fish infected after day 10 from the control (58%) and treated (65%) groups ($\lambda^2 = 0.03$, $df = 1$, $p > 0.05$).

Relative percent survival (Ellis, 1988) of treated fish was 59.2% higher than the controls among the 16 unsampled fish ($\lambda^2 = 14.57$, $df = 1$, $p < 0.0005$) (Fig. 4).

3.2.3. Inflammatory response

The effect of time after injection, fish weight and treatment on the area of acute inflammation, area of granulomatous response, total inflamed area and ratio of area of acute inflammation to granulomatous response were assessed. Only fish in which hyphae were present were used.

There was no significant difference between control and treated fish in terms of either the area of acute or total inflammation (ANCOVA, $p > 0.05$), nor was there any effect of time from injection on either variable (F-test, $p > 0.05$).

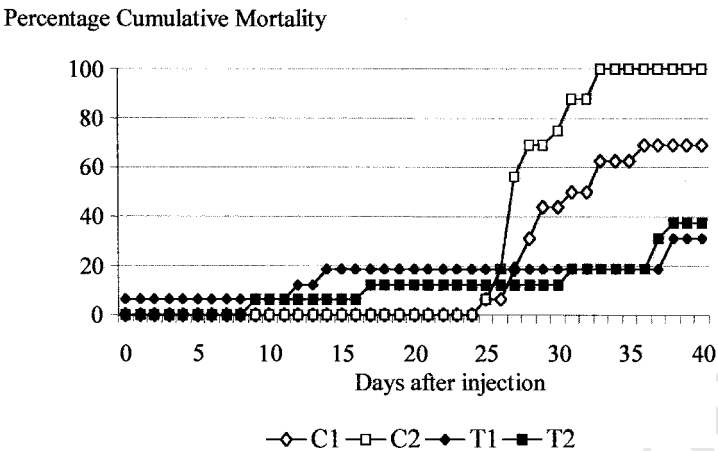

Fig. 4. Cumulative mortality of unsampled striped snakeheads from two tanks fed on feed coated with 2 g kg^{-1} Salar-bec before challenge (T1 and T2) compared to fish fed on uncoated control feed (C1 and C2) after injection with *A. invadans*.

Fig. 5 shows that treated fish developed a larger area of chronic granuloma tissue over time from infection than untreated fish, though statistical significance is equivocal ($r^2 = 0.32$, $F = 4.74$, $df = 1$, $p = 0.055$). A stronger relationship is evident between the ratio of the areas of acute inflammation and granulomatous tissue and time ($r^2 = 0.46$, $F = 6.72$, $df = 1$, $p < 0.05$) (Fig. 6). No such relationship is observed in the case of

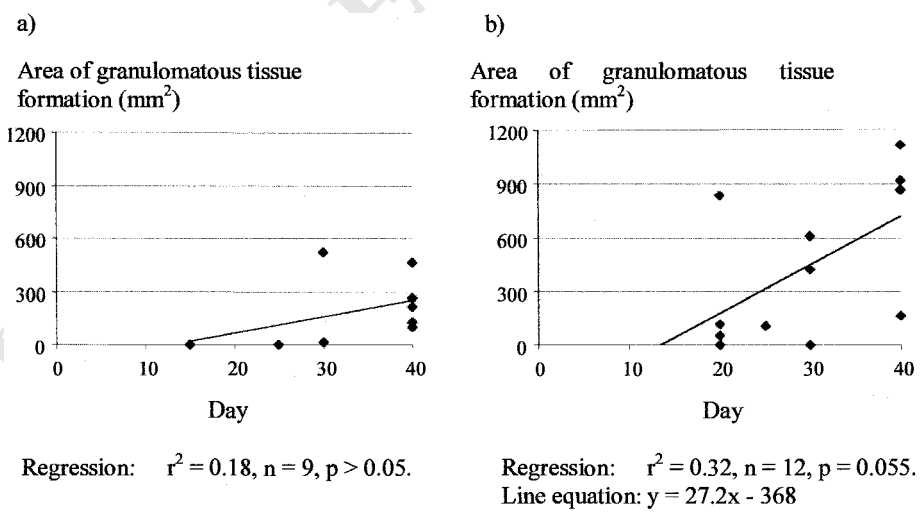

Fig. 5. Plots of area of chronic granulomatous tissue against day after challenge in striped snakeheads injected with *A. invadans* in (a) control fish and (b) fish fed with 2 g kg^{-1} Salar-bec for 14 days before injection.

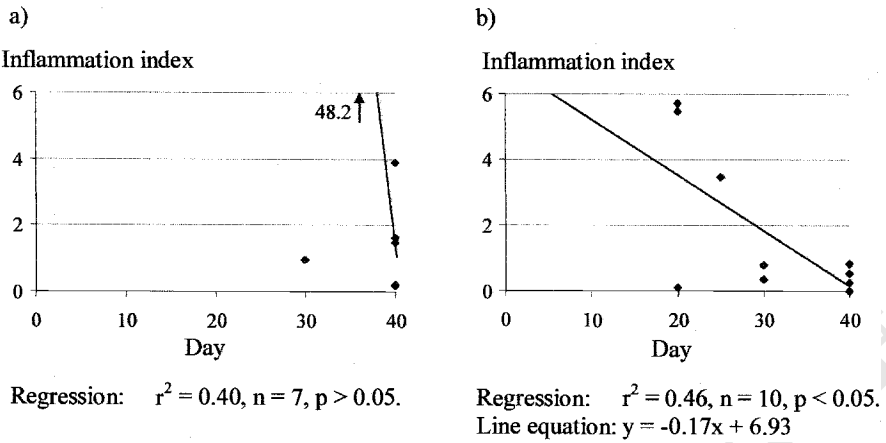

Fig. 6. Plots of inflammation index calculated by dividing area of acute inflammation by area of granuloma formation in striped snakeheads injected with *A. invadans* against day after injection in (a) control fish and (b) fish fed with 2 g kg^{-1} Salar-bec for 14 days before challenge.

control fish (F-test, $p > 0.05$) and there is no effect of fish size on either variable (F-test, $p > 0.05$). Treated fish had more granulomatous tissue by area than control fish (ANCOVA, $F = 4.78$, $df = 1$, $p < 0.05$) (Fig. 5), but there was no difference in the ratio of acute inflammation to granulomatous tissue (ANCOVA, $p > 0.05$) as shown in Fig. 6.

3.2.4. Relative antibody concentration

The relative anti-*A. invadans* antibody concentration of each fish was assessed. Fish treated with Salar-bec had higher concentrations than control fish (t-test, $t = -2.45$,

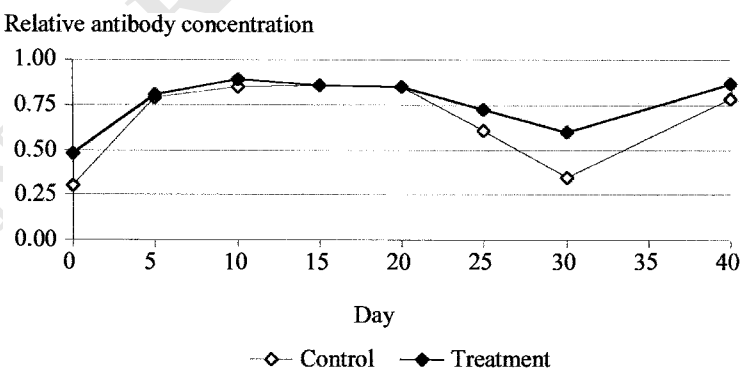

Fig. 7. Plot of relative antibody concentrations of striped snakeheads against days after injection with *A. invadans* of fish fed with feed coated with 2 g kg^{-1} Salar-bec and fish fed control feed. Each point is derived from the mean of three fish.

$df = 77$, $p < 0.05$) (Fig. 7). There was no significant correlation with antibody concentration with time or weight in the case of either treatment or fish (F-test, $p > 0.05$).

4. Discussion

Five substances were evaluated for their ability to stimulate the immune response of striped snakehead to *A. invadans*. The most effective, Salar-bec, was selected for a challenge experiment. The remaining four (Ergosan, Lysoforte, Betamak C85 and Oro glo layer dry) were not pursued as they showed more limited effectiveness.

Fish injected with Ergosan showed improvement in the ability of macrophages to inhibit growth and the ability of serum to inhibit both growth and germination. Such alginates first came to the attention of the aquaculture industry as binders for pelleted feed, but immunomodulatory properties have been found in extracts from phyophaecaetes such as *Laminaria digitata*, the source of the alginates in Ergosan (Dalmo et al., 1998; Gabrielson and Austreng, 1998). Ergosan itself shows a range of immunostimulatory effects on Atlantic salmon *Salmo salar* (Hall, 1998).

Like most alginates, Ergosan contains polyuronic acids that attach to certain cells and act as phase transfer catalysts, facilitating the uptake of oxygen by the cell and improving its vitality (Elorisan-Biostimulatoren, unpublished data). Dalmo et al. (1998) attributed the immunomodulatory properties of a similar alginate, laminaran, to the fact that it contains β glucans. Ergosan also contains β glucans, but not in a form available to the fish (Elorisan-Biostimulatoren, unpublished data).

Lysoforte appeared to improve the ability of serum to inhibit growth of *A. invadans* in the preliminary trial when results were pooled, but this result must be interpreted cautiously as inhibition was only shown conclusively in one individual fish. The lysophospholipids in Lysoforte act as biosurfactants to improve feed conversion and are used in mammal and poultry farming (Schwarzer and Adams, 1996), though their effect on the immune system has not been studied.

The yeast extract Betamak C85 was the least promising of the four immunostimulants assessed in vitro, showing no improvement in any of the parameters measured. Many studies have shown yeast extracts containing β glucans and mannans similar to Betamak C85 to have immunostimulatory properties (Sakai, 1999). Several extracts afford protection against a wide range of pathogens though few studies have examined activity against eukaryotes. In one of the few studies on eukaryotes, a similar extract gave sand whiting *Sillago ciliata* some protection against *A. invadans* (E.S. Catap, personal communication) but no such benefits were shown in this study. Robertsen et al. (1994) suggested that β glucans are most effective against opportunistic pathogens, which may explain their lack of efficacy against *A. invadans*. They also report that the effect of β glucans is dose dependent as low or high doses afford no improvement in the immune system, and very high doses may be inhibitory. Such inhibition may have occurred here as the appropriate dose rate for striped snakeheads is unknown and may have been exceeded.

Oro glo layer dry, a preparation of yellow xanthophylls used as a pigment enhancer in poultry farming, was rejected because it caused a higher mortality among fish injected with it than any other treatment or the control. The immunomodulatory properties of

xanthophylls are unclear, though other carotenoids such as β carotene are vitamin A precursors, and show a range of immunostimulatory and antioxidant activity (Chew, 1993).

Salar-bec was the most effective immunostimulant in the preliminary study as the effects of macrophages on growth and serum on germination were stronger in fish injected with Salar-bec than any other treatment. Further, serum was more inhibitory of growth than in any treatment except Ergosan. Fish fed on diets impregnated with Salar-bec survived better when challenged with *A. invadans* in the tank trial.

Although incidence of infection was not reduced, the effect on mortality suggests that Salar-bec improves the ability to control the spread of the mycelium, especially as hyphae appeared later in treated fish than in controls. There was also evidence for faster granuloma development in fish fed on Salar-bec. Chinabut et al. (1995) found that striped snakeheads injected with *A. invadans* at 26°C or above developed granulomata relatively quickly and recovered, while fish at lower temperatures showed only acute inflammation and died. A rapid granuloma response has also been suggested as a characteristic of species resistant to EUS (Wada et al., 1996). Consequently, it appears that immunostimulation in this study induced an immune response that is typical of fish resistant to EUS.

Stimulated fish also showed higher anti-*A. invadans* antibody concentrations. Previous studies (unpublished data) suggest that the antibody response elicited in striped snakeheads may afford some protection, and it is possible that the higher antibody concentrations elicited by Salar-bec contributed to the ability of the fish to contain the infection.

Several previous studies have shown the value of orally administered vitamin supplements similar to Salar-bec as immunostimulants including vitamin C in channel catfish *Ictalurus punctatus* (Durve and Lovell, 1982; Li and Lovell, 1985), *S. salar* (Erdal et al., 1991; Hardie et al., 1991) and rainbow trout *Oncorhynchus mykiss* (Navarre and Halver, 1989; Wahli et al., 1995) and vitamin E in *I. punctatus* (Wise et al., 1993) and turbot *Scopthalamus maximus* (Pulsford et al., 1995). Of particular interest is Durve and Lovell's (1982) observation that vitamin C improved the resistance of *I. punctatus* to edwardisiellosis at low temperature, implying that it may be used to counter temperature mediated increases in susceptibility such as are commonly seen in EUS outbreaks.

No laboratory-based trial can exactly replicate the situation in a pond where fish are exposed to a wide range of environmental variation and a complex microbial biota. Similarly, injecting the pathogen into the fish does not replicate a natural outbreak. A further concern is that the fish in the trial were fed on pelleted feed, in which the vitamin content is likely to deteriorate rapidly during processing and storage (Soliman et al., 1987). Adding fresh vitamins may improve pelleted feed more dramatically than it would in the case of fresh trash fish, which is used on farms, where vitamin loss would be less severe.

While some caution should be exercised in extrapolating the results of this study to the likely effect of immunostimulants on farms, there is strong evidence that Salar-bec may be of use in controlling EUS subject to optimisation of dose regime and assessment in pond trials.

Acknowledgements

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APPENDIX TWENTY FOUR

Paper seventeen - Khan, M.H., and Lilley, J.H. (2001) Risk factors and socio-economic impacts associated with epizootic ulcerative syndrome (EUS) in Bangladesh. In: Proceedings of DFID/FAO/NACA Asia Regional Scoping Workshop "Primary Aquatic Animal Health Care in Rural, Small-Scale Aquaculture Development in Asia". Dhaka, 26-30 September 1999. (in press)

RISK FACTORS AND SOCIO-ECONOMIC IMPACTS ASSOCIATED WITH EPIZOOTIC ULCERATIVE SYNDROME (EUS) IN BANGLADESH

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ABSTRACT

An interview-based questionnaire survey of a fish farmer and a fisher randomly selected from each of the 64 districts of Bangladesh was carried out to study risk factors associated with outbreaks of epizootic ulcerative syndrome (EUS). The survey was undertaken during the EUS season, December 1998 to April 1999. Data showed that there is a significantly higher relative risk of EUS occurring in farmed fish when wild fish are present in the pond; EUS occurred in the previous season; pond embankments are not high enough to prevent in-coming flood water; ponds are connected to natural waters; ponds are not dried or limed prior to stocking; ponds are not limed post-stocking; nets are not dried or disinfected; and pond water colour is black, indicating high levels of organic waste. Of the wild caught fish, those sampled from haors[1] had a significantly higher relative risk of getting EUS. Fish from rivers and flood plains were at a lower risk of EUS infection.

Out of 64 districts, fish with lesions were recorded from fish farms in 32 districts (50%), and 30 (47%) were confirmed EUS positive, and from wild fisheries 52 districts (81%) demonstrated lesions and 49 (77%) were confirmed as EUS-positive. However, the percentage of infected fish was quite low in some sites. A total of 6,408 wild fish and 6,414 farmed fish were examined for lesions, and average prevalence was 16.0 and 15.5%, respectively. Thirty-one species of fish were confirmed as being EUS-positive out of 47 recorded with lesions.

Eighty-eight percent of farmers interviewed had between one and four ponds. These small-scale farmers, in particular, are at risk from suffering serious financial difficulties from sudden disease losses, or from reduced production levels due to disease. Losses in wild fisheries could deprive the poorer sections of the community from access to cheap sources of animal protein.

The present study demonstrated that EUS is still the most damaging disease among freshwater fishes in Bangladesh, and probably has significant effects on fish production, although no direct information on mortalities was obtained. Eighty-six percent of farmers and 89% of fishers interviewed considered EUS to be a major problem. Total fish loss due to EUS for 1998-99 is estimated as 39,797 mt and US$ 3.97million using the prevalence data obtained from this study.

[1] Depressions in floodplains between two or more rivers, which function as internal drainage basins

INTRODUCTION

There is a Bengali proverb "Mache bhate Bangalee," which means Bangladeshi people cannot survive without fish and rice. Approximately 1.4 million people earn their livelihood from fisheries, and another 11 million people are involved in seasonal or part-time fishing and other ancillary activities (Mazid 1995). Eighty percent of the population lives in villages and catch wild fish from ditches, canals, rice fields, floodplains, beels[2], haors, and baors[3] for their normal diet. Fish is the main source of protein for the rural poor, but they don't have enough money to purchase it for daily consumption. Most of the urban people also prefer a diet of wild fish.

With an increase in unemployment, small-scale fish farming is becoming very popular among the unemployed as a source of earnings. Some of the small-scale farmers have their own ponds, but most of them rent ponds for fish culture business. Consequently, fish disease, and epizootic ulcerative syndrome (EUS) in particular, has a severe socio-economic impact on public life, and especially on rural life.

EUS was a very new phenomenon at the time of the first outbreaks in Bangladesh, and it caused great concern because of the perceived dangers to both staple food crops and to human life. The widespread fear of disease transmission to consumers, although unfounded, led to a drastic decrease in market demand for food fish, including marine species, which were not affected by the disease. Usually, the only animal protein available to accompany the rural people's rice diet is derived from fish, and therefore an inadequate intake of fish could result in nutritional deficiency. It has been estimated that 250 million families in the Southeast Asian Region depend on rice as a main crop, and much of the incidental fish harvests from these paddies are an important part of the family's diet (Macintosh 1986). The economic loss due to EUS was estimated at 118.3 million Taka (US$ 3.4 million; 1 US$=35 Taka) during 1988-89. In the second year the disease occurred with lower severity, and the economic loss was estimated at 88.2 million Taka (US$ 2.2 million). Fish price dropped to 25-40% of the pre-disease level during the first outbreak (Barua 1994).

Since 1988, EUS has been considered the most serious epidemic disease affecting freshwater fish in Bangladesh. As with most other diseases, there is strong evidence that EUS outbreaks occur only when a number of determinants or causal factors combine. A number of factors are considered to be acting at the same level and ultimately lead to the exposure of dermis. These exposed sites could provide the point of attachment and entry for spores of *Aphanomyces invadans*, regarded as the essential component in all EUS outbreaks (Lilley *et al.* 1998). Recent studies suggest that there are a number of other sufficient causes for EUS outbreaks. Although every set of sufficient causes for EUS is different from one another, each combination has the common result of exposing the dermis and allowing entry of *A. invadans*. Callinan *et al.* (1996), reported outbreaks of EUS in estuarine fish in Australia associated with acid-sulphate soil areas, and reproduced EUS by exposing susceptible fish to acid water and spores of *A. invadans*. Kanchanakhan (1996) has shown that EUS can be reproduced when susceptible snakeheads (*Channa* sp.) are injected with a particular strain of rhabdovirus and bathed in spores of *A. invadans*. Demonstration of the highly invasive abilities of EUS fungus in tissues like bone, gizzard and spinal cord provides an indication that under certain circumstances, the fungus may be able to invade the healthy skin of fish (Vishwanath *et al.* 1998).

The epidemiology of EUS is poorly studied in many affected countries, including Bangladesh. However, a number of factors have been hypothesised as either risk factors or determinants for EUS outbreaks in Bangladesh. These factors are based on observations of the mode of disease transmission, the species, habitats and culture systems affected by EUS; human interventions; movements of animals; and seasonality of EUS outbreaks. Identifying true risk factors for EUS allows rational control measures to be developed. EUS research requirements, as recommended by FAO (1986), included the need for a greater

[2]Floodplain lakes, which may hold water permanently or dry up during the winter season.
[3] Oxbow lakes.

understanding of the influence of environmental factors and pollutants on the disease and the identification of causative agent(s). During an EUS survey of Bangladesh, Roberts *et al.* (1989) stressed the need for an epidemiological study of individual waters to collect information on disease transmission, relative species susceptibility, mortality and recovery rate in different species and ages of fish, fish losses and economic impact. The present cross-sectional survey aimed to quantify the degree of the present EUS problem, and also identify risk factors that affect outbreaks. Fish farmers and fishers were interviewed and the information was used to measure the strength of association between EUS and hypothesised risk factors.

MATERIALS AND METHODS

A cross-sectional, interview-based survey was conducted in a thana[4] selected from each of the 64 districts of Bangladesh from December 1998 to April 1999. This period is the recognised "EUS season." Three M.Sc. students from Bangladesh Agricultural University interviewed a fish farmer and a fisher randomly in each thana and examined 100 fish for EUS-type ulcers.

Survey Areas

One thana known to have adequate fisheries resources was randomly selected from each of the 64 administrative districts. In August 1998, a letter was sent to the Thana Fisheries Officers (TFO) requesting a list of categorised fish farms (both registered and unregistered) and wild fisheries areas in their respective thanas. From these lists, one fish farm and one wild fishery were randomly selected.

Development of Questionnaire

The questionnaire development procedure followed the methods described by Thrusfield (1995). Both fish farmer and fisher questionnaires were designed to record information in a standard format with in-built error checks. Closed questions were used, wherever possible, to give data in a yes/no/don't know or categorical format to facilitate ease of coding and analysis. Attempts were made to make wording unambiguous, brief, polite and non-technical. Both questionnaires were prepared in English and Bengali, and the Bengali version was used for interviewing. Before starting the survey, questionnaires were pre-tested two times by interviewing target people to identify ambiguous and irrelevant questions.

