Final Report to the Overseas Development Administration

Bacterial Disease in Frog Hatcheries and Evaluation of Strategies to Reduce Hazards
Association with Antibiotic Usage (AAHRI/IASU-Frog Project)
(R6206Cb)

Supranee Chinabut
Aquatic Animal Health Research Institute
Department of Fisheries
Kasetsart Univ. Campus
Jatujak, Bangkok 10900
Thailand

Final Report to the Overseas Development Administration
Reporting period :   1 November 1994 - 31 March 1997

Bacterial Disease in Frog Hatcheries and Evaluation of Strategies to Reduce Hazards
Association with Antibiotic Usage (AAHRI/IASU-Frog Project)
(R6206Cb)

Appendix 1: Diseases of cultured frog in Thailand
Appendix 2: Comparative study on the integumentary system of tadpole and adult frog (*Rana tigerina*)
Appendix 3: Minimal inhibitory concentrations of oxytetracycline and nitrofurantoin against *Aeromonas hydrophila* and *A. sobria*
Appendix 4: Challenge of culture frogs (*Rana tigerina*) with Aeromonas spp.
Appendix 5: Regeneration and repair of superficial wounds in the skin of frog, *Rana tigrina* Cantor
Appendix 6: Pathogenesis of *Aeromonas hydrophila* infection in cultured frog (*Rana tigerina*)
Appendix 7: Comparative study of cutaneous wound healing between frog (*Rana tigerina*) and hybrid catfish (*Clarias macrocephalus* X *C. gariepinus*)
Appendix 8: Programme for Workshop on the Health profile of Farmed Aquatic Species

Executive Summary

The objectives of this project are to:

1. Understand of the susceptibility of frogspawn and tadpoles to bacterial infection throughout their development.
2. Determine optimum conditions which will avoid outbreaks of bacterial disease in hatcheries.
3. Determine efficacy of antibacterial chemotherapy to control disease with minimum use of drugs.
4. Establish a model bacterial challenge for adult frogs to support related studies.

To achieve the objectives various research activities have been carried out:

- A survey of bacterial diseases of tadpoles and frogs.
- A challenge of tadpoles and frog with various species of pathogenic bacterial species isolated from diseased frogs.
- Investigation of *in vivo* virulence of bacteria on juvenile frogs.
- Investigation of the effects of some chemicals on tadpoles.
- Establishment of *in vitro* susceptibility of bacterial isolates to antibacterial drugs.
- The minimal inhibitory concentration (MIC) of *A. hydrophila* and *A. sobria* to oxytetracycline and nitrofurantoin.

Results indicated that many species of bacteria could be isolated from eggs and tadpoles. The total bacterial count in the water of the hatcheries was high compared to the LC_{50} values. Changes of water temperature can induce mortalities in both tadpoles and frogs. One strain of unidentified Aeromonas from diseased frogs exhibited very high virulence to experimentally infected frogs. *Aeromonas* spp showed significantly resistance to some antibacterial drugs.

The knowledge gained from this project has proved to be very useful and has been utilised in recommendations to frog farmers in relation to the proper management of farms and reduction of disease incidence. Outputs from this project have been disseminated to scientists in the region through a workshop organised at AAHRI with collaboration from Institute of Aquaculture, University of Stirling (IASU) staff. Three research papers will be published in the peer reviews journal.

ODA Report For Research Project R620Cb

Bacterial Disease in Frog Hatcheries and Evaluation of Strategies to Reduce Hazards Associated with Antibiotic Usage

Aquatic Animal Health Research Institute, Department of Fisheries

Kasetsart Univ. Campus, Jatujak, Bangkok 10900, Thailand

Background

The major constraint to the continuation and extension of the frog farming industry in South East Asia is loss through disease, particularly due to disease of bacterial origin. Fundamental to successful on-growing is the efficient production of high quality tadpoles and juvenile frogs with an enhanced resistance to bacterial infection. Antibiotics are widely used, without proper knowledge, in both hatcheries and grow out farms, to control losses from bacterial diseases. Efficacy has not been evaluated and heavy usage at this stage may be the source of later problems encountered with failure of treatment.

Objectives

- determine the critical phases in the development of juvenile frogs with respect to their susceptibility to bacterial infection and to reduce their impact.
- optimise the health status of juveniles.
- critically assess, antimicrobial chemotherapy in hatcheries.

The stated objectives of this proposal will be met by the following protocol:

i)   The bacteriology of the egg and the first free swimming stage will be investigated in relation to the environmental microflora. Particular interest will be taken in the antimicrobial protection afforded by the egg gel.

ii)   The susceptibility of the developing frog to pathogens of different virulence will be assessed throughout the growth cycle, under a range of physical and chemical conditions.

iii) Efficacy of chemotherapy and associated risks of selection of resistant variants will be determined by:

- Phamacokinetic studies in frog tissues
- Relating these results to susceptibilities (drug, minimum inhibitory concentration) of hatchery pathogenic isolates.

iv)   Intervention strategies applied at the pre-determined critical phases in hatchery trials will be evaluated for their contribution to reduction of losses.

Further, to support ODA-funded work already being carried out at IASU, information gathered relating to chemical and physical stressors on the developing stages of frogs was applied to provide a model challenge in adult frogs, effective by bath challenge and oral application rather than injection. This is essential for evaluation of all therapeutic and prophylactic interventions and is work for which the AAHRI groups are well qualified.

Some work is already in progress at Stirling, but it is constrained by poor supply of frogs. Preliminary work has been done at AAHRI and it is proposed to build on this to the benefit of both groups and the project as a whole.

Contributing personnel

Academic staff

Dr Supranee Chinabut, AAHRI staff
Dr Temdoung Somsiri, AAHRI staff

Supporting staff

Mr. J. Intiseth, Project Assistant.
Ms. M. Wannaprapha, Project Assistant.

Research Activities

Summary of work carried out during the project

The following trails have been conducted to fulfil the objectives of the project.

I.   *To determine the critical phases in the development of juvenile frogs with respect to their susceptibility to bacterial infection and to reduce it's impact*

I.1   Survey of bacterial diseases of frogs.

A survey of the bacterial flora of cultured frogs was conducted in eight provinces, Kampheng Phet, Phichit, Nakorn Pathom, Nakorn Rachasima, Udon Thani, Petchaburi and Nakorn Sri Thammarat. A total of 120 isolates from both normal and diseased frogs was collected. These were identified as *Aeromonas hydrophila, A. sobria, Flexibacter columnaris, Pseudomonas* sp, *Proteus* sp, *Serratia* sp, *Citrobacter* sp, *Achromobacter* sp and Dipplococus. Typically, *A. hydrophila, A. Sobria* and an unidentified Aeromonas were found in poorly managed farms. Among these 120 isolates there were only 29 virulent strains, of which 4 were *A. hydrophila*, 10 were *A. sobria* and 15 were unidentified *Aeromonas*.

The result of this work will be present as an oral at the Department of Fisheries Annual Seminar between 17-19 September 1997 in Bangkok, Thailand.

Twenty strains of unidentified *Aeromonas* (15 virulent strains and 5 non-virulent strains) were sent to the Institute of Aquaculture, Stirling for identification using the polymerase chain reaction (PCR) technique.

I.2.   Survey of bacterial diseases of tadpoles

An investigation of bacterial species from eggs and 1-7 day old tadpoles from the AAHRI hatchery and various private farms in Nakorn Pathom and Petchaburi Provinces was conducted. Isolation and identification of bacteria was carried out using standard bacteriological techniques. Species of bacteria from eggs and tadpoles from the AAHRI hatchery, 5 farms in Nakorn Pathom and 5 farms in Petchaburi Provinces are shown in Table 1.

Table 1 Bacteria isolated from eggs and tadpoles

Rana tigerina	**Bacterium**
Egg	*A. hydrophila*
	A. sobria
	Pseudomonas sp.
	Pseudomonas putida
	Proteus sp.
Tadpoles	*A. hydrophila*
	A. sobria
	Pseudomonas sp.
	Pseudomonas putida
	Pseudomonas fluorescen
	Proteus sp.
	Providencia rettgeri
	Citrobacter freundii
	Streptococcus sp.
	Flexibacter columnaris

Table 2. Water quality and number of bacteria in frog hatcheries

Hatchery	**Water quality**				**Total bacteria count**	
	pH	Alk.	Hard.	NH_3	R.S.	P.C.A.
AAHRI	7.0-7.8	90-180	90-100	0.1-0.8	10^3-10^5	10^5-10^7
Petburi	6.7-7.5	129-195	68-170	0.2-1.8	10^4-10^5	10^6-10^9
Nakorn Pathom	6.8-8.0	95-224	86-320	0.8-2.8	10^4-10^5	10^7-10^{10}

I.3   Challenge of cultured frogs (*Rana tigerina*) with *Aeromonas* spp.