Interviewing and Sampling

The three interviewers were trained together to minimise differences in technique. Training also included examination of fish for EUS-type ulcers and sampling for histology. Each interviewer covered one third of the total districts. TFOs were requested to aid and co-operate with the interviewers. Each interviewer carried with him the required number of questionnaires, fish sampling sheets, photographs of EUS-affected fish, 10% buffered formalin, vials, marking pen, scalpels, cast net and hapa. After completion of the interview, at least 100 susceptible fish from each farm or fishing site were examined for EUS ulcers, irrespective of species, and information recorded on the sampling sheet. One fish of each species recorded with lesions was sampled for histology. Tissue samples were fixed in 10% buffered formalin. In case a sampling net was unavailable, the interviewer supplied his own net for catching fish. During farm visits, in order to avoid re-counting the same individuals, fishes, once examined for ulcers, were separated into the hapa until 100 individuals had been examined. Nets were disinfected between sites. A fish farm or wild fishery was classified as affected with EUS if the presence in one or more fish of any species of characteristic mycotic granulomas was confirmed histologically.

Database Preparation and Analysis

[4] Sub-district

Two MS Access™ databases (for fish farmer and fisher data), and two MS Excel™ spreadsheets (for fish species data) were used to enter the information. Univariate analyses were undertaken using Epiinfo™ to examine the association between EUS occurrence and putative risk factors using crude relative risk (RR) as the measure. Fish farm and wild fishery data were analysed separately.

Histology

Formalin-fixed blocks of lesions and underlying muscle were processed, embedded in paraffin wax and sectioned at 5 µm. The sections were stained with haematoxylin and eosin (H&E) to visualise granulomas, and Grocott's silver stain was used to confirm the mycotic involvement.

RESULTS AND DISCUSSION

Variables were analysed for their effect on the relative risk (RR) of EUS (Tables 1 and 2). RR > 1 indicates that the variable is a putative causal factor of EUS; RR = 1 indicates no association exists between the factor and EUS; and RR < 1 indicates the variable is a sparing factor for EUS (i.e., that it reduces the chance of EUS occurring). Where the lower confidence limit is above 1, there is 95% confidence that the variable is a risk for EUS; where the upper confidence limit is below 1 there is 95% confidence that the variable is a sparing factor for EUS.

Data from Fishfarmer Interviews

Pond connections

The analyses showed that there was over 10 times more chance of EUS occurring in culture ponds containing wild fish. This was the highest RR measured out of the variables examined. The data also show that there was a significantly lower RR (0.39) of EUS occurring on farmed fish when pond embankments were high enough to prevent incoming waters. Similarly, ponds that had been flooded that year showed a significantly higher RR (2.33). Fish farms directly connected to water bodies that allowed the entry of wild fishes also showed a significantly higher RR (2.63) of EUS. Each type of connecting water body (i.e., rice-field, ditch and beel) provided a similar level of risk. Ponds containing water sourced from underground wells or only from rain were at much lower risk of EUS (RR=0.91, 0.52), compared to ponds with water sourced from ricefields (RR=2.36). These results equate with those of Hossain *et al* (1992) which, when recalculated for RR, show a significantly lower risk of EUS-type lesions occurring on fish from rainfed ponds (0.65) than from flooded or irrigated ponds.

Floodwater and entry of wild fish are risk factors probably because they are routes of entry for pathogens (Kabata 1985). Roberts *et al.* (1989) described floodwater as a powerful means for spreading EUS throughout Bangladesh. Changes in water quality and agricultural run-off due to floods may cause stress for the farmed fish, and may be a component cause for EUS. There is an absence of parasites and microbial flora in underground water, and the exclusive use of rainwater and underground water would reduce the risks described above (Munro and Roberts 1989).

Table 1. Variables affecting relative risk (RR) of EUS in fish farming areas, giving 95% confidence limits (lower<RR<upper). RR>1 indicates the factor is a putative causal factor of EUS; RR=1 indicates no association exists between the factor and EUS; and RR<1 indicates the factor as a sparing factor for EUS.

Variable	Relative Risk (RR)	Lower Limit	Upper Limit
POND CONNECTION			
Wild fish observed in ponds [1]	10.09	1.62	74.27
Pond has high embankment	0.39	0.25	0.62
Holes observed in the pond bank	1.79	1.08	2.98
Pond connected to other water body allowing entry of wild fish	2.63	1.69	4.08
Other water body = ricefield	2.73	1.81	4.13
Other water body = ditch	2.26	1.70	2.99
Other water body = beel	2.21	1.68	2.91
Water supply = ricefield water	2.55	1.84	3.53
Water supply = only rainfed	0.52	0.31	0.88
Water supply = underground	0.91	0.49	1.71
Pond is close to other water body	1.84	0.94	3.62
Floodwater enters the pond	2.33	1.27	4.29
PRE-STOCKING POND PREPARATION			
Water is drained from pond	0.55	0.30	1.01
Pond is dried	0.41	0.18	0.92
Bottom mud is removed	0.17	0.03	1.09
Pond is limed	0.42	0.21	0.83
Pond is fertilised	0.50	0.21	1.21
POST-STOCKING MANAGEMENT			
Pond is limed	0.46	0.30	0.71
Pond is fertilised	0.93	0.40	2.17
Black water colour (high organic debris)	2.21	1.68	2.91
Transparent pond water	1.07	0.26	4.38
Greenish water colour (phytoplankton)	0.84	0.47	1.51
Reddish water colour (zooplankton)	0.74	0.32	1.70
HYGIENE			
Fry source water released in pond	2.00	1.09	3.66
Cattle wash/drink at pond after grazing or ploughing in the field	2.90	1.46	5.77
Farm nets are dried/disinfected	0.59	0.35	1.01
Buyers use dried/disinfected nets	0.14	0.02	0.90
Parasites observed on fish	2.65	1.45	4.86
CLIMATE / SEASONALITY			
EUS occurred in the previous season	3.00	1.64	5.49
Temperature unusually low prior to disease	3.83	2.36	6.23
Rain unusually heavy prior to disease	2.48	1.81	3.40

[1]62 farmers answered this question (64 farmers answered all other questions)

Table 2. Variables affecting relative risk (RR) of EUS in 64 fishing areas, giving 95% confidence limits (lower<RR<upper). RR>1 indicates the factor is a putative causal factor of EUS; RR=1 indicates no association exists between the factor and EUS; and RR<1 indicates the factor as a sparing factor for EUS.

Variable	Relative Risk (RR)	Lower Limit	Upper Limit
TYPE OF HABITAT			
River	0.54	0.26	1.14
Floodplain	0.63	0.28	1.42
Ricefield	0.98	0.55	1.75
Beel	1.04	0.79	1.37
Haor	1.33	1.15	1.54
STOCKING			
Water body is artificially stocked	1.08	0.81	1.44
HEALTH			
EUS occurred in the previous season	2.19	1.17	4.11

Pond preparation

Complete draining of pond water, drying, bottom mud removal and liming during pond preparation were found to result in low relative risks of 0.55, 0.41, 0.17 and 0.42, respectively. Fertilisation during pond preparation also resulted in a low, but non-significant, RR (0.50).

Pond preparation techniques described above will exclude *A. invadans*, and many other pathogens, from the pond environment. It is interesting that the "removal of bottom mud" resulted in a very low RR. Unlike other oomycete fungi, *A. invadans* does not appear to show strong negative geotaxis, and may possibly accumulate on the pond bottom, although soil assays have not succeeded in isolating *A. invadans* (Willoughby 1999). *Aphanomyces invadans* can feasibly survive the warmer months of summer in the thick bottom mud of older or derelict ponds, which generally possess a temperature below 31°C, and with declining temperature or rainfall disturbance, the fungus might be activated to grow. This theory is supported by Ahmed and Rab's (1995) study, which showed that fish cultured in previously derelict ponds had a significantly increased probability of EUS.

Post-stocking management and hygiene of habitat

Post-stocking liming also gave a significantly low RR of 0.46, and again, fertilisation after stocking did not significantly affect RR. Pond-water colour indicating high levels of phytoplankton or zooplankton had low, but not significant, RRs. However, ponds black with high levels of organic waste showed significantly higher RR (2.21).

Liming increases pH, hardness, alkalinity and the buffering system of pond water and also reduces stress for fish, thereby reducing the risk of EUS. Exposure of fish to low pH might be one of the causes of skin damage, necessary for fungal entry to cause EUS. In aquarium trials, EUS lesions were induced in fish exposed firstly to acidified water, and then to spores of *A. invadans*, and thus, confirming these two factors in combination as a sufficient cause of EUS (Callinan *et al.* 1996). It is possible that the increase in calcium and magnesium in the pond will also have a more direct effect by benefiting fish skin and inducing encystment in fungal zoospores, thereby making them fall out of suspension.

Hygiene/other disease

Allowing cattle to wash and drink in the pond after ploughing or grazing in other areas gave a high RR (2.90), possibly due to the transport of pathogens with the cattle. Netting with dried or disinfected nets, and requiring buyers to do the same, contributed much lower RR values (0.59 and 0.14, respectively). The use of equipment that has been transported between farms (e.g., by buyers) is likely to provide a source of infective material, and drying or disinfection is recommended.

A high RR (2.65) was also demonstrated in ponds where the farmer said fish were affected by parasites. A number of parasites have been isolated from EUS-affected fish (Tonguthai, 1986) and may either be possible vectors for the pathogen, or a stress-inducing factor in EUS outbreaks. Subasinghe (1993) demonstrated such an association between the level of infection by *Trichodina* sp. and the susceptibility of *Channa striata* to EUS infection. The mechanism of attachment of these parasites can cause skin rupture, and might facilitate infection by the EUS fungus.

Climate/seasonality

Farmers that reported EUS in the previous season were shown to be at higher risk of EUS (RR=3.00). Reports by farmers that there was unusually low temperature or heavy rainfall 3-15 days prior to the interview were also correlated with EUS occurrences.

EUS has been associated with low temperature, and has often occurred after periods of heavy rain. Phillips and Keddie (1990) observed from data from 1988-89 that EUS outbreaks occurred during months when the mean daily temperature was below the annual mean temperature in Bangladesh, China, India and Lao-PDR. However EUS outbreaks in the Philippines and Thailand were also recorded in warmer months. Chinabut *et al.* (1995) challenged striped snakehead (*Channa striata*) by injecting with zoospores of *A. invadans* and found a weaker inflammatory response, higher mortality rate and more extensive fungal invasion in fish held at 19°C compared to fish held at 26 and 31°C.

Data from Fisher Interviews

Types of habitat

Among the different types of fish habitat sampled, haors showed the highest RR (1.33) and rivers showed the lowest RR (0.54). A haor is the biggest natural depression between two or more rivers, and is lower than the adjacent floodplains. It functions as a small internal drainage basin and receives upland runoff water (Khan 1997). Chemicals, waste and pathogens may enter the haor through the river systems. At the onset of the dry season, the water level of the haors decrease and the aquatic animals and plants are concentrated, often resulting in stressful conditions for fishes. The presence of a wide range of EUS-susceptible fishes under these circumstances make haors susceptible areas for EUS outbreaks. The active movement of the water in rivers may lessen the chances of the fungal pathogen attaching to fish, thereby resulting in the lower RR recorded for EUS in rivers. There was no significant association between artificial stocking of natural water bodies and occurrence of EUS. Water bodies that fishermen reported had been affected the previous season, were at higher risk of EUS (RR = 2.19).

General Observations

During the period of survey, interviewers recorded farmers' general observations and opinions, which are summarised here. Some of these points have been demonstrated by the present study, whereas others require further work investigate possible associations.

- EUS outbreaks have occurred every year since 1988 and affect most of the freshwater fish. Initially the severity of disease was very high, but this has shown a decreasing trend.

- Wild fisheries are much more affected than farmed fishes. Farmers and fishers from beel areas reported that an outbreak occurs every year in their local beels as the temperature begins to fall. It affects snakehead, *Puntius*, *Mastacembelus*, escaped farmed fish and others. Later, fish farms very close to those affected wild fisheries become affected, and then more distant farms are affected.

- EUS often occurs in culture ponds directly connected to ricefields through drainage, but other nearby ponds not linked through drainage systems are usually unaffected.

- Some farmers and fishers opined that aquatic birds, fish-eating birds, reptiles and mammals might transmit the disease from one place to another by preying on easy to catch EUS-affected fish and dropping uneaten portions in unaffected water bodies. They may spread the pathogen by repeatedly preying and washing alternately between affected ponds and unaffected ponds.

- Most farmers and fishers believe that floods play a vital role in spreading EUS throughout the country.

- Some observers recorded that moderately affected snakeheads, walking catfish and climbing perch might transmit the disease by entering an unaffected water body.

- Some farmers commented that their unaffected pond became affected after they spread duckweed from a wild water body.

- A remarkable number of older ponds with very high bottom deposition and shade were reported to be repeatedly affected over several years, although not in anyway connected to wild or affected fisheries. It appears, therefore, that the fungus may survive in isolated water bodies under particular conditions.

- Ponds with a high stocking density were observed to be more affected by EUS.

During a separate case-control study of ponds in Mymensingh District undertaken concurrently with the cross-sectional study, the following observations were made:

- A series of adjacent fish ponds were found affected with EUS in late winter, and it was difficult to find an unaffected "control" pond in that area.

- Some ponds, with a common embankment located near to a particular beel, were all found to be affected. However, newly constructed ponds with very high embankments near to the same beel were unaffected.

- On a number of occasions, affected ponds were separated from unaffected ponds with similar culture characteristics by a highway or high embankment.

- Unaffected farms with ponds nearby an EUS-affected beel usually became affected within a week. It was thought this was after cattle and items used in the beel were washed in the pond. Some farmers reported that after fishing in EUS-affected beels, ricefields and other ponds, EUS occurred in their fish farms when they washed nets, wild-caught fishes, and themselves in the ponds.

Socio-economics

Out of the 64 districts, fish with lesions were recorded from fish farms in 32 districts (50%) and 30 (47%) were confirmed EUS positive. From wild fisheries, 52 districts (81%) demonstrated lesions and 49 (77%) were confirmed as EUS positive. Thirty-one species of fish were confirmed as being EUS positive out of 47 recorded with lesions. Totals of 6,408 wild fish and 6,414 farmed fish were examined for lesions, and average prevalences were calculated as 16.0 and 15.5%, respectively. Eighty percent of the ulcerated fish were confirmed as EUS positive.

Box 1. Socio-economic data.

- 95% of farmers and fishers interviewed reported use of EUS-affected water for domestic purposes
- 55% reported a fall in price of table fish during the EUS season
- 59% reported a fall in price of table fish in an EUS-affected locality
- 72% of farmers reported a fall in price of healthy fish fry during the EUS season
- 67% of farmers reported a fall in price of healthy fish fry in an EUS-affected locality

EUS commonly affects small wild fishes e.g., *Channa* spp., *Puntius* spp., *Mastacembelus* spp, *Colisa* spp., *Mystus* spp., *Nandus* sp., *Anabas* sp., *Heteropneustes* sp., *Clarias* sp. and *Ambassis* spp. Rural poor people catch these species of fish as a main source of animal protein, as they usually cannot purchase fish or other animal products. People involved in this activity range from small children to professional fisherfolk. Of the fishers interviewed, 89% considered EUS to be a major problem.

Major carps are the most significantly affected farmed fish. Once an outbreak occurs in a carp pond, EUS can damage the entire crop and, as a result, small-scale poor farmers can fall into serious economic crisis, particularly farmers who rent ponds. Of the farmers interviewed, 86% considered EUS to be a major problem. The majority of farmers said that the price of fish dropped during the EUS season, and in EUS affected localities (Box 1). Fish prices given by interviewees indicated that prices dropped by more than 50% when fish were slightly ulcerated, or when when there is an EUS outbreak in the locality (Table 3). Despite the potential dangers, most of the farmers interviewed considered fish farming to be a profitable business

Economic loss was estimated by relating the present prevalence of lesions on fish to five-months projected fish production data for 1998-99 obtained from the Fisheries Resources Survey System, Directorate of Fishery, Bangladesh (FRSS 1998). The estimate excludes hilsha, shrimp and production from the Sunderbans area. It is also adjusted to exclude the 20% of fish with lesions that were not EUS-affected; another 20% of EUS-affected fish that would be consumed anyway; and a further 10% to allow for recovered fish. Total fish loss for 1998-99 is estimated at 39,797 mt, at a value of US$ 3.97m (at Tk 50/kg fish and 1 US$=50 Tk). Farmed fish account for 18,140 mt of this figure, and wild fish account for 21,657 mt. The estimated loss is higher, although the severity of the disease is lower, as compared to 1988 and 89, due to the two-fold increase in fish production over the last 10 years.

Table 3. Average fish prices indicated by interviewees

Subject	Price (Tk)	Comment
Table-size healthy fish per kg	64	-
Table-size slightly affected fish per kg	29	54% price fall in slightly ulcerated table fish
Price of healthy fish fry per thousand	577	-
Price of healthy fish fry per thousand in EUS-affected locality	236	59% price fall in EUS-affected locality

CONCLUSIONS AND RECOMMENDATIONS

Prevention of disease is always more economical than cure. On the basis of risk analysis and general observation, the following precautionary measures could be adopted to prevent EUS:

- Repair and raise pond embankments above the flood level and close inlets and holes on the bank to prevent entry of flood water.
- Dry and lime ponds.
- In the case of old and derelict ponds, bottom mud should be removed.
- Ponds should be supplied with rainfed, underground or purified water.
- Suitable resistant species might be substituted for susceptible species in severely affected areas.
- Ponds should be stocked with healthy hatchery-reared fish fry. Wild fry should be avoided.
- Fish fry could be treated with 2.5% NaCl for at least 15 minutes before release in ponds. Fry source water should not be released into ponds.
- All wild fish (e.g., snakeheads, catfish, *Puntius* spp, *Mastacembelus* spp and *Anabas* sp.) should be removed and excluded from ponds.
- Avoid washing of ploughing equipment, cattle and people in fish ponds following work in other water-filled areas (e.g., ditches, paddy fields or beels) in winter.
- Nets and other equipment should be disinfected (e.g., using bleaching powder or iodophore), or sun dried, prior to re-use.
- Ideally, feet/boots of farm workers and visitors should be disinfected at the farm entrance.
- Good plankton bloom should be maintained. The water should not be allowed to go black with high organic waste.
- Periodic liming during stocking should be done (depending on pH and alkalinity).
- Severe parasitic infestations should be treated.

Winter is the most common period for EUS outbreaks, therefore particular measures should be taken at this time. Fish farms should be monitored regularly. Liming prior to winter (at

1kg/decimal[5]) is recommended. Awareness of fish health management should be created among fish farmers. Regulations concerning transportation of fish from affected/suspected affected zones to unaffected zones might be effective, although the present study demonstrates that EUS is endemic to a large proportion of Bangladesh. Regulations against the indiscriminate use of chemicals and antibiotics against EUS are necessary to prevent detrimental effects to the environment, fish and ultimately, the consumer.

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[5]1 decimal = 40.48 m^2

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APPENDIX TWENTY FIVE

Paper eighteen - Khan, M.H., Lilley, J.H., Majumder, B., Sarker, M.G.A., Alauddin, M., Hoque, A., Ahmed, G.U., and Chowdhury, M.B. (2001) Cross-sectional survey of epizootic ulcerative syndrome (EUS) cases in Bangladesh. Diseases in Asian Aquaculture IV (in press)

Cross-sectional survey of epizootic ulcerative syndrome (EUS) cases in Bangladesh

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Abstract
An interview-based questionnaire survey of one fish farmer and one fisherman randomly selected in each of the 64 districts of Bangladesh was carried out to study prevalence of EUS, and risk factors associated with EUS outbreaks. The survey was carried out during the EUS season, December 1998 to April 1999. At each site, 100 fish were examined for lesions, and one fish of each species with lesions was sampled for histological diagnosis. A fish farm or wild fishery was classified as affected with EUS if one or more fish of any species had a positive diagnosis based on the presence of characteristic mycotic granulomas in histological sections. Univariate analyses were used to examine the association between EUS occurrence and putative risk factors using crude relative risk (RR) as the measure.

Out of 64 districts, fish with lesions were recorded from fish farms in 32 districts and 30 were confirmed EUS positive, and from wild fisheries 52 districts demonstrated lesions and 49 were confirmed as EUS-positive. In total, 6434 wild fish and 6401 farmed fish were examined, and average prevalence of lesions was calculated as 16.0% and 15.5% respectively. Although disease was more widespread in the wild fisheries, the percentage fish with lesions was generally higher at farm sites (8-45%) than wild fisheries (3-32%). A total of 471 fish with lesions was sampled for histology from the 84 affected sites and 80% of these were diagnosed as EUS-positive. Thirty-one species of fish were confirmed as being EUS-positive out of 47 recorded with lesions. Combining data on affected species from both farmed and wild fisheries sites, the highest prevalences of EUS were recorded in *Channa marulius* (30%); *Glossogobius sp.* (25%); *Cirrhinus mrigala* (24%); *Channa striata* (22%); *Channa punctata* (21%); and *Anabas testudineus* (20%); and the lowest prevalence was in *Lepidocephalus guntea* (3%).

Data showed that there is a significantly higher relative risk of EUS occurring in farmed fish when wild fish are present in the pond; EUS occurred in the previous season; pond embankments are not high enough to prevent in-coming flood water; ponds are connected to natural waters; ponds are not dried or limed prior to stocking; ponds are not limed post-stocking; nets are not dried or disinfected and pond water colour is black indicating high levels of organic waste.

Of the wild-caught fish, those sampled from haors had a significantly higher relative risk of getting EUS. Fish from rivers and flood plains were at a lower risk of EUS infection.