Rana tigerina were challenged with two species of bacteria, *Aeromonas hydrophila* and *A. sobria* thought to be responsible for 'red leg' syndrome. Injection and immersion challenges were used in frogs of different ages.

In the immersion challenge, tadpoles at the ages of 5, 10, 15, 20, 25 and 30 days were exposed to different concentrations of both *A. hydrophila* and *A. sobria*. When the temperature was reduced to 20°C, mortalities occurred at bacterial concentrations of 1×10^6, 1×10^8, and 1×10^{10} cfu/ml. Mortalities were also induced in two groups of frogs weighing between 25 - 30g and 120 - 150 g. They were artificially wounded and exposed to 1×10^2 and

$1x10^5$ cfu/ml, at 20°C. Attempts to induce infections in frogs stressed by high levels of ammonia or pH fluctuations in combination with the bacterial challenge were not successful.

Groups of frogs weighing between 25-30 g and 120 - 150 g were injected intraperitoneally with a variety of bacterial concentrations and the LD_{50} was consistently higher for the larger frogs.

This work presented at World Aquaculture'96, held in Bangkok between 29 January and 2 February 1996 (Appendix 1)

I.4   Challange of tadpoles with *Flexibacter columnaris*

Columnaris disease, caused by a gliding bacterium *Flexibacter columnaris*, typically presents as an acute infection. An experimental challenge using this bacterium was conducted on different age groups of tadpoles (10, 20 and 30 days). In immersion challenge tadpoles were exposed to different concentrations of *F. columnaris* at 20°C and 30°C. The mortalities rate of tadpole at 20°C was higher than at 30°C. LD_{50} at 96 hours was calculated by Reed and Muench (1938) method. Attempts to induced infection in tadpoles stressed by transportation was successful.

2.   *To optimise the health status of juvenile frogs.*

2.1 LD_{50} of some bacteria on frogs.

The study on LD_{50} of bacterial isolated from frogs was conducted to assist in identifying those which are virulent. Frogs were injected intraperitoneally with bacterial suspensions at various concentrations to observe the mortality rate at 96 hours. The LD_{50} at 96 hours (28-30°C) was calculated using the Litchfield and Wilcoxon (1949) method. The results of this experiment are shown in Table 3

Table 3. LD_{50} of some bacteria on frogs

Size of frog	LD$_{50}$ (cells/ml)		
	A. hydrophila	*A. sobria*	unidentified Aeromonas
25-30 g	$4.01X10^5$	$3.96X10^6$	$5.01X10^3$
120-150 g	$8.49X10^5$	$7.6X10^6$	$1.68X10^4$

2.2   Investigation of *in vivo* virulence of bacteria on juvenile frogs

Three species of bacteria, *A. hydrophila, A. sobria* and unidentified Aeromonas isolated from eggs, tadpoles, and water from hatcheries were used for this experiment. Frogs weighing 25-30 g were immersed in 1×10^5 cells/ml at 28-30°C. The mortality rate was observed at 96 hours. The results are shown in the Table 4.

Table 4. The virulence of *Aeromonas* spp. on juvenile frog

Bacteria	Number of isolates		% virulence
	Total	Virulence	
A. hydrophila	14	4	28.57
A. sobria	20	6	30
Unidentified *Aeromonas*	22	18	81.81

Among the tested bacteria, unidentified *Aeromonas* gave the highest percentage of virulence. The extracellular product of these bacteria was determined for virulence by electrophoresis technique using 12% SDS-polyacrylamine gel and silver stain.

This work will be presented at the Department of Fisheries Annual Seminar held between 17 and19 September 1997 in Bangkok, Thailand.

2.3   The effect of some chemicals on tadpoles

The LC_{50} of formalin, povidone-iodine, benzylkonium chloride (BKC), malachite green, potassium permanganate and salt, which are the chemicals commonly used in frog farms, were determined for tadpoles aged 10, 20 and 30 days. Tadpoles were challenged with various concentrations of these chemicals and the mortality rate at 96 hours recorded. LD_{50} at 96 hours was calculated using the Litchfield and Wilcoxon (1949) method. The results are shown in Table 5.

Table 5. LC$_{50}$ of some chemicals on tadpoles

Age of tadpoles (days)	96h - LC$_{50}$ (ppm)					
	BKC	Formalin	Povidone iodine	Malachite green	Potassium permanganate	Salt
10	1.65	44.0	68.0	0.44	4.2	>10ppt
20	2.10	52.0	112	0.47	5.8	>10ppt
30	3.70	62.0	139	0.49	6.4	>10ppt

3   *To critically assess, antimicrobial chemotherapy in hatcheries*

3.1   *In vitro* susceptibility of bacterial isolates to antibacterial drugs.

In vitro drugs was tested using sensitivity discs (BBL) on Muller Hinton Agar. The clear zone diameter was measured after incubation at 30°C for 24 hours. The antibacterial drugs used for this test were oxytetracycline 30 μg, chloramphenicol 30 μg, erythromycin 15 μg, nitrofurantoin 300 mcg, sulfamethozaxole with trimethoprim 23.75 mcg/1.25 μg and oxolinic acid 2 μg. Eighty percent of bacteria isolated from tadpoles were susceptible to nitrofurantoin and oxolinic acid and 70% of bacteria were susceptible to sulfamethozaxole with trimethoprim.

3.2   The minimal inhibitory concentration (MIC) of *A. hydrophila* and *A. sobria* to oxytetracycline and nitrofurantoin.

The MIC of oxytetracycline and nitrofurantoin against 15 strains of *A. hydrophila* and 20 strains of *A. sobria* isolated from frogs was tested using the agar dilution susceptibility test (Barry, 1991) on Muller Hinton Agar. The results show that the MIC of nitrofurantoin against bacteria isolated from frogs was 40 g/ml. *Aeromonas* spp showed significant resistance to Oxytetracycline. Nitrofurantoin can be used as a chemotheraputic drug against *Aeromonas* spp infection in frogs with greater effect than oxytetracycline.

The results of this experiment were presented at the Department of Fisheries Annual Seminar in September 1995 in conjunction with the results of similar work on snakehead fish and hybrid catfish. The title of the presented paper is "Minimal inhibitory concentration against *Aeromonas hydrophila* and *A. sobria*".

Table 5. LC$_{50}$ of some chemicals on tadpoles

Age of tadpoles (days)	96h - LC$_{50}$ (ppm)					
	BKC	Formalin	Povidone iodine	Malachite green	Potassium permanganate	Salt
10	1.65	44.0	68.0	0.44	4.2	>10ppt
20	2.10	52.0	112	0.47	5.8	>10ppt
30	3.70	62.0	139	0.49	6.4	>10ppt

3   *To critically assess, antimicrobial chemotherapy in hatcheries*

3.1   *In vitro* susceptibility of bacterial isolates to antibacterial drugs.

In vitro drugs was tested using sensitivity discs (BBL) on Muller Hinton Agar. The clear zone diameter was measured after incubation at 30ºC for 24 hours. The antibacterial drugs used for this test were oxytetracycline 30 μg, chloramphenicol 30 μg, erythromycin 15 μg, nitrofurantoin 300 mcg, sulfamethozaxole with trimethoprim 23.75 mcg/1.25 μg and oxolinic acid 2 μg. Eighty percent of bacteria isolated from tadpoles were susceptible to nitrofurantoin and oxolinic acid and 70% of bacteria were susceptible to sulfamethozaxole with trimethoprim.

3.2   The minimal inhibitory concentration (MIC) of *A. hydrophila* and *A. sobria* to oxytetracycline and nitrofurantoin.

The MIC of oxytetracycline and nitrofurantoin against 15 strains of *A. hydrophila* and 20 strains of *A. sobria* isolated from frogs was tested using the agar dilution susceptibility test (Barry, 1991) on Muller Hinton Agar. The results show that the MIC of nitrofurantoin against bacteria isolated from frogs was 40 g/ml. *Aeromonas* spp showed significant resistance to Oxytetracycline. Nitrofurantoin can be used as a chemotheraputic drug against *Aeromonas* spp infection in frogs with greater effect than oxytetracycline.

The results of this experiment were presented at the Department of Fisheries Annual Seminar in September 1995 in conjunction with the results of similar work on snakehead fish and hybrid catfish. The title of the presented paper is "Minimal inhibitory concentration against *Aeromonas hydrophila* and *A. sobria*".

Dissemination of outputs

The results of this research work have provided very good information which can be utilised by frog farmers to improve management in the farms and to avoid production losses due to diseases. This information has been disseminated through newsletters (Both Thai and English) and directly to the farmers during provision of diagnostic services.