Introduction
Since 1988, epizootic ulcerative syndrome (EUS) has been considered the most serious disease affecting freshwater fish in Bangladesh (Ali, 1997). During initial outbreaks, EUS was identified by the presence of ulcerative lesions and was associated with high mortalities. Lesions are still commonly observed on fish in Bangladesh, but are not necessarily associated with high mortalities. Prevalence studies of EUS-like lesions on fish in Bangladesh have rarely incorporated a pathological diagnosis and therefore may have included a number of other ulcerative diseases. There is growing consensus that EUS should be diagnosed by the presence of mycotic granulomas associated with *Aphanomyces invadans* (=*A. piscicida*) hyphae (Fraser *et al*. 1992; Hatai, 1994; Roberts *et al*. 1994a; Vishwanath *et al*., 1998), and it is this feature that was used to define EUS lesions in the present study.

As with most other diseases, there is strong evidence that EUS outbreaks occur only when a number of component causes combine. Several environmental and biological factors are thought to be capable of affecting fish skin and exposing the underlying dermis. These exposed sites could provide the point of attachment and entry for *A. invadans* spores, regarded as the essential component in all EUS outbreaks (Lilley *et al.*, 1998). Recent challenge studies have demonstrated particular sets of sufficient causes that lead to EUS (Callinan *et al.*, 1996; Kanchanakhan, 1996). In Bangladesh, *A. invadans* can be readily isolated from EUS-affected fish (Willoughby and Roberts, 1994), but other component causes have not been conclusively identified.

A number of potential risk factors for EUS in Bangladesh have been identified during observations of outbreaks. Identifying true risk factors for EUS allows rational control measures to be developed. During a survey of early EUS outbreaks in Bangladesh, Roberts *et al.* (1989) stressed the need for an epidemiological study of particular water bodies to collect information on relative species susceptibility, fish losses, economic impact and factors affecting disease transmission. Ahmed and Rab (1995) applied a logit model to data on the occurrence of lesions on fish in 257 ponds in Gazipur District in Bangladesh and showed that presence of *Puntius gonionotus*, culture in reddish sandy soils and use of piscicide (instead of drying and netting) increased the probability of lesions on fish. They also found that culture in newly excavated ponds and use of lime post-stocking reduced the risk of lesions. The present study also aimed to measure the strength of association between EUS and the hypothesised risk factors. In this study, wild and culture fisheries were investigated, an increased number of variables were studied, and the geographical study area was widened to cover all the districts of Bangladesh.

Materials and Methods

Survey areas:

One thana (subdistrict), known to have adequate fisheries resources, was randomly selected from each of the 64 administrative districts in Bangladesh. In August 1998 a letter was sent to the Thana Fisheries Officers (TFO) requesting a categorised list of fish farms (both registered and unregistered) and wild fisheries areas in their respective thanas. From these lists, one fish farm and one wild fisheries area was randomly selected.

Development of questionnaire:

Fish farmer and fisherman questionnaires were designed using closed questions wherever possible to give data in a yes/no/don't know or other categorical format to facilitate coding and analysis. Both questionnaires were prepared in English and Bengali, copies of which are available from the authors. The Bengali version was used for interviews. Before starting the survey, questionnaires were pre-tested two times by interviewing target people to identify ambiguous and irrelevant questions.

Interviewing and sampling:

The study was carried out from December 1998 to April 1999, which is the recognised "EUS season" in Bangladesh. Three interviewers were trained in interviewing techniques and fish sampling and identification. Each interviewer covered one third of the total districts. After the completion of the interview, at least 100 susceptible fish from each farm or fisheries site were examined for EUS-like lesions, irrespective of species. After examination, fish were separated into hapas, to prevent individuals being recounted. Catch nets and hapas were disinfected between sites. At least one fish of each species recorded with lesions at each site was sampled for histology. A fish farm or wild fishery was classified as affected with EUS if one or more fish of any species had a histologically confirmed diagnosis based on the presence of characteristic mycotic granulomas. The sample size of 100 fish per site would detect presence of EUS with 95% confidence if 3% of fish had lesions.

Database preparation & analysis:

Two MS Access™ databases (for fish farmer and fisherman data), and two MS Excel™ spreadsheets (for fish species data) were used to enter the information. Univariate analyses were undertaken using Epiinfo™ to examine the association between EUS occurrence and putative risk factors using crude relative risk (RR) as the measure. Fish farm and wild fishery data were analysed separately.

Histology:

Formalin-fixed blocks of lesions and underlying muscle were processed, embedded in paraffin wax and sectioned at 5µm. The sections were stained with haematoxylin and eosin (H&E) to visualise granulomas and Grocott's silver stain was used to confirm the mycotic involvement.

Results

A total of 36,736 unique datapoints were entered into the databases. Summary frequency data and the factors that showed a significantly higher risk of EUS are presented here.

Site prevalence of EUS

In the farm survey, 6401 farmed fish were examined for lesions, and the average prevalence of lesions, irrespective of species, was 15.5%. Ninety-five fish with lesions were sampled for histology and 85% of these were EUS positive. Fig. 1 shows the frequency distribution of the percentages of fish affected at fish farm sites, which ranged from 8-45%.

In the wild fishery survey, 6434 wild fish were examined for lesions, and average prevalence was 16%. Three hundred and seventy-six fish with lesions were sampled for histology and 79% of these were EUS positive. Fig.

2 shows the frequency distribution of the percentages of fish affected at fisheries sites, which ranged from 3-32%.

Species prevalence of EUS

In the farm study, 11 species were examined. Of these, seven species were confirmed with EUS, two species (mirror carp and common carp: both *Cyprinus carpio*) suffered from non-EUS lesions, and two species (silver carp *Hypophthalmichthys molitrix* and grass carp *Ctenopharyngodon idella*) had no lesions. The percentage occurrence of EUS in each affected species (as confirmed in at least one fish at each site) was: *Channa punctata* 53%; *Puntius ticto* 39%; *Cirrhinus mrigala* 25%; *Labeo calbasu* 21%; *Puntius gonionotus* 17%; *Labeo rohita* 13%; and *Catla catla* 5%.

In the wild fisheries, 45 species were examined for lesions. Of these, 30 were confirmed as EUS positive, 8 had non-EUS lesions and 7 had no lesions. Among the EUS positive fishes, the highest prevalence was in *Channa marulius* 30%; followed by *Glossogobius sp.* 25%; *Channa striata* 22%; *Channa punctata* 21%; *Anabas testudineus* 20%; *Wallago attu* 20%; *Colisa chuna* 18%; *Puntius sophore* 16%; *Cirrhinus mrigala* 16%; *Channa orientalis* 15%; *Colisa fasciata* 15%; *Mastacembelus armatus* 13%; *Glossogobius giuris* 12%; *Cirrhinus reba* 12%; *Macrognathus aculeatus* 11%; *Mystus vittatus* 11%; *Clarias batrachus* 10%; *Mystus cavasius* 9%; *Nandus nandus* 9%; *Clarias gariepinus* 9%; *Labeo calbasu* 9%; *Puntius gonionotus* 9%; *Mastacembelus pancalus* 8%; *Lepidocephalus sp.* 8%; *Ambasis ranga* 7%; *Mystus tengra* 6%; *Labeo rohita* 6%; *Heteropneustes fossilis* 4%; *Lepidocephalus guntea* 3%; and *Catla catla* 2%.

Data analyses

Variables were analysed for their effect on the relative risk (RR) of EUS (Tables 1 and 2). A RR > 1 indicates the variable is a putative causal factor of EUS; RR = 1 indicates no association exists between the factor and EUS; and RR < 1 indicates the variable is a sparing factor for EUS. Where the lower confidence limit is above 1, there is 95% confidence that the variable is a risk factor for EUS; where the upper confidence limit is below 1 there is 95% confidence that the variable is a sparing factor for EUS.

Pond connections

The analyses showed that there was over 10 times more chance of EUS occurring in culture ponds containing wild fish. This was the highest RR measured out of the variables examined. There was also a significantly higher RR (2.56) of EUS occurring on farmed fish when pond embankments were not high enough to prevent incoming water. Similarly, ponds that had holes in the banks showed a high risk of EUS (1.79). Ponds that were reportedly flooded that year also showed a significantly higher RR (2.33). Fish farms directly connected to water bodies that allowed the entry of wild fishes also showed a significantly higher relative RR (2.63) of EUS. Each type of connecting water body (i.e. rice-field, ditch and beel) provided a similar level of risk. Ponds containing water sourced from underground wells or only from rain were at much lower risk of EUS (RR = 0.91, 0.52), compared to ponds with water sourced from ricefields (RR = 2.36).

Pond management

Failure to drain and lime ponds prior to stocking resulted in significantly higher relative risks of EUS of 2.44 and 2.38 respectively. Ponds that had not had the bottom mud removed also showed an increased likelihood of EUS. Ponds that had not been fertilized prior to stocking were also more affected by EUS, but not to a significant extent.

Farms that did not apply lime post-stocking also had a significantly higher probability of EUS (R = 2.17). Pond water colour indicating high levels of zooplankton (reddish) were at lowest risk of EUS. When RRs for other ponds were calculated with respect to the reddish ponds, those with transparent water and water green with high levels of phytoplankton were found to be not significantly different. However, ponds black with high organic debris were shown to be almost three times as high risk of EUS (RR = 2.75).

Hygiene / health in culture ponds

During fry release, the addition of fry source water into ponds gave a significantly high RR (2.00). Allowing cattle to wash and drink in the pond after ploughing or grazing in other areas also gave a high RR (2.90). Failure to dry or disinfect farm or trader nets gave an increased risk of EUS (RR = 1.69, 7.14). A high RR was also demonstrated in ponds where the farmer said fish were affected by parasites (2.65) or were affected by EUS in the previous season (3.00).

Wild fisheries sites

Of the various wild fish habitats sampled, rivers had the lowest RR (0.48). Therefore, the RRs of EUS for the other water bodies were analysed in respect to the RR in rivers. Table 2 shows that haors[6] were significantly (2.50 times) more at risk of EUS than rivers. The data also showed no significant association between artificial stocking of natural water bodies and occurrence of EUS. Water bodies that fishermen reported had been affected the previous season, were at higher risk of EUS (RR = 2.19).

Discussion

Prevalence of EUS

The number of wild fisheries sites affected by EUS was higher than the farm sites. This corresponds with the results of the data analyses, which showed that ponds containing wild fish or connected to natural water bodies were at higher risk of EUS. The disease can therefore be considered to be endemic in the natural waters, and methods of excluding wild fish and other potential carriers from farms are likely to be effective in reducing the incidence of EUS. Although EUS was more widespread in the wild fisheries, the percentage of fish with lesions was generally higher at farm sites. This is probably due to the greater fish densities in ponds, which result in higher rates of disease transmission.

In total, 31 species were confirmed as affected by EUS, including 17 species confirmed as susceptible by other workers (Lilley *et al.,* 1998) and 14 species not previously confirmed as affected by EUS. The species prevalence data from the farm study shows that two incidental species from the wild (*Channa punctata* and *Puntius ticto)* had the highest prevalence of EUS, and these were calculated to have relative risks for EUS of 2.17 and 2.26 respectively. The results showed some variation in the susceptibility of the Indian major carp species, whereas none of the Chinese carps contracted EUS. This is in agreement with other reports that have indicated that Chinese carps are totally resistant to EUS (Roberts *et al*. 1994b).

Pond connections

The variables measured consistently showed that ponds receiving water from natural water bodies had a higher risk of EUS. These results equate with those of Hossain *et al* (1992) which, when recalculated for RR, show a significantly lower risk of EUS-type lesions occurring on fish from rainfed ponds (0.65) than from flooded or irrigated ponds. Roberts *et al*. (1989) described floodwater as a powerful means for spreading EUS throughout Bangladesh. The reason for this is likely to be due to the spread of propagules of the fungal pathogen, *A. invadans;* but floods may also cause changes in water quality that impact on farmed fish, making them more susceptible to infection. There is an absence of parasites and microbial flora in underground water and the exclusive use of rainwater and underground water would reduce the risks described above (Munro and Roberts, 1989).

Pond management

The importance of draining and liming ponds prior to stocking was demonstrated in this study. These pond preparation techniques would help to exclude *A. invadans* and many other pathogens from the pond environment. Similarly, removal of the bottom mud during pond preparation reduced the risk of EUS in study ponds, but it is acknowledged that it may not be practical to employ this procedure before every crop. It is advised that removal of bottom mud should be undertaken if EUS occurred during the previous crop. Ahmed and Rab (1995) have shown that fish cultured in newly excavated ponds, as opposed to ponds that had been left standing, had a significantly lower probability of EUS. The possibility that the pathogen, *A. invadans*, may accumulate on the pond bottom has been considered, although soil assays have not succeeded in recovering *A. invadans* (Willoughby, 1999).

Applications of lime during the culture period also reduced the probability of EUS. Liming is already considered a useful means of reducing disease over the winter season, but there has been little published data to demonstrate the importance of this practice. Most of the farmers interviewed indicated that they thought the lime they used was mainly calcium carbonate, which increases pH, hardness, alkalinity and the buffering system of the water, but is not known to show activity against pathogens. It is likely, therefore, that the effect of treatments on water quality and fish physiology may be at least as important in controlling EUS as chemical treatments of the pathogen.

[6] A haor is the largest natural depression between two or more rivers

Hygiene / health in culture ponds

Increased risks of EUS were shown in ponds where precautions were not taken against entry of pathogens via the water or equipment of fish traders, and in ponds in which livestock were bathed. Therefore, the drying or disinfection of equipment that may provide a source of infective material is recommended.

In the present study, ponds containing fish with parasite infestations showed a higher risk of EUS. Although no causal association can be assumed from these results, they do provide an indication that control of ectoparasite infestations may reduce the risk of EUS. Other studies have identified a number of parasites from EUS-affected fish. Subasinghe (1993) demonstrated an association between the parasitic load of *Trichodina* sp. and the susceptibility of *Channa striata* to EUS infection. It is possible that the physical presence and feeding mechanism of these parasites can cause skin rupture, and might facilitate infection by the EUS fungus.

Wild fisheries sites

Rivers had the lowest prevalence of EUS out of the wild fisheries sites sampled in this study. This may be due to the lotic characteristics of rivers, which may lessen the chances of the fungal pathogen attaching to fish. Haors and beels showed the highest prevalence of EUS. A haor is a large natural depression, lower than the adjacent floodplains. It functions as a small internal drainage basin and receives upland runoff water (Khan, 1997). Chemicals, waste and pathogens may enter the haor through the river systems. At the onset of the dry season, the water level of the haors decrease and the aquatic animals and plants are concentrated, often resulting in stressful conditions for fishes. The presence of a wide range of EUS susceptible fishes under these circumstances make haors high-risk areas for EUS outbreaks. Similarly, fishes are concentrated in beels, or flood plain lakes, during the dry winter season providing a similar high-risk environment. Particular beels are presently being developed as over-wintering fish sanctuaries, but the risk of EUS has been an important consideration in site selection (FAP6, 1994).

There have been concerns that the artificial stocking of natural water bodies in Bangladesh provides a risk for disease transmission, but in the case of EUS, no association was found in the present study. Subasinghe and Hossain (1997) studied EUS in three floodplain areas in Bangladesh and showed that disease prevalence was generally lower in artificially stocked fish sampled from the natural water bodies, than in wild fish. Given the widespread prevalence of EUS in Bangladesh, it is unlikely that stock enhancement under present conditions would increase the risk of EUS in the water body.

Acknowledgements

Grateful thanks to Dr K. Morgan, Liverpool University, for assistance with the study design and Dr C. Baldock, AusVet, for commenting on a draft of this paper. The work was supported by a grant from the Department for International Development of the UK (DFID) and the British Council.

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Fig.1 Frequency distribution of EUS at fish farm sites

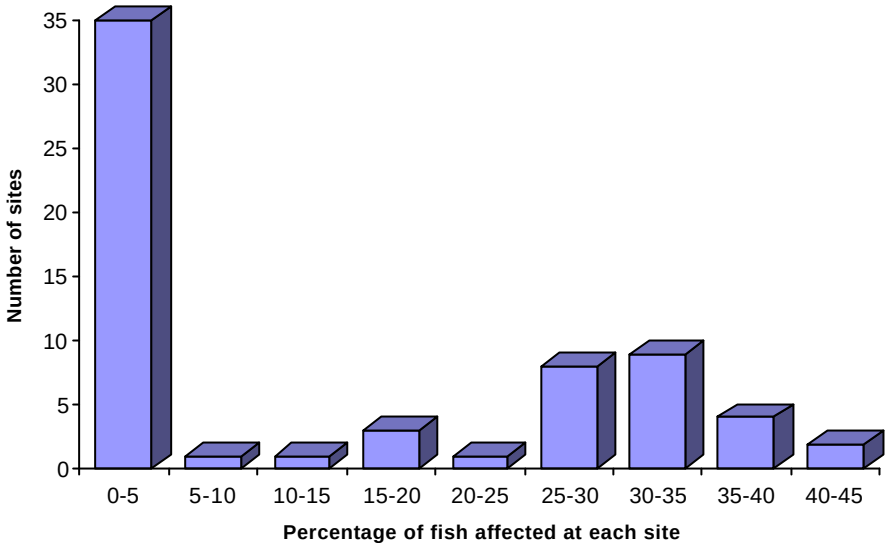

Fig.2 Frequency distribution of EUS at wild fisheries sites

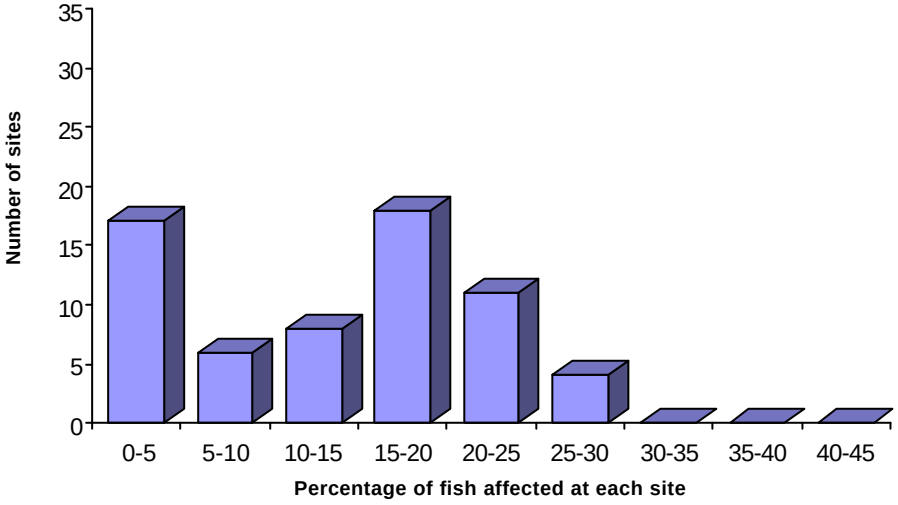

Table 1. Variables affecting relative risk (RR) of EUS in fish farming sites, giving 95% confidence limits (lower < RR < upper)

Variables	Relative risk RR	Lower limit	Upper limit
POND CONNECTION			
Wild fish observed in ponds*	10.09	1.62	74.27
Pond embankment not high enough to prevent entry of outside water	2.56	1.61	4.00
Holes observed in the pond bank	1.79	1.08	2.98
Pond connected to other water body through drain	2.63	1.69	4.08
Other water body = ricefield	2.73	1.81	4.13
Other water body = ditch	2.26	1.70	2.99
Other water body = beel	2.21	1.68	2.91
Water supply = only rainfed	0.52	0.31	0.88
Water supply = underground	0.91	0.49	1.71
Water supply = ricefield water	2.55	1.84	3.53
Pond is close to other water body	1.84	0.94	3.62
Floodwater entered the pond during the previous rainy season	2.33	1.27	4.29
PRE-STOCKING POND PREPARATION			
Pond was not dried	2.44	1.09	5.56
Bottom mud was not removed	5.88	0.92	33.33
Pond was not limed	2.38	1.20	4.76
Pond was not fertilized	2.00	0.83	4.76
POST-STOCKING MANAGEMENT			
Pond is not limed	2.17	1.41	3.33
Pond is not fertilized	1.08	0.46	2.50
Compost is prepared in another water body before applying to pond	1.43	0.28	7.14
WATER COLOUR, RELATIVE TO REDDISH PONDS**			
Black water colour (high organic debris)	2.75	1.26	6.01
Transparent pond water	1.38	0.28	6.75
Greenish water colour (phytoplankton)	1.24	0.54	2.87
HYGIENE/HEALTH			
Fry source water released in pond	2.00	1.09	3.66
Cattle wash or drink at the pond	2.90	1.46	5.77
Farm nets are not dried/disinfected	1.69	0.99	2.86
Buyers do not use dried/disinfected nets	7.14	1.11	50.00
Parasites observed on fish	2.65	1.45	4.86
EUS occurred in the previous season	3.00	1.64	5.49

RR > 1, indicates the factor is a putative causal factor of EUS
RR = 1, indicates no association exists between the factor and EUS
RR < 1, indicates the factor as a sparing factor for EUS

*62 farmers answered this question
**the lowest RR of EUS was in ponds with reddish water colour (0.74), therefore RR given here are calculated in respect to RR in red ponds

Table 2. Variables affecting relative risk (RR) of EUS in wild fishery sites, giving 95% confidence limits (lower < RR < upper)

Variable	Relative risk RR	Lower limit	Upper limit
TYPE OF HABITAT, RELATIVE TO RIVERS*			
Floodplain	1.25	0.41	3.77
Ricefield	1.88	0.73	4.83
Beel**	2.03	0.93	4.40
Haor***	2.50	1.17	5.34
STOCKING			
Water body is artificially stocked	1.08	0.81	1.44
HEALTH			
EUS occurred in the previous season	2.19	1.17	4.11

RR > 1, indicates the factor is a putative causal factor of EUS
RR = 1, indicates no association exists between the factor and EUS
RR < 1, indicates the factor as a sparing factor for EUS
*the lowest RR of EUS was in rivers (0.48), therefore RR given here are calculated in respect to RR in rivers
**floodplain lake
*** largest natural depression between two or more rivers

APPENDIX TWENTY SIX

Paper nineteen - Khan, M.H., Lilley, J.H., Corsin, F., Majumder, B., Sarker, M.G.A., Alauddin, M., Hoque, M.A., Ahmed, G.U., Chowdhury, M.B.R., Morgan, K. and Chinabut, S. (2001) Risk factors associated with epizootic ulcerative syndrome (EUS) in Bangladesh. Diseases of Aquatic Organisms. (in preparation, photographs from study shown, hard copy of figures available from ARP Manager)

CROSS-SECTIONAL STUDY OF FARMED AND FISHING SITES IN BANGLADESH

A

A. Pre-testing farmer interview

B

B. Pre-testing fish sampling on farm sites

C

C. Pre-testing fisherman interview

D

D. Pre-testing fish sampling at wild fishery sites

APPENDIX TWENTY SEVEN

Paper twenty - Lilley, J.H., Khan, M.H., Corsin, F., Hoque, M.A., Ahmed, G.U., Chowdhury, M.B.R., Morgan, K. and Chinabut, S. (2001) Case-control study of epizootic ulcerative syndrome (EUS) in Mymensingh, Bangladesh. Journal of Fish Diseases. (in preparation, selected results shown)

CASE-CONTROL STUDY RESULTS – POND-LEVEL STUDY

Rows in bold indicate a significant difference between cases and controls
An odds ratio (OR) of 1represents no association. OR>1 indicates that the exposure variable is a putative risk factor for EUS, and an OR<1 indicates it is a sparing factor against EUS

Variable	Case ponds (containing 1+ EUS affected fish)	Control ponds (no EUS affected fish in 100 sampled)	Statistic	P value	Odds ratio
Ponds	50	50			
Date	**Nov-98 – 2%** **Dec-98 – 8%** **Jan-99 – 24%** **Feb-99 – 28%** **Mar-99 – 2%** **Dec-99 – 16%** **Jan-00 – 18%** **Feb-00 – 2%**	**Nov-98 – 2%** **Dec-98 – 6%** **Jan-99 – 20%** **Feb-99 – 22%** **Mar-99 – 0%** **Dec-99 – 6%** **Jan-00 – 10%** **Feb-00 – 34%**	**Chi square 19.32**	**0.007**	
Time	09:00 - 09:29 - 10% 09:30 - 09:59 – 6% 10:00 - 10:29 – 26% 10:30 - 10:59 – 10% 11:00 - 11:29 – 30% 11:30 - 11:59 – 2% 12:00 - 12:29 – 16% 14:00 – 0%	09:00 - 09:29 – 10% 09:30 - 09:59 – 2% 10:00 - 10:29 – 34% 10:30 - 10:59 – 20% 11:00 - 11:29 – 20% 11:30 - 11:59 – 4% 12:00 - 12:29 – 6% 14:00 – 4%	An expected value is < 5. Chi square not valid.		
Thana	Mymensingh S. – 90% Phulpur – 2% Phulbaria – 0% Gouripur - 4% Tangail Sadar – 2% Kisher – 2%	Mymensingh S. – 84% Phulpur – 2% Phulbaria – 4% Gouripur – 8% Tangail Sadar – 2% Kisher – 0%	An expected value is < 5. Chi square not valid		
Pond type	Beel – 4% Floodplain – 2% Pond – 92% Ricefield – 2%	Beel – 0% Floodplain – 0% Pond – 100% Ricefield – 0%	An expected value is < 5. Chi square not valid		
Soil	Clay – 44% Loam – 30% Sand – 26% Sandy clay – 0%	Clay – 56% Loam – 28% Sand – 14% Sandy clay – 2%	An expected value is < 5. Chi square not valid		
Soil (with sandy clay deleted)	Clay – 45% Loam – 29% Sand – 27%	Clay – 57% Loam – 29% Sand – 14%	Chi squared 2.52	0.284	
Size (hectares)	mean – 0.47 max – 8.00 min – 0.01	mean – 0.09 max – 0.40 min – 0.01	Kruskal-Wallis H 1.749	0.186	
Size (categorical)	0.008 - 0.039 – 24% 0.04 - 0.19 – 56% 0.2 – 8 – 20%	0.008 - 0.039 – 36% 0.04 - 0.19 – 44% 0.2 – 8 – 20%	Chi square 1.92	0.383	
Depth (metres)	**mean – 0.98** **max – 1.70** **min – 0.50**	**mean – 1.27** **max – 2.20** **min – 0.80**	**T-test 4.746**	**0.000**	
Depth (categorical)	**0.5 - 0.99 – 54%** **1 - 1.49 – 38%** **1.5 - 2.2 – 8%**	**0.5 - 0.99 – 16%** **1 - 1.49 – 58%** **1.5 - 2.2 – 26%**	**Chi square 17.16**	**0.000**	
Stocking density (x100 per hectare)	mean – 97 min – 10 max – 700	mean – 62 min – 4 max – 250	Kruskal-Wallis H 3.799	0.051	
Stocking density (categorical)	4 – 20 – 8% 21 – 99 – 66% 100 – 700 – 26%	4 – 20 – 18% 21 – 99 – 68% 100 – 700 – 14%	Chi square 3.74	0.154	
Water source	**rain only – 23%** **ricefield – 73%** **underground – 5%**	**rain only – 66%** **ricefield – 10%** **underground – 24%**	**Chi square 38.92**	**0.000**	
Outbreak	at sample site only– 4% in local area – 16% a national problem – 80%				

Variable	Case ponds (containing 1+ EUS affected fish)	Control ponds (no EUS affected fish in 100 sampled)	Statistic	P value	Odds ratio
Start of outbreak	Nov 98 - 24% Dec 98 – 22% Jan 99 – 16% Oct 99 – 2% Nov 99 – 14% Dec 99 – 16% Jan 00 – 6%				
Loss (% fish)	mean – 25.2% max – 80% min – 3%				
Loss (Taka)	mean – 3251 Taka max – 24000 Taka min – 220 Taka				
Unusual temperature?	Yes – 96% No – 4%				
Unusual rainfall?	Yes – 98% No – 2%				
Fish introductions?	Yes – 9% No – 91%				
History of EUS?	**70%**	**43%**	**Yates corrected 5.83**	**0.016**	**3.03**
Treatments used	Lime – 16% Lime and KmnO4 – 16% Lime and salt – 11% Ash – 5%	Lime – 0% Lime and KmnO4 – 0% Lime and salt – 3% Ash – 0%			
Pre-stock liming	**20%**	**69%**	**Yates corrected 15.04**	**0.000**	**0.17**
Did pond flood?	**62%**	**6%**	**Yates corrected 28.17**	**0.000**	**25**
Connection to wild water	**66%**	**4%**	**Yates corrected 38.72**	**0.000**	**50**
Temperature	mean – 21.7 min – 17.4 max – 26.3	mean – 21.8 min – 18.5 max – 25.2	Kruskal-Wallis H 0.261	0.610	
Turbidity	mean – 20.8 min – 7.0 max – 49.0	mean – 21.4 min – 10.0 max – 48.0	Kruskal-Wallis H 0.362	0.547	
Conductivity	mean – 0.508 min – 0.010 max – 0.990	mean – 0.485 min – 0.026 max – 1.540	Kruskal-Wallis H 1.506	0.220	
Salinity	mean – 0.8 min – 0.4 max – 0.9	mean – 0.8 min – 0.5 max – 1.3	Kruskal-Wallis H 1.107	0.293	
pH	mean – 7.3 min – 5.6 max – 8.7	mean – 7.4 min – 6.8 max – 8.2	Kruskal-Wallis H 2.042	0.153	
DO	mean – 3.7 min – 0.8 max – 8.0	mean – 3.8 min – 1.6 max – 6.7	t-test 0.459	0.647	
Alkalinity	mean – 86.6 min – 16.0 max – 217.0	mean – 105.6 min – 32.0 max – 272.0	Kruskal-Wallis H 3.019	0.082	
Hardness	mean – 76.4 min – 14.0 max – 175.0	mean – 92.9 min – 21.0 max – 300.0	Kruskal-Wallis H 2.167	0.141	
Ammonia	**mean – 1.3 min – 0.1 max – 3.2**	**mean – 0.7 min – 0.1 max – 3.6**	**Kruskal-Wallis H 6.841**	**0.009**	

Variable	Case ponds (containing 1+ EUS affected fish)	Control ponds (no EUS affected fish in 100 sampled)	Statistic	P value	Odds ratio
Labeo rohita	74%	84%	Yates corrected 0.96	0.326	0.543
Cirrhinus cirrhosus	72%	80%	Yates corrected 0.49	0.482	0.641
Puntius sophore	**70%**	**30%**	**Yates corrected 14.44**	**0.000**	**5.556**
Catla catla	64%	74%	Yates corrected 0.75	0.387	0.625
Barbodes gonionotus	42%	32%	Yates corrected 0.69	0.407	
Channa punctata	**30%**	**2%**	**Yates corrected 12.57**	**0.000**	**20**
Glossogobius giuris	24%	12%	Yates corrected 1.58	0.209	2.273
Trichogaster chuna	18%	4%	Yates corrected 3.55	0.060	5.26
Labeo gonius	14%	4%	Fisher exact	0.160	
Amblypharyngodon mola	12%	4%	Fisher exact	0.269	3.226
Anabas testudineus	12%	4%	Fisher exact	0.269	3.226
Puntius ticto	10%	2%	Fisher exact	0.204	5.44
Mystus tengara	10%	0%	Fisher exact	0.056	
Cirrhinus ariza	8%	0%	Fisher exact	0.117	
Macrognathus pancalus	8%	0%	Fisher exact	0.117	
Ambassis ranga	8%	0%	Fisher exact	0.117	
Ambassis nama	8%	2%	Fisher exact	0.362	
Morulius calbasu	6%	2%	Fisher exact	0.617	3.125
Colisa fasciatus	6%	0%	Fisher exact	0.242	
Macrognathus aculeatus	6%	0%	Fisher exact	0.242	
Mystus vittatus	6%	0%	Fisher exact	0.242	
Xenentodon cancila	6%	0%	Fisher exact	0.242	
Hypophthalmichthys molitrix	4%	2%	Fisher exact	1.000	
Ctenopharyngodon idellus	4%	4%	Fisher exact	1.000	
Clarias gariepinus	4%	0%	Fisher exact	0.495	
Heteropneustes fossilis	4%	0%	Fisher exact	0.475	
Channa orientalis	4%	0%	Fisher exact	0.495	
Lepidocephalichthys guntea	4%	0%	Fisher exact	0.495	
Clarias batrachus	2%	0%	Fisher exact	1.000	
Clarias hybrid	2%	0%	Fisher exact	1.000	
Channa striata	2%	0%	Fisher exact	1.000	
Mastacembelus armatus	2%	0%	Fisher exact	1.000	
Nandus nandus	2%	0%	Fisher exact	1.000	
Badis badis	2%	0%	Fisher exact		
Pangasius suchi	2%	0%	Fisher exact		
Tetraodon fluviatilis	2%	0%	Fisher exact		
Monopterus cuchia	2%	0%	Fisher exact		
Wallago attu	2%	0%	Fisher exact		
Common carp	0%	0%	Chi squared	1.000	
Mirror carp	0%	0%	Chi squared	1.000	
Channa marulius	0%	0%	Chi squared	1.000	
Lepidocephalus spp.	0%	0%	Chi squared	1.000	

CASE-CONTROL STUDY RESULTS – FISH-LEVEL STUDY

Rows in bold indicate a significant difference between cases and controls
An odds ratio (OR) of 1represents no association. OR>1 indicates that the exposure variable is a putative risk factor for EUS, and an OR<1 indicates it is a sparing factor against EUS

Variable	Case fish (with lesions)	Control fish (without lesions)	Statistic	P value	Odds ratio
Ponds	50	50			
Fish	250	250			
EUS granulomas	69% of 75 fish sampled	-			
Fish length (cm)	**mean – 10.4 min – 3.0 max – 56.0**	**mean – 8.4 min – 3.1 max – 19.0**	**Kruskal-Wallis H 8.751**	**0.003**	
Severity of lesions	low = 3% medium = 85% high = 12%	-			
Caudal lesions	44%	-			
Lateral lesions	31%	-			
Dorsal lesions	24%	-			
Ventral lesions	24%	-			
Fin rot	8%	-			
Head lesions	6%	-			
Eye protrusion	3%	-			
Thin (malnutrition)	2%	-			
Fungus (external)	**83% of 250 fish**	**0% of 250 fish**	**Yates corrected**	**0.000**	**160**
Parasites (any type)	**34% of 250 fish**	**13% of 250 fish**	**Yates corrected 29.20**	**0.000**	**3.45**
***Trichodina* spp**	**9.6% of 250 fish**	**4% of 250 fish**	**Yates corrected 5.33**	**0.021**	**2.55**
Trichodina load	low = 87.5% high = 12.5%	low = 100%			
***Chilodonella* spp.**	**6% of 250 fish**	**1% of 250 fish**	**Yates corrected 6.09**	**0.014**	**4.88**
Chilodonella load	low = 100%	low = 100%			
Myxosporidean	2% of 250 fish	1% of 250 fish	Fisher exact	0.681	2.02
Myxosporidean load	low = 100%	low = 100%			
***Apiosoma* spp.**	**13% of 250 fish**	**0.4% of 250 fish**	**Yates corrected 26.96**	**0.000**	**33.95**
Apiosoma load	low = 39.4% medium = 36.4% high = 24.2%	low = 100%			
Epistylis spp.	0.4% of 250 fish	0% of 250 fish	Fisher exact	1.000	
Epistylis load	low = 100%				
Schyphidia spp.	1% of 250 fish	0% of 250 fish	Fisher exact	0.499	
Schyphidia load	low = 100%	-			
Dactylogyrus spp.	1% of 250 fish	1% of 250 fish	Fisher exact	1.000	0.66
Dactylogyrus load	low = 100%	low = 100%			
Gyrodactylus spp.	4% of 250 fish	3% of 250 fish	Yates corrected 0.52	0.471	1.60
Gyrodactylus load	low = 100%	low = 100%			
Monogeneans	1% of 250 fish	1% of 250 fish	Fisher exact	1.000	0.66
Monogeneans load	low = 100%	low = 100%			
Piscicola spp.	1% of 250 fish	0% of 250 fish	Fisher exact	0.499	
Piscicola load	low = 100%	-			
Lernaea spp.	2% of 250 fish	1% of 250 fish	Fisher exact	0.285	3.05
Lernaea load	low = 100%	low = 100%			
Argulus spp.	1% of 250 fish	1% of 250 fish	Fisher exact	0.623	0.33
Argulus load	low = 100%	low = 100%			
Ergasilus spp.	0% of 250 fish	0.4% of 250 fish	Fisher exact	1.000	0.00
Ergasilus load	-	low = 100%			

Variable	Case fish (with lesions)	Control fish (without lesions)	Statistic	P value	Odds ratio
***Aeromonas* spp.**	**82% of 150 fish**	**21% of 150 fish**	**Yates corrected 110.49**	**0.000**	**17.49**
A. hydrophila	27% of 49 fish with *Aeromonas*	41% of 26 fish with *Aeromonas*	Yates corrected 0.87	0.350	0.52
A. veronii biovar *veronii*	18% of 49 fish with *Aeromonas*	27% of 26 fish with *Aeromonas*	Yates corrected 0.29	0.592	0.60
A. sobria biovar *sobria*	37% of 49 fish with *Aeromonas*	18% of 26 fish with *Aeromonas*	Yates corrected 1.65	0.200	2.61
A. caviae	0% of 49 fish with *Aeromonas*	0% of 26 fish with *Aeromonas*			
A. schuberti	10% of 49 fish and *Aeromonas*	14% of 26 fish with *Aeromonas*	Fisher exact	0.700	0.72
A. jandaei	8% of 49 fish with *Aeromonas*	0% of 26 fish with *Aeromonas*	Fisher exact	0.300	

APPENDIX TWENTY EIGHT

Overview of Nepal cross-sectional study (hard copy of figures available from ARP Manager)

USING EPIDEMIOLOGY TO LINK FARMERS AND LABORATORY STAFF IN THE STUDY OF FISH DISEASE IN NEPAL

Background

- Epizootic ulcerative syndrome (EUS) is considered the most important fish disease affecting culture and capture fisheries in Nepal.
- There remains a fear of EUS outbreaks among fish farmers, which may limit further development of the aquaculture sector in Nepal.
- Staff at the Fisheries Development Division (FDD) Nepal have trained at the Aquatic Animal Health Research Institute (AAHRI), Bangkok in histological techniques for the diagnosis of EUS. FDD also has a laboratory with facilities for diagnosing fish disease.

Objectives

- To measure risk factors for EUS in village carp polyculture grow-out ponds in the Terai area of Nepal, with the aim of providing recommendations for control.
- To provide quantitative data on the prevalence and geographical distribution of EUS in the Terai area of Nepal.
- To assess the current livelihood conditions of participating farmers and identify potential impacts of any fish health problems on the farmers

Pre-testing questionnaire and interview technique (above) and fish sampling technique (right) in Janakpur, southern Nepal

Study design

- A cross-sectional survey of 60 randomly-selected polyculture ponds in 10 Terai districts will be conducted between October – December 2000.
- The survey will involve a semi-structured interview of key informants, and examination of 100 fish, at each site. Diseased fish will be histological processed at FDD and diagnosed EUS+ve or EUS-ve.
- The questionnaire and fish disease data will be compared to determine which factors pose significant risks for EUS outbreaks in Nepal. Data will also provide information on prevalence of EUS, and livelihood conditions of affected farmers.

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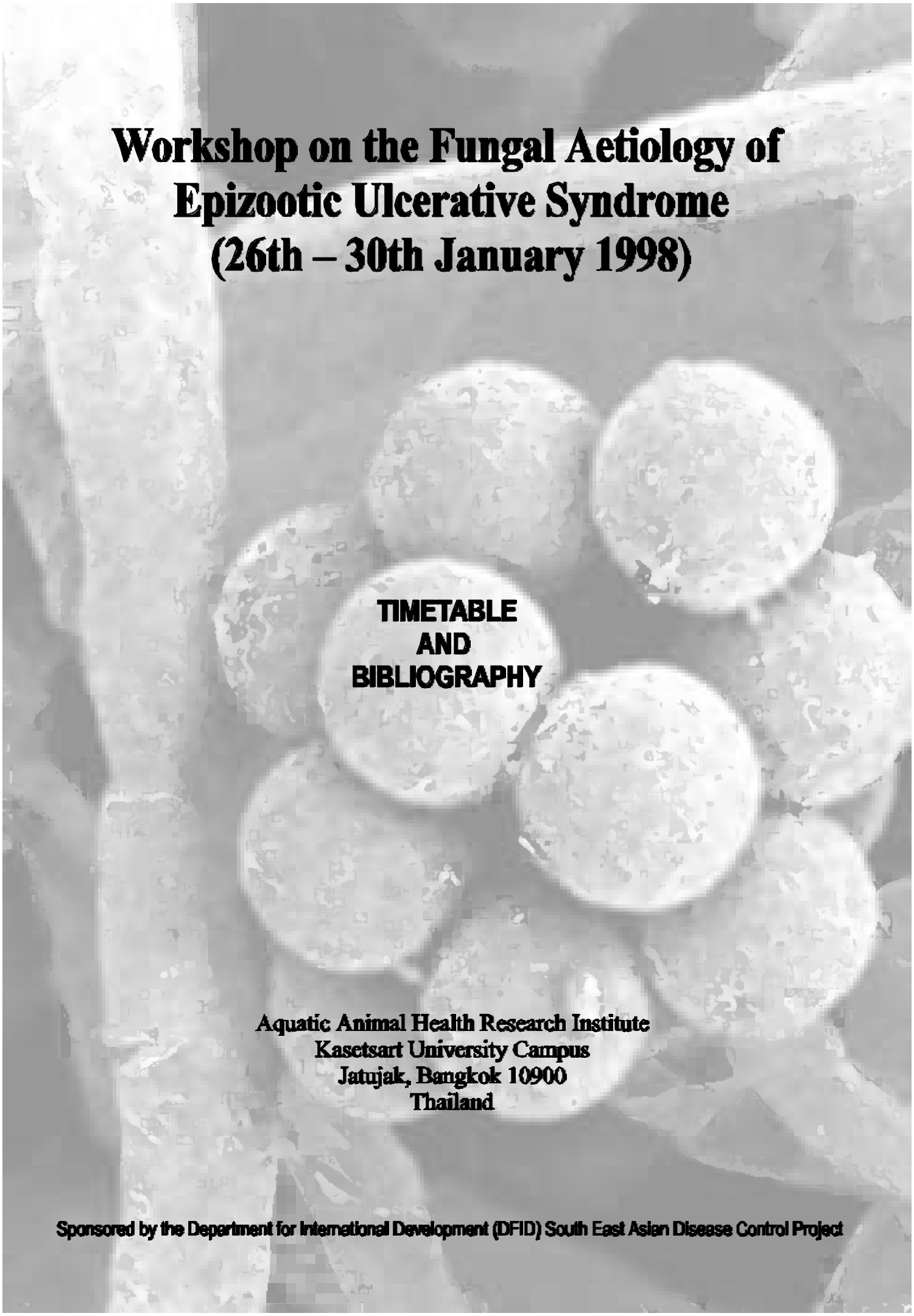
This joint research study was organised using funds from the Department for International Development (DFID) Aquaculture Research Programme (ARP) EUS Project

APPENDIX TWENTY NINE

Workbook from a Workshop on the Fungal Aetiology of EUS, AAHRI, Thailand, 26th - 30th January 1998 (first seven pages)

Workshop on the Fungal Aetiology of Epizootic Ulcerative Syndrome (26th – 30th January 1998)