List of papers

1. Chinabut S. and S. Areekij. 1995. Comparative study on the integumentary system of tadpole and adult frog (*Rana tigerina*). Proceeding of the Department of Fisheries of Thailand Annual Seminar, 1995. Department of Fisheries. Bangkok. p.575-578.

2. Somsiri T., S. Soontornwit and S. Duangsri. 1995. Minimal inhibitory concentration of oxytetracycline and introfurantoin against *Aeromonas hydrophila* and *A. sobria*. Proceeding of the Department of Fisheries of Thailand Annual Seminar, 1995. Department of Fisheries. Bangkok. p.599-603

3. Somsiri T., S. Chinabut and S. Soontornwit. 1996. Challenge of cultured frogs (*Rana tigerina*) with *Aeromonas* spp. In Proceeding of world Aquaculture'96 The 1996 Annual Meeting of the world Aquaculture Society. (in Press)

4. Phanwichien, K. and S. Chinabut. 1996. Regeneration and repair of superficial wounds in the skin of frogs, *Rana tigerina* Cantor. In Proceeding of world Aquaculture'96 The 1996 Annual Meeting of the world Aquaculture Society. (in Press)

5. Chinabut S. and K. Phanwichien. 1996. Pathogenesis of *Aeromonas hydrophila* infection in cultured frog (*Rana tigerina*). Proceeding of the Department of Fisheries of Thailand Annual Seminar, 1996. Deparment of Fisheries, Bangkok. (in press)

6. Chinabut S. and K. Phanwichien. 1996. Comparative study of cutaneous wound healing between frog (*Rana tigerina*) and hybrid catfish (*Clarias macrocdphalus* X *C. gariepinus*). Proceeding of the Department of Fisheries of Thailand Annual Seminar, 1996. Deparment of Fisheries, Bangkok. (in press)

7. Toxicity of some chemical on *Rana tigerina* tadpoles

8. Virulence of *Aeromonas* spp. isolated from diseased frog and tadpole

9. Study on parasite and bacterial diseased of culture frog *Rana tigerina*

10. Study on pathogenesis of red leg disease in tiger frog, *Rana tigerina*

11. Health management of frogs culture (in Thai)

12. Prophylactic treatment for bacterial septicemia disease of tadpoles caused by unidentified *Aeromonas* sp.

13. Effect of oxytetracycline on different stage of tadpole

14. Study of drug resistance of bacteria in frog hatchery

15. Challenge of *Rana tigerina* tadpoles with *Flexibacter columnaris*

Remarks

7 + 8 + 9 will be presented at the Department of Fisheries Seminar from 17-19 September, 1997

10 will be presented at European Association Fisheries Pathology Conference in Edinburgh, Scotland from 14-19 September, 1997

11 Extension paper

12 - 15 are being written up as a paper to be submitted for publication in 1998

Collaborative work with IASU

Ms Margaret Crumlish, PhD student from IASU, worked at AAHRI between 24 June - 15 October 1996 to repeat some experiments and carry out further work utilising frogs bred at AAHRI.

A workshop on the health profile of farmed aquatic species (fish and frogs) was conducted at AAHRI between 25 September and 4 October with the support of Dr. V. Inglis, Ms M. Pearson and Ms M. Crumlish. The programme of workshop is attached as Appendix 3

Ms Marianne Pearson and Ms Margaret Crumlish. PhD students from IASU, worked at AAHRI between 27 April - 30 May 1997 to repeat some experiments and carry out further work utilising frogs bred at AAHRI.

APPENDIX 1

The article presented at the Fourth Asian Fisheries Forum in Beijing from
16-20 October, 1995

Disease of Cultured Frog in Thailand

Supranee Chinabut and Temdoung Somsiri

Frog culture in Thailand has been in practice for more than 20 years. The number of frog farms throughout Thailand expanded rapidly in 1992 but most farmers had no experience of frog culture and many diseases resulted from poor management. Information on tadpole and frog diseases have been gathered from various frog farms in Thailand. The diseases recorded from tadpole are Trichodiasis, Rust Disease, White Patch Disease, Infectious Dropsy, Indigestion and Gas Bubble Disease. Diseases of adult frog are Red Leg Disease, White Patch Disease, Infectious Dropsy, Intestinal rot, Pale Skin Disease, and Paralysis.

Introduction

Frog culture in Thailand has been in practice for more than 20 years. Farming techniques have been developed gradually. Recently, the success with induced spawning techniques and the availability of commercial pelleted feed stimulated the expansion of frog farming in Thailand. The culture system have been developed in a more scientific way. Concrete ponds were introduced into the farming system in the last decade. They are now accepted as a successful method of raising frogs and are widely employed by farmers. The frogs commonly farmed in Thailand are the local species *Rana tigerina* and *R. rugulosa*. The imported American bullfrog *R. catesbeiana*, which was first introduced in 1980, is mostly culture in the northern part of the country (Pariyanonth and Daorerk, 1995). There are more than 300 frog farms operating on commercial basis around the country. Most of them prefer raising local species rather than the imported one. The rapid expanded of frog culture has led to the stress-related diseases outbreak. Large scale mortality have been associated with poor management as the frog farmers had no experience of frog culture.

The diseases of frog started from the hatcheries to the growout ponds. Information on tadpole and frog diseases have been gathered from various frog farms in Thailand. Treatments and preventive methods are based on the resulting recommendations made to farmers.

Parasitic Diseases

Trichodiniasis

Clinical signs: Infected tadpoles have a thin white or opaque film of mucus and petechial haemorrhages on the body surface. In serious cases infected tadpoles exibit gill and fin rot. After infection more tadpoles will die every day. Without proper treatment 100% mortality will occur within 5-7 days.

Causative agent: This disease is caused by ciliate protozoa of the trichodinid group.

Treatment: Formalin at the concentration of 25-30 ppm should be applied on 3 consecutive days following 10% water changes.

Indigestion

Clinical signs: Infected tadpoles exhibit loss of appetite, heavy infected tadpoles show an enlargement of the digestive tract. Affected frogs show swollen abdomen, loss of appetite and lassitude. The digestive tract are enlarged.

Causative agent: Many species of ciliate protozoas are commonly found in the digestive tract of both diseased tadpoles and frogs; *Opalina* sp., *Protoopalina* sp., *Balantidium* sp., *Tritrichomonas* sp., *Nyctotherus cordiformis*.

Treatment: Metronidazole at the rate of 2-3 g/kg of feed is the drug recommended for therapeutic treatment of this disease. Infected tadpoles should be fed with medicated feed for 1 week and it is recommended that water is exchanged daily.

Rust Disease

Clinical signs: Infected tadpoles whirls and wobbles. Approximately 80% of infected tadepoles will die within 24 hr after the infection and without the proper treatment all of them will die later.

Causative agent: Flagelate protozoa, *Oodinium* sp. or *Amyloodinium* sp. are observed from gill and mucus of diseased tadpoles.

Treatment: Treatment with 1 % table salt should be applied on 3 consecutive days following 10% water changes.

Bacterial Diseases

White Patch Disease

Clinical signs: Both tadpoles anf frogs exhibit white patches on the body surface. Heavily infected tadpoles will stay immobile at the bottom of the pond.

Causative agent: Thia disease is caused by the bacterium, *Flexibacter columnaris*, which is commonly known as Columnaris, and usually occurs in ponds with poor water quality.

Treatment: Water should be changed and salt added at a concentration of 0.5%. KMnO4 at the concentration of 2-4 ppm is also recommended for the prolong bath treatment.

Infectious Dropsy

Clinical signs: Infected tadpoles have clear or yellow fluid in the abdomen and haemorrhagic lesions are spreaded over the entire body surface. Death usually occurrs within 24 hours.

Causative agent: Gram negative bacteria, *Aeromonas hydrophila* is isolated from the fluid of infected tadpoles.

Treatment: Water should be changes. Antibiotic, oxytetracycline or nitrofurantoin are commonly applied to the pond as a bath treatment at a concentration of 10-30 ppm for 14 days.

Red-leg Disease

Clinical signs: The external signs of red leg disease include ascites, loss of appetite, lassitude and haemorrhagic lesions on hind legs and abdomen. Internal sign are haemorrhage in most of the viseral organs. Blood and yellow fluid are often found in the abdominal cavity.

Causative agent: *Aeromonas hydrophila* and *A. sobria* have been isolated from diseased frogs. This disease is a good example of a stress-related disease, overcrowding and poor water quality seem to be the major factors involved.

Treatment: The feeding rate should be decreased and 0.5% salt added to the pond. Use of oxytetracycline and nitrofurantoin at a rate of 3-5 g/kg of feed/day for 7-14 days has been a successful in controlling the disease.