TIMETABLE AND BIBLIOGRAPHY

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Kasetsart University Campus
Jatujak, Bangkok 10900
Thailand

Sponsored by the Department for International Development (DFID) South East Asian Disease Control Project

CONTENTS

TIMETABLE

Mon 26th Jan	08:30	Registration
	09:00	Opening Ceremony Tour of AAHRI
	10:30	Coffee
	11:00	Lecture - EUS and its spread across Asia (SC)
	12:00	Lecture - History of research into causative agents of EUS (JL)
	13:00	Lunch
	14:00	Discussion - Present status of EUS in participant's countries
	15:30	Coffee
	16:00	Discussion - Present status of EUS in participant's countries
Tues 27th Jan	09:00	Lecture - Fungal aetiology of EUS (JL)
	10:30	Coffee
	11:00	Practical - Isolation of *Aphanomyces invadans* from fish (JL/RC) Maintenance of laboratory cultures of *A. invadans* (JL/RC)
	12:30	Lunch
	14:00	Practical - Isolation of *A. invadans* from water (JL/RC)
	15:30	Coffee
	16:00	Practical - Sporulation of Oomycete fungi (JL/RC)
Wed 28th Jan	09:00	Lecture - Identification of *A. invadans* and other Oomycete fungi (JL)
	10:30	Coffee
	11:00	Practical - Identification of Oomycete fungi (JL/RC)
	12:30	Lunch
	14:00	Lecture - Histopathology of EUS (SC)
	15:30	Coffee
	16:00	Practical - Examination of EUS histology, including slides prepared from samples brought by participants (SC/JL)
Thurs 29th Jan	09:00	Lecture - Control/treatment of EUS (RC)
	10:00	Coffee
	10:30	Practical - Susceptibility of fungi to chemical treatment (RC/JL)
	12:30	Lunch
	14:00	Lecture - Significance of water quality parameters in EUS outbreaks (MP)
	15:30	Coffee
	16:00	Practical - Measuring water quality parameters (MP)
Fri 30th Jan	09:00	Lecture - Aspects of fungal taxonomy (TF)
	10:30	Coffee
	11:00	Discussion - EUS epidemiology and outbreak investigations (JL)
	12:30	Lunch
	14:00	Discussion - Identification of future research priorities (SC/SK/JL/RC/MP)
	15:30	Coffee
	16:00	Practical - Final examination of fungal isolates & treatment assays (JL/RC)
	18:00	Mahruay Hotel - Dinner and certificate presentation

SC – Dr Supranee Chinabut (AAHRI, Bangkok)
JL – Dr Jim Lilley (Institute of Aquaculture, Stirling University, UK)
RC – Dr Ruth Campbell (Institute of Aquaculture, Stirling University, UK)
MP – Dr Michael Phillips (Network of Aquaculture Centres in Asia-Pacific, Bangkok)
TF - Prof Tim Flegel (National Centre for Genetic Engineering & Biotechnology, Bangkok)

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LECTURE SUMMARIES

Monday 26th

EUS and its spread across Asia (a.m. SC)

Reference: Chapters 1-4 in "EUS Technical Handbook"
The presentation will provide an overview of the main EUS outbreaks during its spread across Asia, the species affected, socio-economic impacts, and aspects of public health. EUS is now known to be the same disease as mycotic granulomatosis (MG) in Japan and redspot disease (RSD) in Australia, and reference to these outbreaks will also be made.

History of research into causative agents of EUS (a.m. JL)

References: Chapter 5 in "EUS Technical Handbook"
The main fungal, viral, bacterial and parasitic agents associated with EUS outbreaks will be discussed in the context of the "Causal Web" diagram (Figure 3 of the "EUS Technical Handbook")

Tuesday 27th

Fungal aetiology of EUS (a.m. JL)

References: Willoughby's "Fungi & Fish Diseases"
Lilley *et al* (1997) Vet. Rec. 140, 11-12
An introduction to the most important group of fish-pathogenic fungi (family Saprolegniaceae, class Oomycetes) will be given, followed by a review of work on the saprolegniacean species associated with EUS (i.e. *Aphanomyces invadans*).

Wednesday 28th

Identification of *A. invadans* and other Oomycete fungi (a.m. JL)

References: Chapter 5 (Fungi) in "EUS Technical Handbook"
Annexes 4-5 in "EUS Technical Handbook"
Willoughby's "Fungi & Fish Diseases"
Oomycete genera are distinguished primarily by their asexual reproductive structures, and fungi are usually identified to the species level on the basis of sexual characters. A short video will be shown highlighting the features of the main saprolegniacean genera (*Aphanomyces, Achlya* and *Saprolegnia*). The EUS pathogen (*A. invadans*) does not, however, produce sexual stages in culture and other means of identification will be discussed.

Histopathology of EUS (p.m. SC)

References: Chapter 7 in "EUS Technical Handbook"
Chinabut *et al* (1995) J. Fish Dis. 18, 41-47
Viswanath *et al* (1997) J. Aqua. Trop. 12, 35-42
Histological identification of distinctive mycotic granulomas, formed by the growth of *A. invadans* through internal fish tissues, is the primary means of diagnosing EUS. Fungal invasion and its associated pathology will be discussed in terms of fish species affected and temperature of infection.

Thursday 29th

Control/treatment of EUS (a.m. RC)

References: Chapter 9 in "EUS technical Handbook"
Ahmed & Rab (1995) J. Fish Dis. 18, 263-271
Lilley & Inglis (1997) Aqua. Res. 28, 461-469
Control of EUS in natural waterways is almost impossible save for prevention of the spread of *A. invadans* to new areas. Strategies for the control of EUS in aquaculture systems include treatment or exclusion of fungal spores in the water, modification of environmental conditions (especially temperature, salinity, acidity), reduction of stress to

the fish, and farming of non-susceptible species. As yet, it is not possible to treat the fungus once it is growing within the fish.

Significance of water quality parameters in EUS outbreaks (p.m. MP)

Reference: Chapter 6 in "EUS Technical Handbook"

EUS outbreaks tend to occur seasonally, in most countries that is during the cool season and after the monsoon season. This is due to the particular environmental conditions occurring during these times. The main water quality parameters that increase the likelihood of EUS outbreaks are: low temperature, low salinity and low pH. Low temperature is also associated with other saprolegniacean infections of fish (e.g. winter saprolegniosis of channel catfish).

Friday 30th

Aspects of fungal taxonomy (a.m. TF)

An overview of recent developments in protistan and fungal taxonomy is given.

EUS epidemiology and outbreak investigations (a.m. JL)

References: Chapter 8 in "EUS Technical Handbook"
Annexes 7 in "EUS Technical Handbook"

A wider, epidemiological, view of EUS causation is explained in terms of "component causes", "sufficient causes" and "necessary causes". The outbreak investigation procedure given in the handbook is discussed.

PRACTICAL SUMMARIES

Tuesday 27th

1. Isolation of *Aphanomyces invadans* from fish (a.m. JL/RC)

Follow the procedure given in Annex 1 of "EUS Technical Handbook". Any fungus growing on plates will be successively subcultured over the next few days to obtain axenic colonies.

2. Maintenance of laboratory cultures of *A. invadans* (a.m. JL/RC)

Techniques given in Annex 3 of "EUS Technical Handbook" will be demonstrated.

3. Isolation of *A. invadans* from water (p.m. JL/RC)

Follow the procedure given in Annex 2 of "EUS Technical Handbook". Spores will be grown from:
(i) Fish-challenge tank water (this will simulate water from an active outbreak)
(ii) Positive control 1: fish-challenge tank water "seeded" with *A. invadans* zoospores
(iii) Positive control 2: APW "seeded" with *A. invadans* zoospores

4. Sporulation of Oomycete fungi (p.m. JL/RC)

You have seven cultures of saprolegniacean fungi growing in GPY broth (labelled A-G).
(i) Wash the nutrients out of the fungi as described in Annex 4 "EUS Technical Handbook". This will induce the production of zoosporangia in the fungi overnight. Tomorrow we will identify each culture according to its sporulation characteristics.
(ii) Also subculture fungi on to GPY agar plates and incubate at 22^{o}C to compare growth rates over the next few days.

Wednesday 28th

5. Identification of Oomycete fungi (a.m. JL/RC)

Identify each culture of saprolegniacean fungus (A-F) under the microscope using features described in Annex 5 of "EUS Technical Handbook" and illustrated in Figures 1-6 of Willoughby's "Fungi & Fish Diseases".

6. Examination of EUS histology, including slides prepared from samples brought by participants (p.m. SC/JL)

Examine slides prepared by the histology unit at AAHRI of any fish samples you have brought to determine whether the fish were suffering from EUS.

Thursday 29th

7. Susceptibility of fungi to chemical treatment (a.m. RC/JL)

You have been given a fungal culture, either *Aphanomyces invadans* or *Saprolegnia* sp. Using a cork borer, transfer agar plugs into each chemical solution in the Replidish. Leave for one hour. After three 10-minute washes in sterile distilled water, dry the plugs on sterile filter paper, and transfer onto maintenance agar. Cultures will be examined for growth inhibition tomorrow.

8. Measuring water quality parameters (p.m. MP/SK)

Use of standard equipment to measure water quality variables will be demonstrated.

Friday 30th

9. Final examination of fungal isolates & treatment assays (p.m. JL/RC)

Final examination of:
Putative *A invadans*. isolates obtained from fish (Prac. 1)
Putative *A. invadans* isolates obtained from water (Prac. 3), calculation of spore densities
Growth of fungal cultures exposed to treatments, compared to controls (Prac. 7)

APPENDIX THIRTY

Callinan, R.B., Chinabut, S., Mohan, C.V. and Lilley, J.H. (1999) Report of EUS Extension Visits to Nepal, India and Sri Lanka. 7-20 June 1999. ACIAR and DFID. 32 pp (pdf or hard copy available from ARP Manager)

Report of EUS Extension Visits to Nepal, India and Sri Lanka
7-20 June 1999

Callinan, R.B.; Chinabut, S.; Mohan, C.V. and Lilley, J.H.

Australian Centre for International Agricultural Research (ACIAR)
Department for International Development of the UK (DFID)
Aquatic Animal Health Research Institute (AAHRI)
College of Fisheries, Mangalore

EXECUTIVE SUMMARY

ACIAR and DFID jointly funded EUS (epizootic ulcerative syndrome) extension visits by an expert team between 7 - 20 June 1999 to the Fisheries Development Division, Ministry of Agriculture, Kathmandu, Nepal; the Central Inland Capture Fisheries Research Institute (CICFRI), Barrackpore, India; the Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India; the Department of Aquatic Biology and Fisheries, University of Kerala, India; and the National Aquatic Resources Research and Development Agency (NARA), Colombo, Sri Lanka.

The team comprised Dr R.B. Callinan (NSW Fisheries), Dr S. Chinabut (AAHRI, Bangkok), Dr J.H. Lilley (IoA, University of Stirling, UK), and Dr C.V. Mohan (College of Fisheries, Mangalore, India). The countries visited were selected on the following bases:

1. EUS continues to be a major problem;
2. information on recent advances in EUS research may not readily accessible;
3. there is sufficient scientific expertise and infrastructure, as well as extension capability, to utilise the information effectively.

The objectives of the visits were:

1. Through structured workshops, to ensure the combined research benefits resulting from the ACIAR, AAHRI and DFID programmes on EUS are embedded in key institutes in India, Nepal and Sri Lanka.
2. Through detailed consultation with scientists and extension officers at these institutes, to develop response strategies to control and prevent EUS outbreaks appropriate to the circumstances in each country.

Accordingly, structured, one-day seminar and discussion sessions were organised in each of the centres visited. In Kathmandu and Colombo, participants from all over the respective countries attended, and in Trivandrum, participants from other states were present. 200 EUS Technical Manuals and 100 Histology Manuals were distributed and in some centres, requests were taken for further copies. Recommendations were formulated during discussion sessions in which further work would be undertaken at local centres and elsewhere, taking into account the presentations on the latest research findings. In some centres, particular joint research proposals were formulated.

In recent years, in the countries visited, EUS has been mainly a problem of wild fishes. Recommendations for the study of wild populations and dependent communities were formulated. With regards to cultured fish systems, recommendations for response strategies and further research focused mainly on Indian major carp culture. The importance of excluding potential sources of fungal entry (eg wild fish and water) was emphasised. Trials of potential, locally-available, prophylactic treatments for use over the EUS season were recommended. In some areas, where plans were underway for the development of aquaculture of other EUS-susceptible species, of the potential risks, and possible intervention measures were discussed.

Recommendations arising from the visits

1. Socio-economic impacts of EUS

Studies are required on the impact of EUS outbreaks on communities dependent on wild fishes, particularly in Kerala and Sri Lanka. Workers at CICFRI also identified the need to study the effect of EUS on fishers and fish farmers. In Nepal, a questionnaire survey is required to identify all the constraints to the development of aquaculture.

2. Impacts of EUS on wild fish populations

An assessment of available inland fish capture statistics in the Kerala backwaters, and other important severely affected fisheries, should be undertaken to identify impacts of EUS on susceptible fish populations.

3. Treatment studies

Tank and pond trials are required to evaluate present treatments, and other candidate safe, affordable, locally available compounds. The pond facilities at CIFA provide an ideal facility for such trials. Bioactive compounds and immunostimulants currently being studied in Kerala could be assessed as treatments for EUS.

4. Ecology and transmission of *Aphanomyces invadans*

Studies on the persistence of *A. invadans* in the environment, and investigations of carrier states in fish or other organisms, are required.

5. EUS determinants
Local causes of dermatitis that lead to EUS should be further examined in the light of the present understanding of EUS causation. Specific reference was made to the study of virological aspects of EUS.

6. Susceptibility of Indian major carps
Further work is required to demonstrate the relative susceptibility of Indian major carps, and any size/age effects on susceptibility. Other immunological aspects of EUS should also be studied, with particular reference to the role of mucus.

7. Resistant strains
The possibility that there are particular strains of susceptible fish that have higher resistance/tolerance to EUS should be investigated.

8. Infrastructure and training
A recommendation is made for a programme of improving laboratory facilities, increased training and establishing collaborative linkages in Nepal. A national Prospective Plan for Fisheries is currently being developed which will encompass fish health issues.

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ACRONYMS

AAHRI -	Aquatic Animal Health Research Institute, Bangkok, Thailand
ACIAR -	Australian Centre for International Agriculture Research
CIBA -	Central Institute for Brackishwater Aquaculture, Madras, India
CICFRI -	Central Inland Capture Fisheries Research Institute, Barrakpore, India
CIFA -	Central Institute of Freshwater Aquaculture, Bhubaneswar, India
CPE -	cytopathic effect
DFID -	Department for International Development, United Kingdom Government, formally ODA
EUS -	epizootic ulcerative syndrome
FAO -	Food and Agriculture Organisation of the United Nations
FFDA -	Fish Farm Development Agencies, India
ICAR -	Indian Council of Agricultural Research
ICSF -	International Collective in Support of Fishworkers, India
IoA -	Institute of Aquaculture, Stirling University, Scotland
MG -	mycotic granulomatosis
NACA -	Network of Aquaculture Centres in Asia and the Pacific, Bangkok, Thailand
NARA -	National Aquatic Resources Research and Development Agency, Colombo, Sri Lanka
NRCCWF -	National Research Centre on Cold Water Fisheries, India
NSW -	New South Wales, Australia
ODA -	Overseas Development Administration of the United Kingdom, present name DFID
OIE -	Office International des Epizooties
RSD -	redspot disease
UM -	ulcerative mycosis (menhaden disease)

ITINERARY

Mon 7 June	Dr Mohan travels Mangalore-Bombay, Bombay-Kathmandu Drs Callinan, Chinabut and Lilley travel Bangkok-Kathmandu TG319 10:30-12:35 Visit Central Fisheries Building, Fisheries Development Division, Ministry of Agriculture, Kathmandu, discussion of programme
Tues 8 June	Preparation of presentations
Wed 9 June	Seminar - Ministry of Agriculture, Kathmandu
Thur 10 June	Wrap-up meeting, Kathmandu Travel Kathmandu-Calcutta RA213 13:30-14:25
Fri 11 June	Seminar - Central Inland Capture Fisheries Research Institute (CICFRI), Barrackpore; discussions and tour of institute
Sat 12 June	Travel Calcutta-Bhubaneswar IC 966 10:15-11:10 Discussion of programme
Sun 13 June	Discussions and tour of institute Seminar - Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar
Mon 14 June	Wrap-up meeting, CIFA Travel Bhubaneswar-Bombay IC7141 16:35-18:40
Tues 15 June	Travel Bombay-Trivandrum IC167 08:40-10:35 Discussion of programme
Wed 16 June	Seminar - Department of Aquatic Biology and Fisheries, University of Kerala
Thur 17 June	Travel Trivandrum-Colombo UL162 09:40-11:05 Discussion of programme
Fri 18 June	Seminar - National Aquatic Resources Research and Development Agency (NARA), Colombo Discussions and wrap-up
Sat 19 Jun	Dr Mohan travels Colombo-Bombay UL141 11:40-13:40 Bombay-Mangalore IC2179 09:25-10:40
Sun 20 June	Report preparation
Mon 21 June	Drs Callinan, Chinabut and Lilley travel Colombo-Bangkok TG308 01:40-06:05

BACKGROUND

Since the early 1970s, outbreaks of epizootic ulcerative syndrome (EUS) have been reported in a wide variety of freshwater and estuarine fish in 18 countries in the Asia-Pacific region. The outbreaks are usually severe, seasonally recurrent and accompanied by high mortality rates. Affected fish typically have one or more extensive cutaneous ulcers.

Since 1992, ACIAR has funded several activities which have substantially improved our understanding of EUS and our ability to control and prevent outbreaks. These included Project 9130 ('Improving fish production in freshwater aquaculture and estuaries by reducing losses due to epizootic ulcerative syndrome'), which identified causes and control measures for EUS. In addition, ACIAR funded a 1996 Master Class ('Epidemiology in Tropical Aquaculture').

DFID has been involved in EUS research since the late 1980s, through collaborative projects between Stirling University and the Aquatic Animal Health Research Institute (AAHRI), Bangkok. AAHRI is currently the only OIE-approved laboratory for the diagnosis of EUS. In 1994 DFID sponsored a Regional Seminar on EUS in Bangkok that brought together most of the researchers on EUS and provided the first accepted case definition of the disease. DFID has also funded two workshops at AAHRI on EUS and fungal diseases of fishes, and a mission to Pakistan in 1998 to provide training and advise on EUS surveys. Recently, DFID project R5779 characterised the fungus associated with EUS, and the current DFID project R6979 is researching control strategies.

ACIAR and DFID have collaborated on several EUS-related activities. In 1997 both organisations supported a technical mission to Pakistan to advise on EUS control and prevention, and recently published a technical manual ('Epizootic Ulcerative Syndrome (EUS) Technical Handbook'). This handbook combines earlier information about EUS with the important body of new knowledge derived from several recently completed research projects, i.e. ACIAR Project 9130 and a number of collaborative DFID/AAHRI research projects on EUS.

Rationale for the present EUS extension visits

ACIAR Project 9130 cost $1.7 million dollars and it has been estimated, based on the most conservative of assumptions, that the project outcomes could yield net benefits (in present value terms) of $56 million (Impact Assessment Series No.7). These significant benefits are a result of the importance of fish production in Australia, Indonesia and the Philippines alone, both as a commercial crop and a source of subsistence income. Note that benefits accruing in EUS-affected countries such as Thailand, India and Bangladesh were not included in this estimate. These benefit estimates were based on the assumption that the knowledge obtained from the project would actually be adopted by fish producers and others. Furthermore, it was proposed in the Project 9130 Impact Assessment document that ACIAR could achieve high leverage from funds devoted to increasing rates of adoption. Distribution within the region of the recently published, ACIAR-funded, EUS technical manual will, in part, assist in this adoption. However, it was considered essential that knowledge derived from Project 9130, together with that derived from recent complementary research projects on EUS conducted by other agencies in the region, is adopted in a broad, sustained way in those countries where EUS remains a major problem. The visits, described in this report, by a small team of technical experts to key institutes and agencies in selected countries was organised to achieve this end.

Methodology

The team of EUS experts, comprising Dr R.B. Callinan (NSW Fisheries), Dr S. Chinabut (AAHRI, Bangkok), Dr J.H. Lilley (IoA, University of Stirling, UK), and Dr C.V. Mohan (College of Fisheries, Mangalore, India) visited India, Nepal and Sri Lanka. In each country, the team disseminated research and other benefits derived from the above ACIAR and DFID research project and activities. The three countries were selected using the following criteria:

a) EUS continues to be a major problem;
b) information on recent advances described above may not readily accessible;
c) there is sufficient scientific expertise and infrastructure, as well as extension capability, to utilise the information effectively.

To achieve the programme's objectives, the knowledge was disseminated through structured workshops covering all key aspects of EUS. Team members and relevant personnel at each institute gave oral presentations (see Appendix 2) detailing advances in knowledge, and providing EUS situation reports. Team members also

coordinated focused, interactive sessions designed to identify appropriate ways of controlling and preventing outbreaks, and to identify major research gaps.

Team members
Each team member has particular expertise appropriate to the objectives.

- Dr R.B. Callinan is Special Veterinary Research Officer (Fish Diseases), NSW Fisheries. He was project coordinator and principal researcher for Project 9130, which included a component in India. He has wide knowledge of the pathogenesis and epidemiology of EUS in Australia and in regional countries. He participated in the ACIAR/AAHRI/DFID/NACA EUS mission to Pakistan in 1997.
- Dr S. Chinabut is Senior Fish Pathologist at AAHRI, Bangkok. She is an authority on EUS, with special interests in its pathogenesis, pathology and histopathology. Dr Chinabut is currently collaborating with Dr Lilley in research at AAHRI on EUS control and prevention measures. She participated in the Consultation on EUS vis`a vis the Environment and the People (International Collective in Support of Fishworkers) in India in 1992 and in the ACIAR/AAHRI/DFID/NACA EUS mission to Pakistan in 1997.
- Dr J.H. Lilley is Research Scientist at University of Stirling, U.K. He is an authority on the causative fungus and is currently researching epidemiology of EUS in Bangladesh, as well as control and prevention measures in collaboration with AAHRI. He participated in the ACIAR/AAHRI/DFID/NACA EUS mission to Pakistan in 1997.
- Dr C.V. Mohan is Associate Professor (Fish Pathology) at College of Fisheries, University of Agricultural Sciences, Mangalore, India. He collaborated in Project 9130 and is an authority on pathology and epidemiology of EUS in India.

Given that AAHRI is the region's key centre for aquatic animal disease research, this institute is likely to retain an ongoing regional role in EUS research and, concurrently, to serve as a source of expertise and information on EUS. In this regard, Dr Chinabut may subsequently act as contact person for EUS-related research matters arising from the visits.

OBJECTIVES

The objectives of the visits were:

1. Through structured workshops, to ensure the combined research benefits resulting from the ACIAR, AAHRI and DFID programmes on EUS are embedded in key institutes in India, Nepal and Sri Lanka.

2. Through detailed consultation with scientists and extension officers at these institutes, to develop response strategies to control and prevent EUS outbreaks appropriate to the circumstances in each country.

REPORT OF ACTIVITIES

Mon 7 June — AM - Dr Mohan travels Mangalore-Bombay, Bombay-Kathmandu.
Drs Callinan, Chinabut and Lilley travel Bangkok-Kathmandu.

Visited Central Fisheries Building, Fisheries Development Division, Ministry of Agriculture, Kathmandu

Meeting with Dr Gopal Shrestha and Shankar Dahal, discussed EUS status and lecture programme.

It was noted that EUS was reported in February 1999 in *Cirrhina mrigala* in farms in the Terai and in wild *Channa punctata, Puntius* sp. and *Anguilla bengalensi.* Histological samples of these fish will be processed later this year.

Tour of facilities - these comprise of basic histopathology, microbiology, photo compound microscopes and water quality. A lack of access to latest scientific information was identified as an important problem.

Meeting with Dr Deep Swar, Chief, Fisheries Development Division. The current EUS situation was briefly discussed and Dr Swar talked about the fish health management needs of Nepal. It was agreed to include a wider consideration, extending beyond EUS and perhaps using it as an example, for a general fish health management programme. There is a farmer belief that all fish are susceptible to EUS, and that discourages them investing in aquaculture activities.

Tues 8 June — Preparation of seminar presentations.

Wed 9 June — **EUS seminar - The seminar hall, Ministry of Agriculture, Kathmandu**

Approximately 60 participants from 22 districts attended the seminar. Participants comprised of fisheries officers from Fisheries Centres and university lecturers. A list of participants is included in Appendix 1. 38 EUS Handbooks and 20 Histology manuals were distributed (Appendix 3). Requests were taken for a further 85 EUS Handbooks.

Mrs Ram Badan Pradhan, Director General, Department of Agriculture - Welcome Address

Dr Jagadish Chandra Gautam (Special Secretary, Ministry Agriculture) - Preliminary Address

Dr Gautam talked of the need to remove the psychological effect of EUS on aquaculture. Nepal has 100 million hectares of water surface but only 1-2% is utilised. A Prospective Plan for Fisheries is currently being developed and should be discussed with visiting team members. Fisheries development should involve the private sector and include coldwater fisheries, with particular emphasis on export markets and foreign investment. Fish health management, and specifically EUS, should be a priority. This should be addressed to ensure it does not become a big problem. In this regard there should be increased collaboration between institutes.

Mr Chakra Prasad Banstola, Minister of Agriculture - Inaugural Address

There is considerable opportunity for fish culture in Nepal. Food Security is an important issue. Various fish disease problems have been identified as present.

Dr Deep Bahadur Swar, Chief, Fisheries Development Division - Vote of thanks

Mr Dharami Man Singh, Chief, Inland Aquaculture & Fisheries Section - Vote of thanks

Dr Mukti Narayan Shrestha, First Secretary, Ministry of Agriculture - Chairman's remarks

Losses of up to 17.2% (NRs 30 million) have been recorded, due to EUS. The needs for addressing the problem include: training, improved disease investigation capability, diagnostic facilities, and formulating a National Fish Disease Control Plan. This Plan would include quarantine systems set up with OIE assistance. Hopes were expressed that the visiting team will come up with recommendations for the control of EUS.

Technical Session 1
(see Appendix 2 for summaries of lecture by visiting team members)

Dr Supranee Chinabut - Introduction and history
Rapporteurs: Mr Resham Raj Dhital, Mr Shankar Dahal

Dr K.G. Rajbanshi, Royal Nepal Academy of Science & Technology - Comments

Dr Gopal Shrestha - EUS in Nepal
Rapporteurs: Mr Bikash Chand Shrestha, Ms Ramola Ranjit
The Fisheries Development Division is responsible for fish disease issues in Nepal. The westwards spread of EUS in Nepal was described. Seventeen fish species have been affected. Liming with $Ca(OH)_2$ at 500kg/ha is the preferred treatment.
Recommendations:
1) Establishment of collaborative programmes
2) Identify treatments with low public health risk
3) Quarantine principles should be followed for international translocation of fish
4) Staff training, basic diagnostic equipment and water quality test kits required to support basic fish disease diagnosis at the provincial level
5) Regional coordination in exchange of information on aquatic animal health
6) A central, well-equipped laboratory should be developed

Dr CV Mohan - Pathology of EUS
Rapporteurs: Mr Resham Raj Dhital, Mr Shankar Dahal

Dr JH Lilley - Mycology of EUS
Rapporteurs: Mr Dharani Man Singh, Mr Shanokaji Pachhai

Dr RB Callinan - Causal factors
Rapporteurs: Mr Shivananda Yadav, Mr Ram Prasad Panta

Lunch

Technical session 2

Dr S Chinabut - Differential diagnosis

Dr JH Lilley - Control of EUS

Dr RB Callinan - Outbreak investigation

Comments from the audience:
There is a lack of expertise in disease diagnosis, control and prevention.
Training is needed in the selection and use of chemicals, and in the availability of chemicals. Indigenous chemicals should be used.
There was concern on public heath issues with regards to chemical use in aquaculture.
Combine research on fish disease with training in academic programmes (specifically Tribuhwan University Aquaculture MSc)
There is a lack of equipment at the central and provincial laboratories
Disease is a new subject for most fisheries workers. Only two senior staff have a disease background.
A clear reporting system should be developed. (There was a question over whether extension officers approach research centres or service centres)
There is a fear of fish diseases amongst farmers that is based on a lack of knowledge; fish health management programmes similar to IPM were suggested.
There is a need for a quarantine system to address problems posed by the movement of fish from India.

Thur 10 June AM - Dr DB Swar, Dr G Shrestha, Mr DM Singh, Mr RR Dhital and Mr S Dahal were among a group that joined the visiting team to formulate recommendations.

Recommendations:

1) There is a requirement for training in disease diagnosis and health management.
2) Diagnostic laboratories at central and provincial level should be set up.
3) Manpower should be developing through training programmes and higher studies.
   a) specialist level (Institute of Agriculture and Animal Sciences, Tribuhwan University, Nepal is offering an M.S. in Aquaculture)
   b) agriculture technicians - Mangalore can provide training but requires funding
   c) fish farmers
4) Training in quarantine is required
5) A fish disease manual, in Nepalese, should be produced aimed at the farmer level (similar to the CICFRI manual).
6) Collaborative linkages between institutes should be developed
7) A collaborative research programme should be considered to address fish health problems and to assist in staff training and equipping central and provincial laboratories.
8) Assistance for Nepal should be sought in gathering data on disease impacts in relation to the NACA/DFID meeting in Dhaka from 27-30 September 1999.

PM - Travel Kathmandu - Calcutta

Fri 11 June **<u>Visit to Central Inland Capture Fisheries Research Institute (CICFRI), Barrackpore</u>**

CICFRI background

The Central Inland Capture Fisheries Research Institute (CICFRI) is the oldest premier research institution in the field of inland fisheries research and training in India. Established in 1947, the institute came under the administrative control of Indian Council of Agricultural Research (ICAR) in 1967.

The main objective of the Institute (until the Sixth Plan, 1987) has been to conduct investigation for proper appraisal of inland fisheries resources in the country and to evolve suitable methods for their conservation and optimal utilisation. At the beginning of the Seventh Plan (1987), three new institutes were established viz., Central Institute for Freshwater Aquaculture (CIFA), Central Institute for Brackishwater Aquaculture (CIBA) and National Research Centre on Cold Water Fisheries (NRCCWF). The Institute was renamed CICFRI and its mandate modified, giving emphasis to capture fisheries resources in India. It is presently mandated to :

1) Study fish population dynamics of exploitable inland water bodies exceeding 10 ha in area.
2) Evolve management systems for optimising fish production from such water bodies.
3) Investigate causes, effects and remedies of their degradation/pollution and provide research support for utilisation for conservation of such resources.
4) Study the impact of river valley projects on the fisheries of the basins concerned and evolve strategies for their management.
5) Act as national data centre on inland fisheries.
6) Conduct training and provide extension/consultancy services.

The research activities of the Institute are organised in seven divisions (Riverine, Reservoir, Floodplain Wetlands, Estuarine, Environmental Monitoring, Fish Health Protection and Hilsa, Resource Assessment).

CICFRI is functionally linked with eleven regional research centres and four survey centres in other parts of India.

During the Seventh and Eighth Plan CICFRI has developed a number of technologies relating to environmental conservation and sustainable development of fisheries resources. In addition, CICFRI has developed expertise on fishery management in open water systems in a number of areas, notably on fish diseases and their causes, and on identification of indicators of stress in fish.

AM
The team arrived at CIFRI and met briefly with the Director, Dr M. Sinha. The team were presented with complimentary copies of CICFRI publications, including Das, M.K. (1997) Epizootic Ulcerative Syndrome in Fishes - Its Present Status in India. CICFRI Bulletin No.69. 22pp.

Seminar - The seminar room, CICFRI, Barrackpore

Approximately 20 participants from CICFRI attended the seminar, chaired by Dr Sinha. A list of participants is included in Appendix 1. 15 EUS Handbooks and 20 Histology manuals were distributed (Appendix 3).

Dr S Chinabut - Introduction
Dr CV Mohan - Pathology of EUS
Dr JH Lilley - Mycology of EUS
Dr RB Callinan - Causal factors
Lunch
Dr S Chinabut - Differential diagnosis
Dr JH Lilley - Control of EUS

Dr Manas K Das presented an overview of the work done on EUS at CICFRI.
He noted that major epizootics had occurred from 1988-1993. Outbreaks typically occurred in the post-monsoon periods, except in Kerala where outbreak periods were prolonged because of the extended monsoon period. About 30 species were affected. Outbreaks were most severe in Assam. Low alkalinity and hardness were reported to be associated with more severe outbreaks. EUS had grave biological and socio-economic consequences. Rivers and large water bodies were affected most in the initial stages, with heavy mortalities of valuable stocks of fish. As a result, depletion of fisheries is evident with consequent negative impact on dependent fishing communities. Women fish vendors were particularly disadvantaged and forced into employment as agricultural labourers, head-load and quarry workers.
Work at CICFRI:
Thirty species of bacteria have been isolated. *Aeromonas hydrophila* was the most consistent. Artificial infection was achieved with bacterial isolates, but Koch's postulates were not fulfilled. Myxozoan infections were commonly mistaken for EUS. The advised prophylactic treatment is 50kg/ha lime and 0.5ppm bleaching powder. The advised therapeutic treatment 100kg/ha lime and 1ppm bleaching powder.
Some of the recommendations of the 1994 DFID EUS Seminar in Bangkok have been addressed at CICFRI. Studies on the normal physiology of *Labeo rohita* were undertaken with a view to using variations in these parameters as indicators of stress and disease status. The CICFRI mandate allows interaction with CIFA and involvement in aquaculture studies.

Discussion:
The following issues were raised:
Dr Das - Capture data was shown indicating a lower catch of susceptible species between 1988 and 1991, which could possibly be attributed to EUS outbreaks during that period.
Dr Das - Mucus is produced and sloughed off in stressed fish, resulting in a "rough skin" condition, which may predispose fish to disease such as EUS.
Dr Sinha - Fear of EUS has discouraged participation in aquaculture.
Dr Sinha - EUS is not currently a problem, but it is unpredictable. Therefore a monitoring programme is needed and further work on control and prevention.
Dr Sinha - Current research programmes at CICFRI include work on fish parasites and environmental stressors. CICFRI is interested in collaboration in health management programmes.
Dr Das considers *Catla catla* more susceptible to EUS than other Indian major carps.
Prevalence of EUS is higher in NE India, and a relationship with acidic soils is suspected.
Sporozoans and trichodinid parasites are thought to cause major losses in farmed fish in West Bengal, and considered priority areas for fish disease research in India.

Recommendations:

Laboratory and pond related research

1) More work is required on virological aspects of EUS.
2) More work is required on immunological aspects of EUS, with particular reference to the role of mucus.
3) Treatment trials are required to examine the efficacy of: CaO, bleaching powder, Levamisole and CIFAX.
4) Species susceptibility, and size-related effects, with particular reference to Indian major carps.
5) Studies on the persistence of *Aphanomyces invadans* in the environment, and investigations of carrier states in fish or other organisms.
6) Physiological stress parameters of fish in relation to EUS.

Field research

1) Epidemiological and socio-economic studies with regards to outbreak cycles in wild fish populations. There is currently little reliable information on susceptible wild fish population levels and the impact of outbreaks on dependent communities.
2) The significance of EUS as a cause of fish losses should be compared to other fish diseases, with particular reference to Indian major carps. This could be linked with an FAO programme for disease reporting which recently started.

Sat 12 June AM - Travel Calcutta-Bhubaneswar
PM- Meeting with Dr R.K. Dey and Dr Sahoo, schedule arranged

Sun 13 June **Visit to Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar**

CIFA background

Central Institute of Freshwater Aquaculture (CIFA) was formally established in 1987, having been the Pond Culture Division of CICFRI since 1949, and the Freshwater Aquaculture Research and Training Centre since 1977.

CIFA is the NACA Regional Lead Centre for carp farming, and the Centre of Advanced Studies in Freshwater Aquaculture, offering post-graduate degree programmes. CIFA's mandate includes research, training and extension in freshwater aquaculture. Research is undertaken on Indian major carps, Chinese carps, common carp, catfishes, prawns and molluscs. It's facilities include 500 ponds and laboratories and wetlabs equipped for research within each of the following Divisions: Production technology, Soil-water environment, Fish Physiology, Nutrition, Genetics, Pathology, Engineering, Economics & Statistics and Aquaculture extension.

AM - Meeting with Dr S. Ayyappan, Director, CIFA.
Tour of CIFA pond facilities.

Seminar -The seminar room, CIFA, Bhubaneswar

Approximately 15 participants from CIFA attended the seminar. A list of participants is included in Appendix 1. 30 EUS Handbooks and 20 Histology manuals were distributed (Appendix 3).

Dr S Ayyappan - EUS in India and CIFA initiatives
CIFA has been involved in EUS research since it was first identified in India in 1988. Control measures are suggested and a monitoring procedure is in place. The country is demarcated into zones and Fish Farm Development Agencies, each with a small laboratory, are responsible for monitoring their zone. Training programmes, publications and audio cassettes are provided by CIFA. However, insurance agencies are currently reluctant to fund aquaculture ventures because of the threat of disease, largely due to earlier EUS outbreaks. A recognised problem with EUS research has been the lack of information exchange.

Dr RB Callinan - Introductory remarks
Dr CV Mohan - Pathology of EUS

Dr S Chinabut - Differential diagnosis
Dr JH Lilley - Mycology of EUS
Dr RB Callinan - Causal factors
Dr JH Lilley - Control of EUS
Lunch

Dr MK Mishra - Microbiological studies on EUS
The spread of EUS in India was described. In studies at CIFA, the presence of all biological agents was investigated. Sampling for viruses resulted in CPE in 4 cell lines (FHM, BB, EPC and SCT). TEM of tissue sections showed rhabdovirus particles. Many different strains of bacteria were isolated. A number of different fungi were isolated and identified, including *Aspergillus* in liver and kidney samples. Lime is the preferred treatment, but requirement varies between ponds. Generally for prophylactic treatments, 3 doses of 200 kg/ha CaO are given 30 days apart. For therapeutic treatments 3 doses of 200 kg/ha CaO are given 7 days apart. Calcium carbide (CaC_2) increases the pH more rapidly than CaO and 3 doses of only 5 kg/ha are required. CaO (100 kg/ha) + fresh turmeric (10 kg/ha) has also provided good results. CIFAX is applied at 1 litre/ha. It has been noted that EUS occurs at pH 7-8, but does not occur above pH 8.4.

Dr RK Dey - Pathological studies on EUS
Descriptions of the gross signs and pathology of EUS in different species were presented. Slides were presented showing that mycotic granulomas had been identified in the early outbreaks.

Discussion:
Dr Mishra - The main fungal disease problem is considered to be aflatoxicosis in *Catla catla*. Three strains of *Aspergillus* have been identified.
Dr Callinan - The histology of *Aspergillus* infection would be very different from that of *A. invadans* infection, and would be a separate disease problem from EUS using our current definition of EUS.
Dr Dey - Cohabitation studies in 1988-9 using EUS-affected fish and water from EUS-affected ponds did not reproduce EUS in healthy fish.
Dr Callinan - Enquired about published studies on the use of CIFAX.
Dr Mishra - Efficacy of CIFAX has been demonstrated by farmer feedback and demand, and by tank, pond and farm trials. Impacts on plankton have also been assessed.
Dr Dey - Ulcers have been shown to heal within 7-10 days of application.
Dr Mohan - With regard to Indian major carps, studies are being initiated to investigate comparative resistance of the different species to infection by a variety of pathogens, including *A. invadans*.

Mon 14 June AM - Wrap-up meeting with Dr Ayyappan, Dr Mukherjee, Dr Dey, Dr Mishra, Dr Sahoo. Discussions centred around priorities for fish disease research, and proposed trials at CIFA to assess efficacy of identified treatments in prevention and treatment of *Aphanomyces invadans* infection. Dr Mukherjee pointed out that *A. hydrophila* was isolated from 75% of ulcers of diseased fish, and that CIFAX is formulated to treat bacterial ulcers.

Recommendations and follow-up:
1) Dr Mohan and Dr Lilley will provide Dr Mishra with cultures of *A. invadans* for further work.
2) Dr Lilley will apply for funds so that treatment trials against *A. invadans* can be undertaken at CIFA with the institute's 48x 1000-litre tanks, over the winter season 1999-2000.

PM - Travel Bhubaneswar-Bombay

Tues 15 June AM - Travel Bombay-Trivandrum
PM - Meeting with Dr P. Natarajan, discussion of programme

Wed 16 June **Seminar-Department of Aquatic Biology and Fisheries, University of Kerala**

Seventeen participants attended the seminar, comprising of directors and research scientists from universities in Kerala and Tamil Nadu. Participants are listed in Appendix 1. 50 EUS Handbooks and 13 Histology manuals were distributed (Appendix 3). Participants supplied the team with some literature on EUS including: Natarajan, P., Rameshkumar, B., and Dhevendaran, K. (1999) Epizootic ulcerative syndrome - a review. Chapter 18. Advances in Aquatic Biology and Fisheries (Pp. 221-243). Department of Aquatic Biology and Fisheries, University of Kerala.

Dr P Natarajan - Introductory remarks
The Department of Aquatic Biology and Fisheries, Faculty of Science, University of Kerala was established in 1938 and is one of the oldest Fisheries Departments in India. A wide variety of disciplines are covered and 1600 publications have been produced, of which 400 were concerned about fish disease. Several department staff, and attending participants from other research centres, have undertaken research on EUS.

Dr RB Callinan - Purpose of mission
Dr S Chinabut - Introduction and history of EUS
Dr CV Mohan - Pathology of EUS

Questions/comments:
It was commented that there is a much reduced population of *Channa*, which has been replaced by other species. However with changes in irrigation systems, pollution etc, other species have also declined.
Dr Devika Pillai mentioned the isolation of paramyxovirus from *Mystus* with lesions grossly consistent with EUS. This was re-injected into *Mystus* and resulted in dermatitis.

Lunch and change of venue
Dr JH Lilley - Mycology of EUS
Dr RB Callinan - Causal factors
Dr S Chinabut - Differential diagnosis
Dr JH Lilley - Control of EUS

Dr P Natarajan - EUS research in Kerala
A survey of EUS in Kerala backwaters revealed:

- Of 18 species examined, only 15 were affected.
- Maximum prevalence in *Anabas, Channa, Etroplus, Puntius sophore, P. amphibious* and *P. filamentosus.*
- *Glossogobius giurus, Labeo rohita and Mystus gulio* were unaffected.
- Among the 15 affected species, 10 are freshwater species, 5 brackishwater.
- Among the 15 affected species, 9 are bottom living species, 4 column fish and 2 live at the surface.
- EUS occurred during the monsoon months May - September. Maximum prevalence was from June - August.
- Maximum prevalence was in the 10-30 cm size group. Smaller fish were less affected.
- Female fish were marginally more affected than males.
- Bacteria were more commonly isolated from affected fish than unaffected fish. 12 strains were obtained.
- In infection trials using a combination of 3 species (*Aeromonas hydrophila, Vibrio anguillarum* and *Pseudomonas fluorescens*) lesions appeared after 24h and 100% mortality occurred after 120h. With *A. hydrophila* alone, lesions appeared after 48h and 80% mortality was recorded. With *V. anguillarum*, lesions appeared after 48h and 40% mortality was recorded. With *P. fluorescens* alone, no lesions appeared.
- Bacterial sensitivity tests were carried out. Strains were sensitive to most of the 19 antibiotics tested.
- Histopathologically fungus was seen in skin tissues. Internal organs are currently being studied.