Intestinal Rot

Clinical signs: Affected frogs exhibit swollen abdomen, loss of appetite and lack of movement. The intestine is enlarged and the lumen contains a clear liquid and undigested food. Protrusion of posterior intestine from cloaca may be found in some affected frogs.

Causative agent: As yet unknown, but most of the affected farms use decayed chicken offal and trash fish as frog feed.

Treatment: Farmers should stop feeding the frogs for 3-5 days, change 50% of the pond water and add 0.1% salt to the pond. Feeding should then changed to a commercial pellet or fresh trash fish/fresh chicken offal. Oxytetracycline should be mixed with the diet at a rate of 3-5 g/kg of feed for 7 days.

Non-infectious Diseases

Gas Bubble Disease

Clinical signs: Affected tadpoles have a swollen abdomen and floating at the water surface.

Causative agent: This disease is caused by insufficient water changes and usually occurs in farms the use fresh unaerated supersaturated underground water as a supply.

Treatment: There is as yet know effective treatment for this disease. The best method is prevention. Underground water should be held and aerated for at least one day before use. Water exchanges should not be carried out too quickly.

Pale Skin Disease

Clinical signs: Diseased frogs exhibit loss of colour, sluggishness and loss of appetite. Death usually occurs within one week.

Causative agent: Poor water quality especially low pH water (<5)

Treatment: Use lime to increase the pH of the water in the pond.

Paralysis

Clinical signs: Affected frogs cannot move as hind legs have atrophied. The liver has degenerated and body weight is decreased. Mortality rate is around 60-80%.

Causative agent: As yet unknown.

Treatment: Vitamin B complex should be mixed with the diet at a rate of 1 g/kg of feed/day for 1-2 weeks.

References

Putsatee Pariyanonth and Veerote Daorerk. 1995. Frog farming in Thailand. INFOFISH International Vol.3, p 25-28.

APPENDIX 2

<div align="center">

Comparative study on the integumentary system

of tadpole and adult frog (*Rana tigerina*)

</div>

<div align="center">

Supranee Chinabut

Suparporn Areekij

</div>

<div align="center">

Abstract

</div>

The integumentary systems of tadpoles and adult frogs is composed fo skin which covers the whole body. This skin is composed of the 3 layers, epidermis, dermis and hypodermis. The epidermis of 1-19 day-old tadpoles is composed of cell layers as follow : the outermost is a layer of apical cells with microvilli on the surface, followed by a skein cell layer and basal cell layer respectively. By 20 days old, tadpoles have developed skin similar to the adult frog. The apical cessl differentiate to become erithelial cell. The skein cells and basal cells differentiate to become stratum granulosum and stratum germinativum respectively. The stratum corneum can be first noticed in 20 day old tadpoles. The dermis of tadpoles is thinner than the of the adult frog and cannot be differentiated into stratum spongiosum and stratum copactum. Mucous and serous glands can be first noticed in the dermis of 30 day old frogs.

APPENDIX 3

The article presented at Department of Fisheries of Thaiand Annual Seminar, 1995

Minimal inhibitory concentration of oxytracycline and nitrofurantoin against
Aeromonas hydrophila and *A. sobria*

Temdoung Somsiri

Suriyan Soontornwit

Seksan Duangsri

Abstract

Minimal inhibitory concentration (MIC) of oxytetracycline and nitrofurantoin against 33 of Aeromnoas hydrophila and 48 strains of A. sobria isolated from frog, snakehead fish and hybrid catfish and hybrid catfish were tested. MIC of oxytetracycline against bacteria isolated from snakehead fish was 50 yg.ml and MIC of nitrofurantoin against bacteria isolated from frog was 40 yg/ml. Oxytertracycline showed highly resistace to Aeromonas sp. Nitrofurantoin cna be used as chemotherapuetic drug for Aeromonas sp. infection in frog, snakehead and hybrid catfish better than oxytetracycline.

APPENDIX 4

The article presented at the 1996 annual meeting of the World Aquaculture Society in Bangkok from 29 January-2 February 1996

Challenge of cultured frogs (*Rana tigerina*) with *Aeromonas* spp.

Temdoung Somsiri*, Supranee Chinabut and Suriyan Soontornvit

Abstract

Rana tigerina were challenged with two species of bacteria, *Aeromonas hydrophila* and *A. sobria* thought to be responsible for 'red leg' syndrome. Injection and immersions challenges were used in frogs of different ages.

In the immersion challenge, tadpoles at the ages of 5, 10, 15, 20, 25 and 30 days were exposed to different concentrations of both *A. hydrophila* and *A. sobria*. When the temperature was reduced to 20°C mortalities occurred at bacterial concentrations of 1×10^6, 1×10^8, and 1×10^{10} cfu/ml. Mortalities were also induced in two groups of frogs weighting between 25-30g and 120-150g. They were artificially wounded and exposed to 1×10^2 and 1×10^4 cfu/ml, at 20°C. Attempts to induce infections in frogs stressed by high levels of ammonia or pH fluctuations in combination with the bacterial challenge were not successful.

Groups of frogs with weighting between 25-30g and 120-150g were intraperitoneally injected with a variety of bacterial concentrations and the LD_{50} was consistently higher for the larger frogs.

Key word : *Rana tigerina, Aeromonas hydrophila, A. sobria*

Introduction

Frog culture expanded rapidly throughout Thailand in 1994 due to the tremendous increase in demand from the international market for frog legs. Since then many farmers businesses have failed as they did not have experience in frog culture. The other limiting factor in frog production is bacterial disease. Red leg syndrome (Tayler, S, unpubl. data) is a serious septicaemic infection of wild and cultured frogs. *Aeromonas hydrophila* and *A. sobria* have often isolated from diseased frogs with the distinct clinical signs of this disease, petechial haemorrhages of thighs and abdomen (Nymen, 1986). Outbreaks of this disease are often associated with water pollution, sudden changes in environmental factors and other stressful conditions.

The purpose of this study was to evaluate the effect of bacterial concentrations, temperature, pH and ammonia levels on susceptibility to bacterial infection of tiger frogs (*Rana tigerina*) of different ages.

Materials and methods

Experimental animals

Tadpoles at the ages of 5, 10, 15, 20, 25 and 30 days, and frogs with weights between 25-30 g and 120 - 150 g were obtained from the Aquatic Animal Health Research Institute's hatchery. They had been reared in dechlorinated water at 28-32°C, with daily water changes. The tadpoles were fed with waterflea and commercial powder feed. Frogs were fed with commercial pellet which contained 30% protein.

Challenge bacterium

The bacteria used for challenging the frogs were *A. hydrophila* strain no. 94/B4 and *A. sobria* strain no. 94/B11. These were isolated in 1994 from diseased frogs with clinical signs of red leg syndrome. Stock cultures of these bacteria were kept on glass beads at -70°C. The bacteria were grown on Tryptic Soy Agar (TSA) at 30°C for 18 hours and a suspension of bacteria mixed with a suitable volume of dechlorinated water for immersion challenge or 0.85% sterile NaCl for injection challenge. The numbers of bacteria in the challenge suspension were estimated by the drop plate method. Briefly, each sample of suspension of 10 µl spotted on

TSA (10 drops per plate). Colonies of bacteria were counted after incubation at 30°C for 18 hours.

Susceptibility studies

Immersion challenge

Tadpoles of different ages were immersed in 3 L of challenge suspension containing 1×10^6, 1×10^8 or 1×10^{10} cfu per ml for 4 days. Frogs of different weights, both experimentally wounded (5 mm surgical wound was made on left hind leg of each frog) and unwounded, were exposed to 1×10^2 and 1×10^4 cfu per ml in 10 L. of challenge suspension for 4 days.

pH

Tadpoles and frogs were immersed in dechlorinated water with a pH 6, 7 and 8, adjusted with 1N NaOH and 1N H_2SO_4.

Temperature

The temperatures used for this experiment were 20 and 30°C.

Ammonium level

The ammonium concentrations of challenge suspension were adjusted to 0, 2 and 4 ppm. using NH_4Cl and NH_4OH (Sprague, 1969)

- Each experimental trial had two replications, with 20 tadpoles or frogs per replicate. Control groups were not challenged with any bacteria. Mortalities and clinical signs were recorded daily for four days. Kidney samples of moribund frogs were taken for bacteriological cultivation on Rimler-Shotts medium.

Injection challenge

Virulence assays were performed to compare the susceptibility to *Aeromonas* spp. of frogs weighing 25 - 30 g and 120 - 150 g. Experimental frogs were intraperitoneally injected with a variety of bacterial concentrations. Mortalities at 24, 48 and 96 hours were observed and median lethal dose (LD_{50}) was determined by the method of Litchfield and Wilcoxon (1949).

Results

In immersion challenges, no mortalities were observed in both tadpoles and frogs stressed by high levels of ammonia or pH fluctuations in combination with various concentrations of bacteria. No bacteria were isolated from the kidneys of survivors at the end of the experiment. Tadpoles aged of 5, 10, 15, 20, 25 and 30 days were exposed to bacterial concentrations of 1×10^6, 1×10^8 and 1×10^{10} cfu/ml and the temperature reduced to 20°C (Table 1).

Frogs weighting between 25-30 g and 120-150 g were wounded and exposed to 1×10^2 and 1×10^4 cfu/ml at 20°C also suffered mortalities (Table 2). Unwounded frogs suffered no mortalities.

The LD_{50} for *A. hydrophila* and *A. sobria* injected intraperitoneally are shown in Table 3. The first death occurred at 12 h post injection. The frogs that survived after 96 h. appeared to recover, judged by the normal behaviour observed. Signs of moribund frogs included light-reddish fluid in the body cavity and haemorrhage on the skin of abdomen and thigh.

Table 1 Effect of temperature on mortality rate of tadpoles experimentally infected with *A. hydrophila* or *A. sobria*.

Pathogen	Water temp. (°C)	Age of tadpoles (days)	Average mortality (%)		
			1×10^6 cfu/ml	1×10^8 cfu/ml	1×10^{10} cfu/ml
A. hydrophila	20	5	12.5	50.0	100.0
		10	10.0	77.5	100.0
		15	22.5	75.0	100.0
		20	25.0	85.0	100.0
		25	20.0	72.5	100.0
		30	10.0	60.0	100.0
	30	5-30	0	0	0
A. sobria	20	5	30.0	40.0	95.0
		10	32.5	40.0	92.5
		15	35.0	40.0	90.0
		20	40.0	50.0	100.0
		25	32.5	42.5	85.0
		30	20.0	22.5	80.0
	30	5-30	0	0	0

Table 2 Effect of temperature on mortality rate of frogs experimentally infected with *A. hydrophila* or *A. sobria*.

Pathogen	Water temp. (°C)	Weight of frogs (g)	Average mortality (%) 1×10^2 cfu/ml	1×10^4 cfu/ml
A. hydrophila	20	25-30	10.0	77.5
		120-150	7.5	20.0
	30	25-30	0	20.5
		120-150	0	0
A. sobria	20	25-30	12.5	50.0
		120-150	7.5	10.0
	30	25-30	2.5	5.0
		120-150	0	0

Table 3 LD$_{50}$ of frogs challenged with *Aeromonas* spp.

Pathogen	Size of frog (g)	LD$_{50}$ (cells/ml) 24 hrs	48 hrs	96 hrs
A. hydrophila	25 - 30	4.20×10^7	3.95×10^7	4.01×10^5
	120 - 150	8.34×10^7	4.56×10^7	8.49×10^5
A. sobria	25 - 30	5.82×10^7	4.80×10^7	3.96×10^6
	120 - 150	8.44×10^7	6.72×10^7	7.60×10^6

Discussion

In the immersion challenge, tadpoles at the age of 5, 10, 15, 25, 20, 25 and 30 days were exposed to different concentrations of *Aeromonas* bacteria suspension, mortalities occurred at bacterial concentrations of 1×10^6, 1×10^8 and 1×10^{10} cfu/ml when the temperature was reduced to 20°C. Mortalities also occurred in two groups of frogs which were wounded and exposed to 1×10^2 and 1×10^4 cfu/ml at 20°C. Groups of frogs which were not wounded but exposed to the same concentrations of bacteria and temperature suffered no mortalities. These results indicate that susceptibility to *A. hydrophila* and *A. sobria* of tadpoles is affected by temperature. Neither high levels of ammonia or pH fluctuation caused disease in experimental animals. Adult frogs require both stress by low temperature and physical injury before infection occurs. This is possibly due to the fact that the integumentary system of frogs is more advanced than that of tadpoles. Chinabut and Areekij (1995) reported that the dermis of tadpoles (*Rana tigerina*) is thinner than that of adult frogs and mucous and serous glands are first noticed in the dermal layer of 30 days old frogs. Du Pasquier *et al* (1989) studied on immune system of South African frog (*Xenopus* sp.) and found that there was a period of vulnerability to infection just prior to metamorphosis and during the metamorphosis due to switching from larval to adult immune system. In comparable to fish, there were few reports mentioned that at low temperature, the immune and inflammatory response of fish is considerably reduced (Avtalion 1969 ; Avtalion, Wichkovsky & Katz 1980 ; Fryer, Pitcher, Sanders, Rohovec, Zinn, Groberg & McCoy 1976 ; Paterson & Fryer 1974).

From Table 1, the groups of 20 day old tadpoles showed more susceptibility to bacterial infection than other groups. Somsiri *et al* (1995) found that the appendages of tadpoles first appear at 20-23 days and tadpoles often sit at the water's edge. This stage may be a critical period for their health. Tadpoles may expend a great deal of energy completing metamorphosis and this may suppress their immune response.

Hird *et al* (1981) found *A. hydrophila* to be widespread in apparently healthy, wild-caught adult and larval *Rana pipiens* in Minnesota, but the bacterium was more prevalent in tadpoles than in adults. Frogs have a high risk of exposure to this bacterium as *Aeromonas* spp is commonly found in water and sewage. Outbreaks of aeromonad septicaemia have been

variously associated with low temperatures, physical injury, extreme crowding and other stresses (Schott *et al*,1972).

In the intraperitoneal injection challenge, the LD_{50} was consistently higher for larger frogs than for smaller frogs, probably due to more advanced development of the immune system. The immune mechanisms of tiger frog are studying.

A. hydrophila and *A. sobria* both induced the symptoms of red leg syndrome. Age of tadpoles or frogs and water temperature are considered to be the important factors determining severity of the syndrome. Frog farmers should give more attention on take care of tadpoles during the period of metamorphosis due to changing from tadpole to young frog and avoiding stressing frog during the critical period is recommended.

Reference

Avtalion, R.R. 1969. Temperature effect on antibody production and immunological memory in carp (*Cyprinus carpio*) immunised against bovine serum albumin (BSA). Immunology 17 : 927-931

Avtalion, R.R., A. Wichkovsky, and D. Katz, 1980. Regulatory effect of temperature on specific suppression and enhancement of the humoral response in fish. pp. 113-121 *In* M.J. Manning (ed.) Phylogeny of Immunological Memory, North-Holland Elsevier, Amsterdam.

Chinabut, S and S. Areekij, 1995. A comparative study of the integumentary system of tadpoles and adult frogs (*Rana tigerina*). The Seminar on Fisheries, 1995, DOF, Thailand (in press)

Du Pasquier, L., J. Schwager and M.F. Flajnik, 1989. The immune system of *Xenopus*. Ann. Rev. Immunol. 7:251-275

Fryer, J.L., K.S. Pitcher, J.E. Sanders, J.S.Rohovec, J.L. Zinn, W.J. Groberg and R.H. McCoy, 1976. Temperature, Infectious Diseases and the Immune Response in Salmonid fish. U.S. Department of Commerce. National Technical Information Service, PB.253191.

Hird, D.W., S.L.Diesch, R.G. McKinnell, E. Gorham, F.B. Martin, S.W. Kurtz, and C. Dubrovolny. 1981. *Aeromonas hydrophila* in wild caught frogs and tadpoles (*Rana pipiens*) in Minnesota. Lab. Animal Sci. 31 : 166 - 169

Litchfield, J.T. and F.W. Wilcoxon, 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96 : 99 - 113

Nyman, S. 1986. Mass mortality of larval *Rana sylvatica* attributable to the bacterium *Aeromonas hydrophila*. Journal of Herpetology 20 : 196-201

Paterson, W.D. and J.L. Fryer. 1974. Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* endotoxin. Journal of Fisheries Research Board of Canada 31 : 1743-1749

Schotts, E.B., Jr., J.L. Gaines, L. Martin and A.K. Prestwood. 1972 Aeromonas induced deaths among fish and reptiles in an entrophic inland lake. J. Amer. Vet. Med. Assoc. 161 : 603 - 607

Somsiri, T., S. Chinabut, S. Soontornwit and S. Areekij 1995. A study of the development of frog's eggs to larval stage. Thai Fisheries Gazette 48 : 41 - 46

APPENDIX 5

The article presented at the 1996 annual meeting of the World Aquaculture Society in Bangkok from 29 January-2 February 1996

Regeneration and Repiar of Superficial Wounds
in the Skin of Frogs, Rana tigerina Cantor

Kantimanee Phanwichien* and Supranee Chinabut**

Abstract

Incisions, approximately 0.7 -1.0 cm long, 0.2 cm wide and 0.2 cm deep, were made on the ventral left thigh of frogs. Following injury, within 1 hr fibroblast sheet occurred to act as the temporary clouser of the wound. By 1 hr after incision the epidermis detached from the underlying basement membrane and migrated as a sheet toward the wound gap. Re-epithelialization occurred by the fusion of the thin epithelial layer protruded from the fixed zone of both margins of the wound and completed within 18 hr after incision. The injured muscle was replaced by the proliferation of fibroblasts by day 3 after incision. The fibroblast replacement was gradually reduced, whereas the regeneration of muscle developed. Repair of the incised skin and the injured muscle completed by day 26 and day 43, respectively without any sign of external scar, except the newly formed fascia left from the reduction of granulation tissue which could be an indicator of the wounded area.