Clarification of the following points is needed:

- What makes some species susceptible as opposed to others?
- Why particular susceptible species remained unaffected during the Kerala survey?
- Kerala backwaters are polluted with sewage, oil etc. What are the are pollutants that affect fish health?
- More histopathology studies are required of internal organs of affected fish.
- Site specificity of lesions in particular species should be studied.
- Studies on control methods are required (incorporating present studies on marine bioactive compounds).

Discussion:

- Tamil Nadu is formulating a programme to promote/conserve fish as a food resource. The objectives are to (i) provide employment (ii) maximise use of land and water resources (iii) provide a source of nutritious food - primarily fish. Incentives for *Channa striata* and *Clarias batrachus* culture. Breeding techniques have been developed and profit levels are envisaged as being 3-4 times that of carps. The existing network of Fish Farm Development Agencies (FFDA) could assist this programme. There is therefore a chance that EUS will be a restraint to the development of this industry. Chicken slaughter waste could be utilised for *Channa* culture.
- The group was concerned that populations of susceptible fish have declined. Data on catches of susceptible and non-susceptible fish should be collected.
- *Channa striata* strains resistant to EUS are reported to occur. It was suggested they could be used as the basis for restocking programmes.
- Conservation of *Etroplus* spawning habitats as this species is a high cultural value fish.
- More than 90% of fishers are marine or brackishwater. However, the monsoon season, when marine fishing is banned and people rely largely on fish from inland areas, is also the time of highest prevalence of EUS. The International Collective in Support of Fishworkers (Enigma of EUS. 25-26 May 1992. Trivandrum) states that 200,000 people rely on inland fisheries for their livelihood.

Recommendations:

Inland fisheries

1) Assess inland fish capture statistics over the last decade to study impacts of EUS on susceptible fish populations.
2) Assess the socio-economic impact of EUS on susceptible fish in Kerala.
3) Study the possibility of restocking inland waters with resistant strains of *Channa* and other affected species.
4) Identification of environment-related causes of EUS.

Aquaculture

1) Assess locally developed marine bioactive compounds/immunostimulants and herbal remedies as treatments for EUS.
2) Development of EUS vaccine for pond aquaculture.

Thur 17 June — AM - Travel Trivandrum-Colombo
PM - Discussions with Dr P.P.G.S.N. Siriwardena, finalisation of programme

Fri 18 June — **Seminar - National Aquatic Resources Research and Development Agency , Colombo**

Approximately 56 participants attended the seminar. Participants comprised of NARA staff, university staff and students, and private sector workers. A list of participants is included in Appendix 1. 50 EUS Handbooks and 10 Histology manuals were distributed (Appendix 3). Requests were taken for a further 17 EUS Handbooks and 29 Histology manuals.

Chairman of NARA - Opening remarks
Dr P.P.G.S.N. Siriwardena - Introductory remarks
Dr RB Callinan - Purpose of mission
Dr S Chinabut - Introduction and history of EUS
Dr CV Mohan - Pathology of EUS
Tea
Dr JH Lilley - Mycology of EUS

Dr RB Callinan - Causal factors
Dr S Chinabut - Differential diagnosis
Dr JH Lilley - Control of EUS
Dr RB Callinan - Outbreak investigation
Lunch

Discussions

A study of environmental variables associated with EUS in 2 water bodies was described. pH had been around neutral for the study period, but the disease occurred after rainfall and during a period of low temperature. *Trichogaster pectoralis* and *Puntius* spp were affected and tilapia were unaffected.

Impact of EUS

It was noted that EUS normally occurs between December and February. It was recorded in wild fish in 1998, but no outbreaks were reported in 1999. Impacts are also minimised as no EUS-susceptible species are presently farmed on a large scale in Sri Lanka.

Dr Siriwardena - EUS occurs almost every year. Diseased seabass and *Mugil* are also seen, but it is not known if this is due to EUS. There is great interest in the development of aquaculture of these species, along with milkfish.

The question of whether EUS might have affected susceptible fish populations (eg *Channa*) was raised, but there is no known catch data.

There is a large ornamental fish trade in Sri Lanka, and it has been postulated that EUS was introduced with carrier ornamental fish. There is however, no record of a significant impact of EUS on the industry.

Dr Vinobaba - Most fish species in Batticaloa lagoon were affected by an ulcerative disease in 1994. Affected species included *Nematalosa, Mugil cephalus, Siganus lineatus, Siganus javus, Siganus oramin, Sphyranea* spp and *Tylosurus* spp. Ulcerated fish had a poor market value. 10,650 families in the area are dependent on these lagoon fisheries. There is no information on what triggered outbreaks, although affected fish did appear to have a higher trichodinid burden.

Research priorities

Acidic water is released from local rubber factories and it is important to investigate this as a risk factor for EUS. There are also acid sulphate soils in shrimp culture areas and in areas where dams have been built.

Present priorities for aquatic animal health are viral diseases of shrimp and parasitic diseases of ornamental fishes.

Disease diagnosis and quarantine programmes should be set up.

Wild fishes are an important source of protein for some sections of the population, and it is important to gather data on fish populations in inland waterways.

Sat 19 June	Dr Mohan travels Colombo-Bombay, Bombay-Mangalore
Sun 20 June	Report preparation
Mon 21 June	Drs Callinan, Chinabut and Lilley travel Colombo-Bangkok

ACHIEVEMENTS AGAINST OBJECTIVES

1. Structured, one-day seminar and discussion sessions were organised in each of the centres visited. In Kathmandu and Colombo, participants from all over the country attended, and in Trivandrum, participants from other states were present. 200 EUS Technical Handbooks and 100 Histology Manuals were distributed and in some centres, requests were taken for further copies. Recommendations were formulated during discussion sessions in which further work would be undertaken at local centres and elsewhere, taking into account the presentations on the latest research findings. In some centres, particular joint research proposals were formulated.

2. In recent years, in the countries visited, EUS has been mainly a problem of wild fishes. Recommendations for the study of wild populations and dependent communities were formulated. With regards to cultured fish systems, recommendations for response strategies and further research focused mainly on Indian major carp culture. The importance of excluding potential sources of fungal entry (eg wild fish and water) was emphasised. Trials of potential, locally-available, prophylactic treatments for use over the EUS season were recommended. In some areas, where plans were underway for the development of aquaculture of other EUS-susceptible species, of the potential risks, and possible intervention measures were discussed.

3. SUMMARY OF RECOMMENDATIONS

1. Socio-economic impacts of EUS
Studies are required on the impact of EUS outbreaks on communities dependent on wild fishes, particularly in Kerala and Sri Lanka. Workers at CICFRI also identified the need to study the effect of EUS on fishers and fish farmers. In Nepal, a questionnaire survey is required to identify all the constraints to the development of aquaculture.

2. Impacts of EUS on wild fish populations
An assessment of available inland fish capture statistics in the Kerala backwaters, and other important severely affected fisheries, should be undertaken to identify impacts of EUS on susceptible fish populations.

3. Treatment studies
Tank and pond trials are required to evaluate present treatments, and other candidate safe, affordable, locally available compounds. The pond facilities at CIFA provide an ideal facility for such trials. Bioactive compounds and immunostimulants currently being studied in Kerala could be assessed as treatments for EUS.

4. Ecology and transmission of *Aphanomyces invadans*
Studies on the persistence of *A. invadans* in the environment, and investigations of carrier states in fish or other organisms, are required.

5. EUS determinants
Local causes of dermatitis that lead to EUS should be further examined in the light of the present understanding of EUS causation. Specific reference was made to the study of virological aspects of EUS.

6. Susceptibility of Indian major carps
Further work is required to demonstrate the relative susceptibility of Indian major carps, and any size/age effects on susceptibility. Other immunological aspects of EUS should also be studied, with particular reference to the role of mucus.

7. Resistant strains
The possibility that there are particular strains of susceptible fish that have higher resistance/tolerance to EUS should be investigated.

8. Infrastructure and training
A recommendation is made for a programme of improving laboratory facilities, increased training and establishing collaborative linkages in Nepal. A national Prospective Plan for Fisheries is currently being developed which will encompass fish health issues.

9. Other diseases
The following other aquatic animal diseases of importance were identified during discussion sessions: (i) myxozoan infections of Indian major carps, (ii) viral shrimp diseases and (iii) parasites of ornamental fish.

APPENDIX 1. PARTICIPANTS BY COUNTRY

List of persons attending EUS seminar at the Ministry of Agriculture, Kathmandu

Mr Chakra Prasad Banstola
Minister of Agriculture

Dr Mukti Narayan Shrestha
First Secretary
Ministry of Agriculture

Jagadish Chandra Gautam
Special Secretary
Ministry of Agriculture

Mrs Ram Badan Pradhan
Director General
Department of Agriculture

Dr Deep Bahadur Swar
Chief Fisheries Development Officer
Fisheries Development Division
Kathmandu

Mr Dharani Man Singh
Fisheries Development Officer
Inland Aquaculture and Fisheries Section
Kathmandu

Mr Bikash Chand Shrestha
Fisheries Development Officer
Fisheries Development Centre
Dhangadhi, Kailali

Mr Gopal Bahadur Shrestha
Fisheries Development Officer
Inland Aquaculture and Fisheries Section
Kathmandu

Mr Raghavendra P. Pandeya
Assistant Fisheries Development Officer
Kathmandu Central Fish Hatchery
Kathmandu

Mr Ram Prasad Panta
Assistant Fisheries Development Officer
Fisheries Development Division
Kathmandu

Mr Madhav Prasad Dahal
Assistant Fisheries Development Officer
District Agriculture Development Office
Kailali

Mr Ram Bilash Thakur
Assistant Fisheries Development Officer
District Agriculture Development Office
Mahottari

Mr Munni Lal Agrahari
Assistant Fisheries Development Officer
District Agriculture Development Office
Nawalparashi

Mr Jageshore Yadav
Assistant Fisheries Development Officer
Fisheries Development Centre
Bhairahawa, Rupandehi

Mr Gayatri Raj Wagle
Assistant Fisheries Development Officer
District Agriculture Development Office
Kanchanpur

Mr Shano Kaji Panchhai
Assistant Fisheries Development Officer
Fisheries Development Centre
Bhandara, Chitawan

Mr T.P. Pokharel
Director
Nepal Agricultural Research Council (NARC)
Kathmandu

Mr Purusottam Lal Joshi
Senior Scientist NARC
Fisheries Research Division
Godawari, Kathmandu

Mrs Asha Rayamajhi
Technical Officer
Fisheries Research Centre
Godawari

Mr Shankar Dahal
Assistant Fisheries Development Officer
Kathmandu Central Fish Hatchery
Balaju, Kathmandu

Mr Kanti Bahadur Karki
Fisheries Development Officer
Fisheries Development Centre
Bhairahawa, Rupandehi

Mr Ganesh Bahadur Karki
Assistant Fisheries Development Officer
Fisheries Development Centre
Kulakhani

Mr Vishundeo Yadav
Assistant Fisheries Development Officer
District Agriculture Development Office
Shiraha

Mr Kamal Prasad Shaha
Assistant Fisheries Development Officer
District Agriculture Development Office
Bara

Mr Tham Bahadur Darai

Mr Ashok Kumar Shrivastav

Assistant Fisheries Development Officer
District Agriculture Development Office
Rupandehi

Mr Kalyan K.C.
Assistant Fisheries Development Officer
District Agriculture Development Office
Parsa

Mr Gopal Prasad Lamsal
Technical Officer Fisheries Research Centre
Trishuli, Nuwakot

Mr Dilip Kumar Jha
Professor
Department of Fisheries and Aquaculture
Institute of Agriculture and Animal Science (IAAS)
Rampur Campus, Chitwan, Nepal

Mr Yoogal Kishore Tiwari
Assistant Fisheries Development Officer
District Agriculture Development Office, Banke

Mr Janardan Bikram K.C.
Senior Officer
Agriculture Project Service Centre (APROSC)
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Ragbansi

Mr Raja Man Mulmi
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District Agriculture Development Office
Jhapa

Mr Vijaya Kumar Pradhan
Chief
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Mr Rabindra Man Malla
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District Agriculture Development Office
Dhanusha

Mr Shurendra Prasad
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Pokhara

Mr Resham Raj Dhital
Fisheries Development Officer

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Saptari

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Chief Agriculture Communication Division
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Mr Sanjib Ganguly
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Mr Shiva Shundar Shrestha

Mr Kumar Sapkota
Professor
Tribhuvan University
Kirtipur, Kathmandu

Mr Dibakar Paudel
Ministry of Agriculture

Mr Shasi Chitrakar
Fisheries Officer
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Mr Bharat Upadhaya
Chief
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Mr Ram Krishna Mahaseth
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Mr Sanjib Gurung
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Dr Upendra Mishra
Nepal Agriculture Research Council
Khumaltar, Lalitpur

Dr K.G. Rajbanshi
Royal Nepal Academy of Science & Technology
Kathmandu, Nepal

Dr Sunder B. Shrestha
Fisheries expert
Kalimati, Kathmandu

List of persons attending EUS seminar at CICFRI, Barrackpore

Dr Maniranjan Sinha
Director
CICFRI

Dr M.K. Das
Senior Scientist
CICFRI

Dr K. Chandra
Principal Scientist and Division Head
Environmental Monitoring and Fish Health Protection
CICFRI

Dr S. Samanta
Scientist
CICFRI

Dr S.K. Manna
Scientist
CICFRI

Dr A.K. Ghosh
Principal Scientist
CICFRI

Shri R.A. Gupta
Principal Scientist
CICFRI

Dr M.K. Mukhopadhyay
Senior Scientist
CICFRI

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CIFA

Dr R.K. Dey
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CIFA

Dr M.K. Mishra
Senior Scientist
CIFA

Dr S.C. Mukherjee
Senior Scientist
CIFA

Shri P.K. Sahn
Scientist
CIFA

P.B. Swain
Scientist
CIFA

Dr B.K. Das
Scientist
CIFA

Dr P. Patnaik
Technical Officer
CIFA

Dr Radheshyam
Technical Officer

Dr S.K. Sarher
Chief Training Organiser (CTO)

CIFA

Shri S. Nayak
Research Scholar
CIFA

Dr Sahoo
CIFA

CIFA

Dr Gayatri Murjani
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Ms S. Mahapatra
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List of persons attending EUS seminar at Department of Aquatic Biology and Fisheries, University of Kerala, Trivandrum

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College of Fisheries
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Manonmaniam Sundaranar University
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Dr S. Lazarus
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List of persons attending EUS seminar at NARA, Colombo

Dr P.P.G.S.N. Siriwardena, Head
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NARA

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A.R. Mudalige
A.A.C., Dambulla
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D.C.A. Limnosekera
F.I.
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B. Kodituwakku
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Amiradhapura

L.M. Kariyawasam
Aquaculturist, Aquaculture Development Centre
Mdambulla

C.W. Fonseka
Fish Breeding Society

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Mrs V. Pahalnaratharchch
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Panchuka Gurnarde
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Banduma Karunathiam
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Jayson Aquaproduction

Vasandha Jayasuriya
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Mahinda Kintathsok
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Ministry of Fisheries, NAQDA

Dr S.C. Jayamaivie
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APPENDIX 2. SEMINAR PROGRAMME COMPONENTS

The team recognised that the central task of the seminar programme was to present, as clearly and convincingly as possible, the body of work establishing that the fungus, *Aphanomyces invadans*, is an essential cause of EUS.

EUS – Introduction and History (approx 15 min)
Dr Supranee Chinabut

As an essential starting point, a modified definition of EUS, based on the definition recommended at the 1994 DFID-sponsored EUS Seminar at AAHRI was presented, together with a case definition, agreed in advance by the team members.
Brief descriptions of the initial spread and current status of EUS throughout the region in the period 1971-1999 were presented.
EUS, mycotic granulomatosis (MG; Japan) and red spot disease (RSD; Australia) were confirmed as identical conditions, while the status of ulcerative mycosis (UM; USA) in relation to EUS was stated as currently undetermined.

Pathology of EUS (approx 30 min)
Dr C V Mohan
The gross and histopathological features of defined EUS lesion types in representative freshwater and estuarine fishes were described in detail.
The constant presence of invasive fungal hyphae in lesions was emphasised and characteristic differences in responses to fungal invasion by representative fish species, with special reference to Indian major carps and snakeheads, were described.

Mycology of EUS (approx 40 min)
Dr J H Lilley
The findings of previous mycological studies of EUS were presented, with particular focus on studies from several countries in the region describing consistent recovery of an apparently single *Aphanomyces* sp. from affected fish.
Comparative studies of these isolates have confirmed that they represented a single species, *Aphanomyces invadans*, and probably a single genetic clone, which shows that the fungus has spread, and is not a long-term resident in each country.
The various life stages of the fungus and its probable method of fish-to-fish spread were described. Where relevant, similarities with *Aphanomyces astaci*, the cause of crayfish plague, were pointed out.
A brief description of the recommended fungal isolation techniques (with reference to the methods described fully in the manual) was presented. The presumptive and confirmatory diagnostic features of the fungus were given.

Causal factors (approx 40 min)
Dr R B Callinan
The apparent inability of *A. invadans* to initiate lesions in healthy fish of susceptible species, and the ability of spores to initiate lesions on abraded skin or if injected intramuscularly, were emphasised.
To illustrate, the results of two studies identifying component causes for EUS in estuarine fish (*Sillago ciliata* fingerlings, sublethal exposure to runoff from acid sulfate soil areas, *A. invadans* spores) and in freshwater fish (*Channa* sp., exposure to rhabdovirus, *A. invadans* spores) were presented in detail.
A causal web for EUS, illustrating established and hypothesised pathways for lesion induction, was presented.
To further illustrate the diverse pathways which may lead to EUS lesion induction, three sufficient causes for EUS were presented; each included the fungus as a necessary component cause, while the other component causes in each case were different.

Differential Diagnosis (approx 20 min)
Dr Supranee Chinabut
Slides of a variety of ulcerative diseases of fish were shown in order to demonstrate the difficulties in diagnosing EUS using gross signs alone. The importance of histopathology in the differential diagnosis of ulcerative diseases was emphasised.

Control of EUS (approx 40 min)
Dr J H Lilley
Strategies for the prevention of EUS were presented.

The results of *in vitro* trials of various compounds for anti-*A. invadans* activity were described. The potential usefulness of naturally occurring compounds, such as neem seed extract, was pointed out. In addition, results of aquarium trials and pond studies of several compounds for their ability to prevent induction of EUS lesions were presented.
Suggestions for general and specific approaches to control and treatment of EUS outbreaks in fish reared in ponds, in cages in large water bodies, and in the wild were given.

EUS outbreak investigation (approx 15 min)
Dr R B Callinan
Nine basic steps to outbreak investigation were described, with particular emphasis on options for EUS outbreak investigation at pond, farm or regional level. To illustrate, reference was made to a published pond-level study of EUS outbreaks in Bangladesh.