Keywords: Wound healing, Frog, *Rana*

Regeneration and Repiar of Superficial Wounds
in the Skin of Frogs, *Rana tigrina* Cantor

Kantimanee Phanwichien[*] and Supranee Chinabut[**]

[*] Department of Pathobiology, Faculty of Science, Mahidol University,
Rajathevi, Bangkok , 10400 Thailand.

[**] Aquatic Animal Health Research Institute, Department of Fisheries,
Kasetsart University Campus, Chatuchak, Bangkok 10900, Thailand.

Introduction

Wound healing in vertebrates is a complex phenomenon involving three phases; acute inflammation phase, proliferation phase, and remodelling phase (Cotran et al. 1994).

Rana tigrina is now becoming widely cultured in Thailand as a commercial animal and is frequently used as an experimental animal. The skin of cultured frogs in the tank can be easily injured by biting each other because of their carnivorous feeding behavior. Damaged skin is known to be the major route of entry for many kinds of pathogens or the animal may rapidly die due to extensive loss of body fluids and resulting osmotic effects (McVicar & White 1979; Stiffler 1988). Thus it is important that such wounds heal rapidly, or at least that the effective barriers are provided as soon as possible to maintain the osmotic balance. To our knowledge, the regeneration and repair of superficial wounds has not been reported in the frog. Therefore, knowledge of the process of wound healing is important for maintaining cultured frogs in a healthy condition.

This study was investigated in order to define the process by which the frog could heal small superficial wounds which were not complicated by secondary infection.

Materials and Methods

Animals and aquaria holding

Adult healthy frogs, weighting btween 100-170 g obtained from a private farm at Prathumthani Province, Thailand, were used as experimental animals. A total of 120 frogs of both sexes were acclimatized in glass aquaria (45x100x45 cm) in group of 20 animals per aquarium for 2 weeks. Frogs were fed once daily with a nutritionally floating pellet. Well-cleaned aquaria contained tap water of pH 7.2-7.4 to prevent water-borne pathogens of frogs that might contaminate from natural sources. Water was changed once daily, and average water temperature was 28°C.

Wounding procedure

A small longitudinal surgical incision approximately 0.7-1.0 cm long, 0.2 cm wide, and 0.2 cm deep was made into the ventral myotomal muscle of left thigh of each frog while it was anesthetized by chloroform (Fig. 1). This position was selected because the thigh anatomically was considered to have greatest abundance of muscle bundles, and to have smooth skin. The unwounded right thigh was used as a control sample. All frogs were returned to aquaria for further observation.

Sampling procedure

Three incised frogs were anesthetized by chloroform and were sacrificed routinely at 15, 30, 45 min, every hour from 1 to 10 hr; 12, 14, 16, 18, 20 and 24 hr; daily from day 2 to day 10 and for other intervals at day 12, 14, 16, 18, 20, 24, 26, 36 and 43. The cross section of the injured site and the control right thigh were dissected out and fixed in 10% buffered formalin.

Histological porcedure

The fixed cross section was decalcified to make the bone softer. The incised wound was equally divided into three parts; two end parts, and a central part. Only the central part of wound was selected to ensure uniform sampling and to avoid inaccurate impressions of the faster rate of epithelialization which normally occurred near the end of the incised wound. Paraffin infiltrated blocks were serially sectioned at 5 μm and stained with hematoxylin and eosin and to define collagen fiber Van Giesson Masson's Trichrome was used.

Results

I. Regeneration and repair of the epidermis :

15 min to 2 hr

After a period of inactivity of nearly 1 hr after incision, basal cells adjacent to both cut edges enlarged and flattened and then released their usual firm anchorage on the basement membrane resulting in the lifting up of the epidermis from the dermis. Hyperemia was observed in the hypodermis.

3-18 hr

By 3 hr after incision, the excessive number of epidermal cells migrated as a sheet over the inverted dermis. By 8 hr the migrating epidermis with cornified layer already fixed on the basement membrane. The mitotic figure of basal cells in a zone very near the cut edge of inverted epidermis was observed. Migration of epithelial cells from zone of mitosis at both margins was revealed in the thin layers without basement membrane and cornified layer by 9 hr after incision (Fig. 1) and bridged over the wound gap about 18 hr (Fig. 2).

20 hr - 2 days

By 22 hr after incision the newly joined epidermis was thickened which revealed about 8 layers. Newly formed glands observed in the stratum spongiosum by 18 hr completely developed to mature glands by 20 hr. By day 2 the rejoined epidermis revealed the stratum corneum.

3 - 4 days

By day 3 after incision fibroblast proliferation was seen in the breach of the dermis beneath the new epidermis. By day 4 both cut edges of the dermis and the hypodermis were linked by active fibrous tissue in the parallel direction, which had been formed by fibroplasia at day 3. The thickened edematous epidermis partly attached to the fibrosis area of the dermis. The irregular arrangement of fibroblasts in the fibrous tissue replacement of the dermis extended down to join with the underneath fibrous tissue replacement of the muscle lesion. The fibrous tissue replacement referred to as granulation tissue which was due to the proliferation of new blood vessels (Fig. 3).

5 - 7 days

By day 5 after incision fibroblasts in the granulation tissue became more compact and appeared to arrange themselves parallel to the body surface. The size of granulation tissue was decrease by day 7. Wound contraction occurred at this time and caused the mutual approchment of the dermis.

8 - 10 days

By day 8 after incision, the completely rejoined dermis had already separated from the fibromuscular granulation tissue. There was a newly formed fibrous sheet, or fascia, left from the reduction of the granulation tissue in order to hold the skin and the

subcutaneousfascia over the musculature (Fig. 4). In fact, such fascia located only along both sides of the frog body.

12 - 36 days

By day 12 after incision the stratum spongiosum at the rejoined area was slightly edematous, and revealed the normal appearance by day 16. By day 26, the epidermis, dermis and glands had become fully developed and by day 36 the skin lesion appeared normal.

II. Regeneration and repair of the muscle :

15 min - 1 hr

During this time there was a minimal ooze of blood and exudate from the injured vessels covering the surface of exposed muscle. Dilated blood vessels in the injured areas were gorged with erythrocytes and numbers of thrombocytes. Myopathy from the incision was observed restrictly to the edge of the lesion. Strands of fibroblasts migrating from both exposed edges by 45 min after incision were fused to form a continuous sheeth by 1 hr that acted as a temporary closure of the defect.

2 - 4 hr

Neutrophils were increased in number both in the clotted blood and in the injured muscular areas by 2 hr after incision. Few macrophages were found in the injured areas by 3 hr but myophagia could not be observed. New capillaries were observed in fibrotic areas of the injured muscle by 4 hr after incision.

5 - 9 hr

Active myophagia was observed by 5 hr in areas of the injured muscle and was very active by 7 hr. By 6 hr fibroblasts proliferated since 4 hr at the subcutaneous fascia join each other resulting in the closure of the wound gap (Fig. 5).

10 - 22 hr

By 10 hr after incision, myophagic areas was decreased, so that the area of muscle damaged appeared smaller. The appearance of the virtual subcutaneous fascia occurred and the wound gap was disappeared as well. By 16 hr, myofibrils which had been only lightly damaged started to redevelop their peripheral sarcolemmal tubes. These tubes connected to other bundles. Nuclei of muscle fibers were observed migrating along these tubes. New small muscle buds were also seen in small areas where myophagia debridement was complete.

1 - 3 days

The apparent fibroplasia exhibited over the exposed portion of the injured muscle by day 2 after incision. By day 3, lymphocytes were observed in the fibroplastic area as well as the regenerating muscle fibers with round multinuclei derived from the syncytial muscle fibers adjacent to the level of uninjured muscle bundles.

4 - 7 days

By day 4 after incision, granulation tissue was observed and gradually became more compact by day 5. By day 5, there were marked mix of fibrosis, very small areas of regenerating muscle, new muscle buds and new blood vessels in this stage. In addition, macrophages containing lipofuscin in their cytoplasm were observed until day

7 after incision. By day 7, the numbers of macrophages in the granulation tissue was disappeared including lymphocytes and new blood vessels.

8 - 16 days

By day 8 after incision, new muscle bundles became more distinct and extensive. The muscle extension occurred across the lesion into the empty spaces and more obvious by day 16 (Fig. 6).

18 - 43 days

There was little change occurred in this period. The regenerated muscle bundles filled only half of the lesion, but the fibrosis was decreased by day 26 after incision. There were tiny muscle bundles located in the subcutaneous fascia (Fig. 7). By day 43, the muscle lesion revealed the fullness of muscle bundles (Fig. 8). The fibrous tissue, or muscle scar, could not be observed by the end of wound healing. Nevertheless, there was no external evidence of scaring, or any other sign of wound healing.
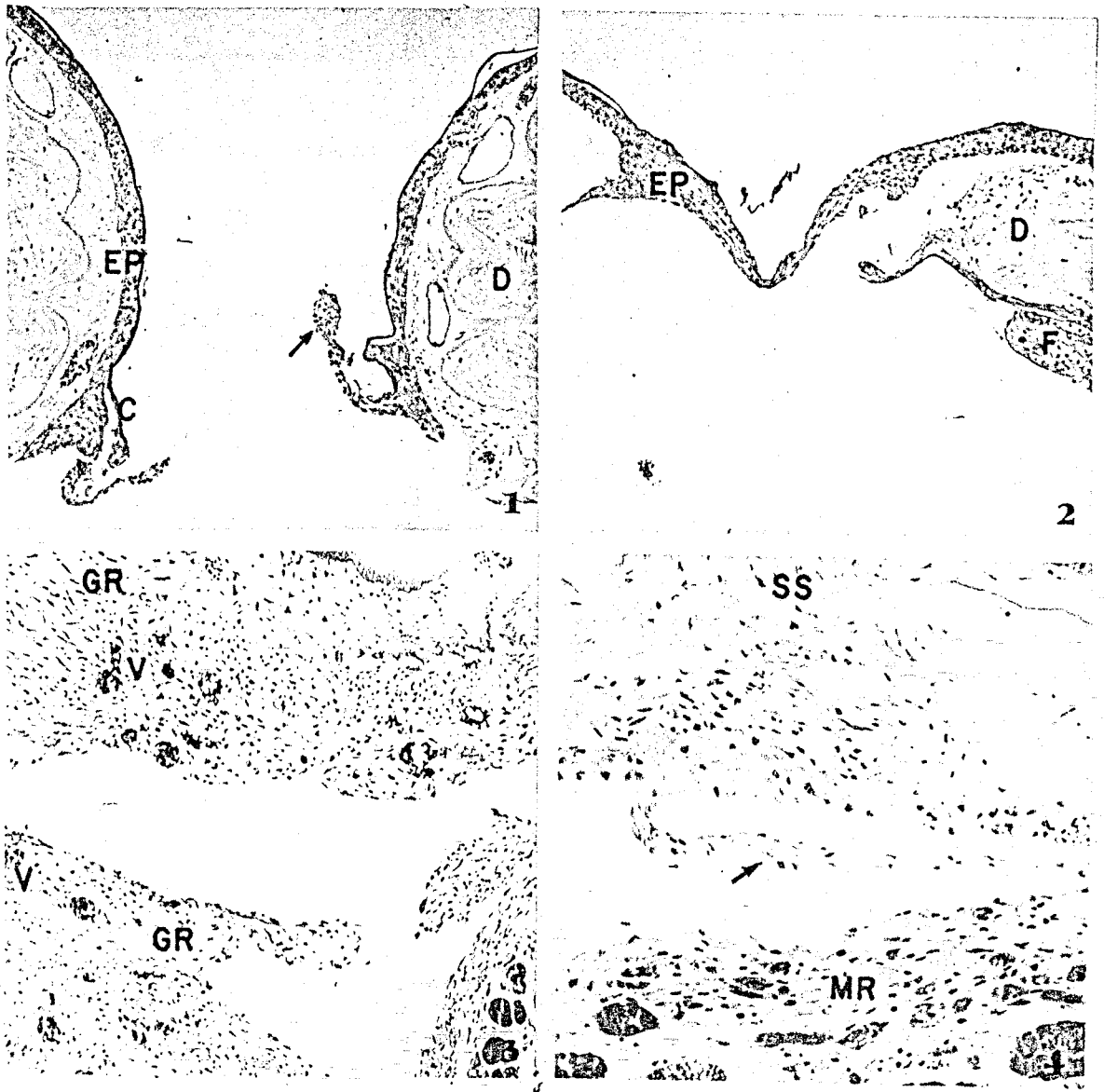
Fig. 1   The protrusive epidermal sheets (arrow) without cornified layer were marked by 9 hours after incision. EP = epidermis, C = cornified layer, D = dermis   H&E x 260

Fig. 2   By 18 hours after incision the epithelialization was already completed without either cornified layer and basement membrane. An accumulation of fibroblasts in extracellular matrix (F) located beneath the hypodermis. EP = epidermis, D = dermis   H&E x 260

Fig. 3   The early stage of granulation tissue (GR) by day 4 after incision showing abundant new blood vessels (V) filled with erythocytes. Masson's Trichrome x 33

Fig. 4   Edematous stratum spongiosum (SS) of the completely rejoined dermis was obvious by day 9 after incision. Arrow = new fascia, MR = regenerating muscle area   H&E x 340
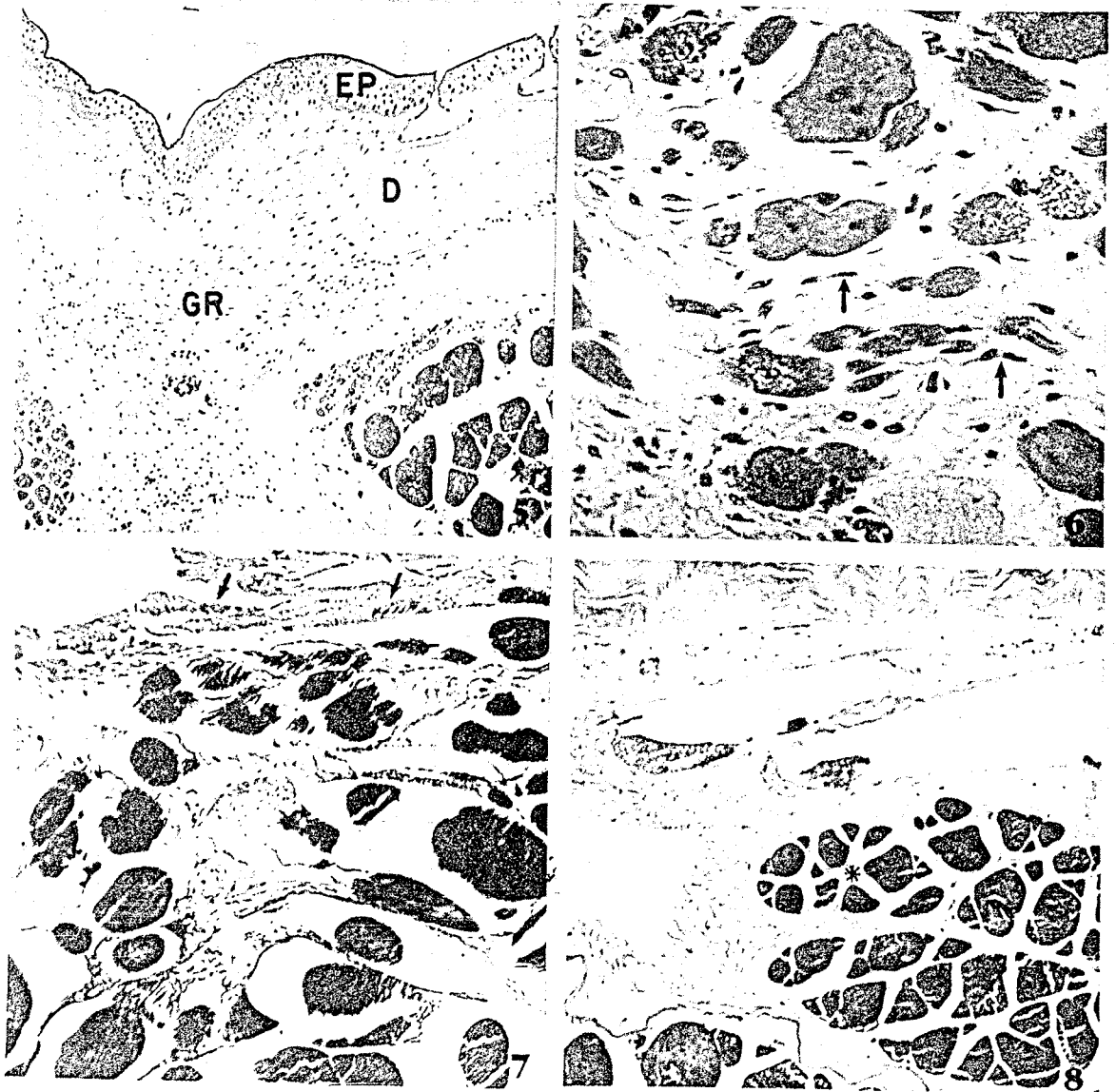
Fig. 5   Reduction of granulation tissue (GR) by day 7 after incision. Two cut edges of the dermis (D) were nearly joined. The rejoined epidermis (EP) was slightly thicker. H&E x 260