APPENDIX 3. MANUAL DISTRIBUTION

	EUS handbook	**Histology manual**
Nepal -	38 (85 more requested)	20
Barrackpore -	15	20
Bhubaneswar -	30	20
Trivandrum -	50	13
Colombo -	50 (17 more requested)	10 (29 more requested)
Dr Mohan - Mangalore College, CIBA (Madras), remaining fisheries colleges	12	12
Dr Callinan - NSW, Queensland	5	5
TOTAL	**200**	**100**

APPENDIX 4. ACQUITTAL OF FUNDS

Activities	US$	Baht	Aust$
Dr CV Mohan			
Airfare	950.00		
Subsistence	1,475.00		
Others	175.00		
Total	2,600.00		
Dr R Callinan			
Airfare	1,858.39		
Subsistence	1,475.00		
Others	380.95		
Total	3,714.34		
Dr JH Lilley			
Airfare	1,038.39		
Subsistence	1,375.00		
Others	258.69		
Total	2,672.08		
Dr S Chinabut			
Airfare	1,038.39		
Subsistence	1,375.00		
Others	170.97		
Total	2,584.36		
Other expenditure during the trip			
DHL Contract to ACIAR	19.18		
Taxi in Kathmandu	5.97		
Telephone Calcutta	6.18		
Overweight charge	19.00		
Taxi in Calcutta	69.71		
Reconfirm ticket	5.00		
Reconfirm ticket	5.71		
Reconfirm ticket	2.38		
Taxi to airport	3.81		
Battery	10.48		
Taxi in Colombo	3.29		
Total	150.71		
AAHRI overhead	456.62		710.00
Grand total	**12,178.11**	**455,217.75**	
Received budget from ACIAR		**436,545.50**	**18,160.00**
Received budget from DFID	**1,608.93**	**60,141.80**	
Total received		**496,687.30**	
Funds remaining		**41,469.55**	**1,725.02**

Exchange rates
1US$ = 42 Indian Rp
1US$ = 67 Nepalese Rp
1US$ = 70 SriLankan Rp
1US$ = 37.38 Baht
1Aust$ = 24.04 Baht

APPENDIX THIRTY ONE

Manual - Lilley, J.H., Callinan, R.B., Chinabut, S., Kanchanakhan, S., MacRae, I.H., and Phillips, M.J. (1998). Epizootic ulcerative syndrome (EUS) technical handbook. Aquatic Animal Health Research Institute, Bangkok. 88pp. (cover) (pdf available from ARP Manager, hard copy available from AAHRI)

EPIZOOTIC ULCERATIVE SYNDROME (EUS) TECHNICAL HANDBOOK

Aquatic Animal Health Research Institute
Department of Fisheries
Kasetsart University Campus
Bangkok

APPENDIX THIRTY TWO

EUS Extension leaflet in Urdu. Produced in collaboration between Pakistan Ministry of Food, Agriculture and Cooperatives, DFID, ACIAR, NACA, and AAHRI. (hard copy available from ARP Manager)

فِش فارمرز
متوجہ ہوں

کمشنر فشریز آفس وزارت خوراک و زراعت
حکومت پاکستان

فش فارمرز متوجہ ہوں

مچھلیوں کی ایک نئی بیماری
(ای۔ یو۔ ایس کے بارے اہم معلومات)

تعارف

ای۔یو۔ایس جلدی زخم اور گلنے سڑنے کی علامات رکھنے والی ایک مہلک بیماری ہے۔ یہ کثیر تعداد میں مچھلیوں کی ہلاکت کا سبب بنتی ہے اور بھاری مالی نقصان کا باعث بن سکتی ہے۔ یہ بیماری جاپان' تائیوان' ملائیشیا' تھائی لینڈ' سری لنکا' برما اور بھارت میں کافی نقصان پہنچا چکی ہے۔ پچھلے برس پاکستان کے مختلف حصوں میں خصوصاً صوبہ پنجاب کے اضلاع' گوجرانوالہ' سیالکوٹ اور بہاولنگر میں بھی اس بیماری کو شناخت کیا جا چکا ہے۔ اور بعد ازاں صوبہ سندھ کے ضلع بدین میں بھی اس بیماری کی موجودگی کی تصدیق ہو چکی ہے۔

اس سلسلے میں آپ تمام حضرات کو مشورہ دیا جاتا ہے کہ سنجیدگی سے اس بیماری پر توجہ دیں۔ لہٰذا پمفلٹ ہذا کا مقصد آپ کو اس بیماری کی شناخت کے بارے میں معلومات فراہم کرنا اور تحفظاتی امور سے آگاہ کرنا ہے۔

اگر آپ کے فارم میں اس بیماری کی علامات پائی جائیں تو آپ فوراً ماہرین مچھلی پروری سے درستی احوال کے لئے رابطہ قائم کریں

بیماری کی علامات

اس بیماری میں سب سے پہلے مچھلی کی جلد پر چھوٹے چھوٹے سرخ نشانات ظاہر ہوتے ہیں جو وقت کے ساتھ ساتھ زخموں کی شکل اختیار کر لیتے ہیں۔ بعد ازاں یہ زخم گہرے اور بڑے ہو جاتے ہیں اور ان میں پیپ بھر جاتی ہے۔ نتیجۃً مچھلی سست اور کاہل ہو کر بے ترتیبی سے تیرنے لگتی ہے۔

عموماً یہ بیماری مچھلیوں کی اشتہا میں اور نمو کو متاثر کرتی ہے بلکہ بھاری دوسری مقامی مچھلیوں یعنی روہو' موری اور تھیلاپیا

پھیلا دیں۔

(ب) اگر آپ کسی بھی وجہ سے قدرتی ذرائع کا بیج مچھلی حاصل کرتے ہیں تو اسے تالاب میں ڈالنے سے پہلے ایک فیصد عام نمک کے محلول میں ایک گھنٹے کا غسل ضرور دیں تاکہ بیماری یعنی فنگس کے جراثیم مرجائیں۔

(ج) جال اور دیگر آلات کے استعمال سے پہلے انہیں ہائپو کلورائٹ (بلیچنگ پوڈر) کے محلول میں اچھی طرح کھنگال لیں۔

(د) اس بیماری سے بچاؤ کے لئے خصوصاً مئی ستمبر سے اپریل کے دوران اپنے تالابوں میں ٹیوب ویل کا پانی استعمال کریں۔

(س) متاثرہ مچھلیوں کو غیر متاثرہ تالابوں میں مت ڈالیں۔

2۔ مچھلی کی جلد کو درست رکھیں

(ا) تالاب میں جال کا بار بار استعمال نہ کریں کیونکہ اس سے مچھلیوں کی جلد کو نقصان پہنچنے کا اندیشہ ہوتا ہے۔

(ب) جلدی طفیلیے مثلاً لرنیا (LERNIA) اور آرگولیس (مچھلی کی جوں) وغیرہ مچھلی کی جلد کو نقصان پہنچا سکتے ہیں اس لئے موسم سرما کی ابتدا

یاد رکھیے:

ای۔یو۔ایس ایک بہت ہی مہلک بیماری ہے اور اس کا کوئی مئوثر علاج ممکن نہیں۔ یہ بہت بھاری مالی نقصان پہنچا سکتی ہے اس سے بچاؤ کی صورت صرف احتیاطی تدابیر کا اختیار کرنا ہے تاہم اس بیماری کے ظاہر ہونے کی صورت میں فنگس ماہی پروری کے ماہرین سے فوری رجوع کریں۔

(اگر آپ کے فارم پر فنگس ماہی پروری کا عملہ بیماری کی تصدیق کیلئے آئے تو آپ اس سے بھر پور تعاون کریں)

سے پہلے اس امر کو یقینی بنائیں کہ ڈپریکس اور فارملین کے استعمال کے بعد آپ کی مچھلی جلدی طفیلیوں سے پاک ہو گئی ہے۔

3۔ تالاب کے ماحول کو صاف ستھرا رکھیں

(ا) تالاب کے پانی کا مندرجہ خصوصیات رکھنا نہایت اہم ہے۔ آلودہ پانی جس میں گھریلو کا گندہ پانی اور فیکٹریوں کا نقصان دہ پانی شامل ہے وہ تالابوں میں نہ ڈالا جائے۔ نہایت کم یا زیادہ تیزاب (PH) والا پانی بھی مچھلی کی جلد کو نقصان پہنچا سکتا ہے۔ جس کی وجہ سے فنگس مچھلی کو متاثر کر سکتی ہے۔

(ب) فنگس سے بچاؤ کے لئے ٹیوب ویل کا پانی سب سے بہتر ہے

4۔ ای۔یو۔ایس۔ مزاحمتی مچھلیاں پالیں۔

اگر آپ کے علاقے میں ای۔ یو۔ایس کے اثرات پائے جائیں۔ تو جاپانی مچھلیاں پالیں جو اس بیماری سے محفوظ رہنے کی صلاحیت رکھتی ہیں مزاحمتی اقسام کے بیج مچھلی کی سٹاکنگ کے لئے اپنے علاقہ کے ماہر ماہی پروری سے رابطہ کریں۔

پھیلا دیں۔

(ب) اگر آپ کسی بھی وجہ سے قدرتی ذرائع کا بیج مچھلی حاصل کرتے ہیں تو اسے تالاب میں ڈالنے سے پہلے ایک فیصد عام نمک کے محلول میں ایک گھنٹے کا غسل ضرور دیں تاکہ بیماری یعنی فنگس کے جراثیم مرجائیں۔

(ج) جال اور دیگر آلات کے استعمال سے پہلے انہیں ہائپو کلورائٹ (بلیچنگ پوڈر) کے محلول میں اچھی طرح کھنگال لیں۔

(د) اس بیماری سے بچاؤ کے لئے خصوصاً مئی ستمبر سے اپریل کے دوران اپنے تالابوں میں ٹیوب ویل کا پانی استعمال کریں۔

(س) متاثرہ مچھلیوں کو غیر متاثرہ تالابوں میں مت ڈالیں۔

2۔ مچھلی کی جلد کو درست رکھیں

(ا) تالاب میں جال کا بار بار استعمال نہ کریں کیونکہ اس سے مچھلیوں کی جلد کو نقصان پہنچنے کا اندیشہ ہوتا ہے۔

(ب) جلدی طفیلیے مثلاً لرنیا (LERNIA) اور آرگولیس (مچھلی کی جوں) وغیرہ مچھلی کی جلد کو نقصان پہنچا سکتے ہیں اس لئے موسم سرما کی ابتدا

APPENDIX THIRTY THREE

Article six - Lilley, J., Chinabut, S., and Khan, M. (2000) Current prevalence of epizootic ulcerative syndrome (EUS) and strategies for control. Aquaculture News, Institute of Aquaculture, University of Stirling 26, 13-16. (hard copy available from ARP Manager)

Current prevalence of epizootic ulcerative syndrome (EUS) and strategies for control

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DFID SUPPORT OF EUS PROJECTS

Collaborative research between the Institute of Aquaculture, Stirling and the Department of Fisheries (DoF) of Thailand began in the mid 1980s following serious outbreaks of the freshwater fish disease, epizootic ulcerative syndrome (EUS) in Southeast Asia. Subsequent outbreaks occurred in Bangladesh and other parts of South Asia (Fig.1). It was partly as a result of the impact of EUS on aquaculture in the region that DFID initiated the Southeast Asia Aquatic Disease Control Project (SEAADCP) to promote research and training on health management of cultured aquatic animals. The project was based at the DoF research centre, The Aquatic Animal Health Research Institute (AAHRI) in Bangkok. AAHRI has since expanded to become an international-class research institute and is currently the only OIE-approved laboratory for the diagnosis of EUS.

In 1994, DFID sponsored an EUS Regional Seminar in Bangkok that brought together most of the researchers on EUS and provided the first accepted case definition of the disease. DFID-funded EUS research projects have combined field surveys, laboratory studies and tank trials to determine the role of various agents in EUS outbreaks. One result of this research is that the oomycete fungus, *Aphanomyces invadans* Willoughby *et al.*, 1995, is now recognised by most workers as the necessary cause of EUS, and granulomas caused by this fungus are used to diagnose cases of EUS. DFID is currently funding a joint Stirling-AAHRI research project that uses knowledge gained from previous projects to identify control strategies against EUS.

PREVALENCE OF EUS

The severity of EUS outbreaks has decreased in recent years, but there is a continued widespread occurrence of the disease. A DFID-funded cross-sectional survey in Bangladesh conducted during the 1998-99 winter season revealed that 16% of 12,822 wild and farmed fish from 128 sites had lesions (Fig. 2). Of these, 471 fish were taken for histology and 80% were diagnosed as EUS-positive (Khan *et al.*, 1999). This shows that EUS is still the largest cause of clinical lesions on freshwater fish in Bangladesh. Earlier data from the Bangladesh Flood Action Plan 17 (FAP17) Project in 1992-94 showed that 26% of 34,451 wild freshwater fish examined had lesions (Lilley *et al.*, 1999) (Fig. 3).

Unlike most fish diseases, EUS has generally had greater impacts on extensive, low input systems and wild fisheries, than on controlled intensive fish culture. In South Asia, this means that poorer fishing communities are at greater risk of suffering impacts. Bhaumik *et al* (1991) also showed that rural traders and consumers have been more affected by the outbreaks than urban/suburban communities.

New occurrences of EUS are still being reported in previously unaffected areas, and in newly developed farming systems. In 1998, EUS occurred for the first time in Sindh province in Pakistan (Lilley *et al.*, 1998) and in the Philippine island of Mindanao (BFAR, unpublished). Earlier, in Jan 1996, up to 30% prevalence in EUS susceptible fish was recorded from both Laguna Lake and Mangabol swamp in central Luzon, Philippines (Callinan, pers comm).

Figure 3: Percentages of fish with lesions from October 1992 – March 1994. Data from FAP17 (1995)

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In 1999, wild and cultured snakehead samples from Cambodia (Sophal, unpublished) and Nepal (Dahal, unpublished) were confirmed at AAHRI as EUS positive. Although EUS has frequently been reported from these countries, affected fish had not previously been confirmed by histological diagnosis. Last year there were also unconfirmed reports of ulcerative disease in cultured fish in southern Vietnam and southern Thailand.

The most recent outbreaks show that EUS is not always strictly seasonal, or always causes high mortalities, but may be prevalent at a low level throughout the year. In 1999, several occurrences of EUS in juvenile giant gouramies (*Osphronemus goramy*) and climbing perch (*Anabas testudineus*) in Thailand were confirmed at AAHRI. These occurred at times outside the usual 'EUS season'.

EUS in Australia remains an important issue in estuarine wild fish and in cultured silver perch (*Bidyanus bidyanus*) in New South Wales, Queensland and Northern Territory. The disease has occurred almost all year round and at prevalences of 20-90% in farmed silver perch (Callinan *et al.*, 1999).

Recent studies have also indicated that the geographical distribution of *A. invadans* may extend beyond the Asia-Pacific region. New evidence has indicated that the invasive *Aphanomyces* involved in ulcerative mycosis outbreaks in Atlantic menhaden (*Brevoortia tyrannus*) in Chesapeake Bay, USA appears to be the EUS fungal pathogen *A. invadans* (Blazer *et al.*, 1999). In Egypt, Shaheen *et al.* (1999) have described the isolation of thermo labile isolates of *Aphanomyces* from diseased mullet, but further work would be required on these isolates to establish the species involved.

STRATEGIES FOR CONTROL

Infection by *A. invadans* occurs in all cases of EUS, but the cause of the initial lesion, by which the fungus invades, may vary between outbreaks. In Australian estuaries, the initial dermatitis is caused by acidified runoff water from acid sulphate soil areas (Callinan *et al.*, 1996). In snakeheads in Thailand, a specific rhabdovirus has been shown to be capable of inducing small lesions that allow penetration by *A. invadans* (Ikanchanakham, 1999). Given that some causal factors have now been conclusively identified, work is now concentrating on developing and implementing strategies for the control of outbreaks.

Control of EUS in wild fish populations would be problematical and expensive, but may be possible in some situations. In the example from Australia given above, better management of estuaries to prevent the release of acidified water would greatly reduce the risk of EUS outbreaks.

Where *A. invadans* is not endemic to a country or area, measures could be taken to prevent the entry of the pathogen. Such measures have been proposed by the FAO/NACA/OIE Regional Programme for the Development of Technical Guidelines on Quarantine and Health Certification, and Establishment of Information Systems for the Responsible Movement of Live Aquatic Animals in Asia. The development of pathogen databases, and recent improvements in EUS diagnostic procedures (Panyawachira *et al.*, 1999; Yoriaca *et al.*, 1999), will assist the implementation of procedures to prevent pathogen entry.

In areas where EUS is already endemic, strategies are required for the control of the disease in pond-reared fish. Some themes for EUS prevention and treatment are listed in Figure 4 and discussed further here.

Figure 1: EUS-affected Indian major carp (*Labeo rohita*) January 1998, Bangladesh (Photo by MH Khan)

CULTURE RESISTANT SPECIES OR STRAINS

A great deal of data is now available on the relative susceptibility of different fish species (Khan *et al.*, 1999). In some situations, it may be acceptable to substitute susceptible fish with resistant species. Alternatively, susceptible fish could be harvested from the pond before the cool season, when fish are at higher risk of EUS. In Bangladesh, EUS-susceptible *Puntius* spp. are often selectively harvested from carp polyculture ponds in this way. Recent evidence suggests that extending this practice to *Cirrhinus mrigala* and *Labeo calbasu* would further reduce the risk of EUS (Khan *et al.*, 1999).

The severity with which EUS affects fish populations in newly affected areas may be an indication that naive fish are generally less resistant than fish from endemic areas. Possible mechanisms for resistance and the potential for inducing resistance, are being investigated by Miles *et al.* (1999) and Catap and Munday (1999).

CONTROL THE INITIAL CAUSE OF SKIN DAMAGE

Aside from those already mentioned, there are likely to be a number of other causes of the initial dermal lesions that allow entry of the fungus. The identification and control of such lesions would reduce the risk of EUS. Presence of high ectoparasite loads were shown to almost double the risk of EUS infections in Bangladesh (Khan *et al.*, 1999). Monitoring parasite burdens and treating severe infestations would therefore further reduce EUS occurrences.

EXCLUDE A. INVADANS FROM THE POND

As *A. invadans* propagules are not resistant to desiccation, they can be eradicated from a pond by drying and liming the pond prior to stocking. Khan *et al.* (1999) also noted that the risk of EUS was much reduced in ponds that had the bottom mud removed prior to stocking.

The presence of wild fish in the pond, direct connection to natural water-bodies, and failure to disinfect nets and equipment before use in the pond are also important risk factors for the occurrence of EUS as they allow the entry of pathogens from outside (Khan *et al.*, 1999). Use of prophylactically treated, hatchery-reared fry, exclusion of wild fish, and use of ranked or tube well water are useful practices to reduce the likelihood of EUS. However, many farms need to take some water from natural water-bodies, thereby creating a possible entry point for the pathogen. Only in the most intensive systems is it possible to sterilise inlet water. Some snakehead farmers in Thailand are able to stop water exchange during periods of cold weather, but snakeheads are air-breathers and can tolerate very low levels of dissolved oxygen

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Other farm systems require inlet water during the cold season, and therefore prophylactic treatments have been recommended over this period.

In order to identify possible preventative treatments, a large number of compounds were screened *in vitro* against *A. invadans* (**Campbell**, unpublished). These studies concentrated on natural products such as neem seed extract (Fig.5). Candidate fungicides were then tested as prophylactic treatments against EUS in tank trials at AAHRI, and are currently being further tested in pond trials.

IDENTIFY RISK FACTORS AND APPLY BEST PRACTICE MANAGEMENT

Managing risk of disease losses from EUS is usually a cheaper and more effective means of control than treatment. The monitoring and control of potential risk factors such as low water quality and high parasite burdens is a useful means of reducing incidences and severity of EUS outbreaks. As an example, post-stocking lime applications have been shown to reduce the risk of EUS (Khan *et al.*, 1999). This measure has a more significant effect on increasing the buffering capacity of the water, and maintaining the integrity of the fish skin, than in the treatment of any pathogens.

As with other fish diseases, EUS outbreaks will be more severe in ponds where fish are overly crowded, and therefore stocking densities should be kept as low as possible over periods of high risk.

Water with high algal content has been shown to contain lower counts of oomycete fungal propagules (Lilley, 1992), and Khan *et al.* (1999) has shown that green or red water (i.e. with high phytoplankton or zooplankton content) is at lower risk of EUS than transparent or black water. The potential to use algal blooms to reduce the risk of EUS is currently being investigated further.

Fig. 4 Strategies for the control of EUS in ponds

TREAT INFECTION OR IMMUNOSTIMULATE FISH

Therapeutic treatment of fish already affected by EUS is problematical because *A. invadans* grows inside the muscle of the fish and is therefore not exposed to water treatments. Attempts to treat naturally infected snakeheads and climbing perch have been unsuccessful. The fish used were severely ulcerated, at which stage the treatment of secondary bacterial and fungal infections is also of great importance. The use of fungicides in early outbreaks should, however, reduce the reservoir of infective propagules and therefore reduce the severity of the disease in the overall pond population. Snakehead farmers in Thailand usually stop all water exchange to and from a pond when EUS

is detected. This not only prevents spread to other ponds, but can also reduce the severity of the outbreak in the affected pond as there is no further entry of pathogens.

Treatment of fish with advanced lesions using orally administered immunostimulants is also not realistic as such fish have very reduced appetites. However, the use of oral immunostimulants has shown potential to convey some protection to challenged fish (Miles, unpublished; Catap and Munday, 1999) and may be useful in early stages of an outbreak.

Further studies have concentrated on stimulating the fish humoral response, and a passive immunisation trial is currently underway to investigate the potential for EUS vaccine development (Miles, unpublished).

Figure5: Neem (Azadirachta siamensis), one of several herbal treatments for EUS currently being investigated (photo by D. Fairweather)

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Prevention better than cure

Fish are as susceptible to disease as any other farmed crop, whether animal or vegetable. Commercial operators are well trained to spot early signs of trouble and take the necessary steps, but for owners of small ponds, keeping their fish healthy may be the first, and sometimes a terminating, challenge.

Often the first sign of ill health is that the fish show less enthusiasm for their food. Other obvious signs include ulcers or spots on the skin, or there may be an overall change in colour or behaviour. Expert advice is obviously essential because it can be very difficult to decide what the problem is and how it should be treated. For example, catfish sometimes develop a liver problem caused by poor quality food. Improving the food solves the problem. There are also some infectious diseases where the life-cycle of the disease-causing agent passes through several different animals or organisms, the host fish being just one part of the life-cycle. Control may be relatively easy if the life cycle can be interrupted before the fish are affected. In some cases, this may require no more than netting the pond to keep birds away.

Not all diseases are so easily controlled. Epizootic ulcerative syndrome, a disease which affects freshwater fish in Asia, is caused by a fungal pathogen that has so far proved impossible to treat once fish are infected. The disease was first recorded in 1971 in Japan, from whence it has spread rapidly westwards, reaching Pakistan in 1996. All kinds of fisheries are affected; rice paddy fish and small scale fisheries as well as intensive culture systems. Over one hundred freshwater species have been recorded as being susceptible to the disease, including the African catfish. Marine species do not, however, appear to be affected.

The fungus can only attack fish that are already suffering some degree of skin damage. Acid water run-off from soils can cause mild skin lesions on fish that are then at risk of the disease. Parasites, or even netting can also cause sufficient skin damage to allow the fungus to penetrate and invade the muscle tissue beneath. Scientists at the Institute of Aquaculture at the University of Stirling in the UK are working on ways to stimulate the non-specific immune system of the fish with techniques that are cheap and appropriate at all levels of fish culture. Non Asian fish are also being tested for susceptibility but, as with other fish diseases, environmental conditions play a major role in the level of resistance.

There is a very real risk that this disease will continue to spread. Within the region currently affected, some 250 million farming families depend on rice and much of the incidental fish harvested from the paddy fields is an important element of their diet. The main months for harvesting rice paddy fish are between September and February when the disease is most prevalent. Although the disease is not dangerous to human health provided the fish are well cooked, ulcerated fish are, of course, difficult to sell and confidence in freshwater fish farming, especially among potential investors, has been badly affected.

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