Fig. 6   The extensive muscle bundles comprised more than half of the lesion by day 16. Regenerative muscle fibers (arrow) were also present in the fibrotic area. H&E x 340

Fig. 7   The very distinct tiny muscle fibers (arrow) were observed in the subcutaneous fascia. Masson's trichrome x 33

Fig. 8   The muscle lesion (*) by day 43 after incision revealing the normal appearance. H&Ex33

Discussion

Soon after the incision in the skin of frogs, blood from the injured vessels plugged the wound which acted as the first temporary closure of the wound. The second temporary closure of the wound occurred in the wound gap was observed by 1 hr after incision by the fusion of the migrating fibroblastic sheath which has not been reported in any animals. The temporary closure of the wound is important in the prevention of the tissue fluid loss which caused the imbalance of body osmoregulation (Cotran et al 1994).

Following injury, epidermal cells start migrating and this results in the bridging of the incised skin which makes the wound gap close. In this study, only the thin epithelial layer moved over the breach and bridge over the wound gap. Many investigators in this field reported that the migration of epithelial layers in fishes (Mittal & Munshi 1974; Chinabut 1989) and mammals (Hunt 1990; Cotran et al 1994) turned downward to the depth of the wound and covered the wound floor by joining together. This different finding in the present study may be due to the natural separation of frog skin from the musculature underneath except at the proper position. This characteristic makes both edges of the incised wound free so that the clotted blood and exudate are easily washed away. The only way to close the wound gap by lacking exudate and fibrin, which the epidermis moves initially through (Damjanov 1996), is to protrude the proliferating epithelial cells from the fixed zone on both margins. Neufeld and coworkers (1996) reported that the advancing cells appeared to be supported only by desmosome contact between adjacent epithelial cells.

The closure of the wound gap in this study occurred by 10 hr after incision by wound strength, or tensile force produced by proliferated fibroblasts in the subcutaneous fascia and in the muscle lesion. Contraction in small wounds has been found to take place without formation of collagen (Peacock 1984).

The occurrence of parallel arrangement of fibroblasts found in the compact granulation tissue by day 5 after incision can explain by the study of Welch and his colleagues (1990). They found that the present of linear bundles of F-actin along the peripheral cytoplasm of all fibroblasts of older granulation tissue caused the parallel arrangement of fibroblasts. Moreover, fibronectin, or deposit connective tissue matrix, was pulled inward by fibroblasts causing the reduction of granulation tissue which resulted in the wound contaction.

The repair of the injured epidermis was completed within 5 days after incision, before the repair of the dermis took place. This evidence can be explained involving the basement membrane and dermal fibers during regeneration. Neufeld and his colleagues (1996) reported that dermal cells of the injured skin did not synthesize tenascin, an extracellular matrix molecule that characterized damaged or rapidly growing tissue, whereas the epidermal cell had this character (Onda et al 1990). So epidermal cells have the ability to migrate and grow, whereas dermal fibers cannot. The new basement membrane, which formed in continuity with the old, provided the foundation and necessary stimulus for dermis formation from the underlying presumtive cells.

Conclusion

Wound healing in frogs involves three phases. The acute inflammatory phase is marked by thrombocyte accumulation, coagulation and leukocyte infiltration. The prolieration phase is characterized by re-epithelialization which completed within 18 hr, angiogenesis, fibroplasia, and wound contraction. Finally, the remodelling phase takes place by the regeneration of muscle over a period of time.

The temporary closure of the wound is characterized by the fibrous sheet and the closure of the wound gap in muscle lesion is taken place by the tensile force produced by proliferated fibroblasts of the subcutaneous fascia.

A new phenomenon revealed in this study is a newly formed fascia which holds between the skin lesion to the muscle lesion.

Acknowledgement

References

Chinabut, S. 1989. Studies on the inflammatory response of the striped snakehead, *Channa striatus* (Fowler). Ph.D. thesis, Institue of Aquaculture, University of Stirling.

Cotran, R.S., V. Kumar and S.L. Robbins. 1994. Robbins Pathologic Basis of Disease. 5[th]ed., W.B. Saunders Company, Philadelphia, 1024 pp.

Damjanov, I. 1996. Histopathology: A Color Atlas and Textbook. Williams & Wilkins, New York, 306 p.

Hunt, T.K. 1990. Basic principles of wound healing. Journal of trauma 30(Suppl): s122 - s128.

McVicar, A.H. and P.G. White. 1979. Fin and skin necrosis in cultivated Dover sole, *Solea solea* (L.). Journal of Fish Disease 2: 557-562.

Mittal, A.K. and J.S.D. Munshi. 1974. On the regenertion and repair of superficial wounds in the skin of *Rita rita* (Ham.) (Bagridae, Pisces). Acta Anatomica 88: 424-442.

Neufeld, D.A., F.A. Day and H.E. Settles. 1996. Stabilizing role of the basement membrane and dermal fibers during newt limb regeneration. Anatomy Record 245: 122-127.

Onda, H., D.J. goldhamer and R.A. Tassava. 1990. An extracellular matrix molecule of newt and axolotl regenerating limb blastemas and embryonic limb buds: Immunological relationship of Mt1-antigen with tenascin. Development 108: 657-668.

Peacock, E.E. 1984. Wound repair. 3rd ed., W.B. Saunders Company, Philadelphia. 526 p.

Stiffler, D.F. 1988. Cutaneous exchange of ions in lower vertebrates. American Zoology 28: 1019-1029.

Welch, M.P., G.F. Odland and R.A.F. Clark. 1990. Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. The journal Cell Biology 110:133-145.

APPENDIX 6

The abstract of the article presented at Department of Fisheries of Thaiand Annual Seminar, 1995

Pathogenesis of *Aeromonas hydrophila* infection in cultured frog *(Rana tigerina)*

Supranee Chinabut

Kantimanee Phanwichien

Abstract

Cultured frogs with an average weight of 130 g. were injected intramusculary into the tights with a suspension of *Aeromonas hydrophila* at the lethal dose concentration of 2.54 x 10^6 cell/ml (LC_{37} 96 hrs). The infected frogs were lethargic, developed ascites and petechiae hemorrhagic lesions occurred on the skin of the abdomen and legs with noticeable swollen muscle around the injected area. Later on ulcerative lesions with abscess developed at the injected sites. Histopathological changes in infected frogs were studied. Inflammation, hemorrhage, congestion and increased production of melanin pigment were observed in live, kidney spleen, pancreas, lung, heart, skin and injected muscle. The stomach and duodenum showed inflammation and congestion. The hepatocytes had cloudy swelling and hydropic degeneration. The glomeruli of the kidney were degenerated and the epithelial lining of the renal tubules revealed cloudy swelling, hydropic degeneration and focal necrosis. Spleen cells located in the red pulp also revealed cloudy swelling. Exocrine pancreas and endocardial cells were necrotized focally. The eosinophilic granular cells were generally found in the inflammatory areas.

APPENDIX 7

The Abstract of the article presented at Department of Fisheries of Thaiand Annual Seminar, 1995

Comparative study of cutaneous wound healing between frog (*Rana tigerina*) and hybrid catfish (*Clarias macrocephalus* x *C. gariepinus*)[*]

Supranee Chinabut

Kantimanee Panwichien

Abstract

A longitudinal incision of 0.7x0.2x0.2 cm was made on the ventral left thigh muscle of frog and a transverse incision of 0.5x0.1x0.3 cm was made on the posterior lateral myotomal muscle of hybrid catfish. The processes of wound healing and repair were found to begin far faster in hybrid catfish compared to frog. The epidermal cells at the edge of hybrid catfish wound began to migrate 15 min after the incision and the epithelialization was complete within 2 hr, whereas the epidermal cells of frog wound began to migrate 1 hr after the incision and rejoined within 18 hr. One hour after incision neutrophils were the first type of white blood cell which accumulated in the frog wounded area, whereas in the hybrid catfish wound macrophages were primarily deserved at the site of incision. There were some differences in the processes of wound repair of frog and hybrid catfish. The repair of damaged muscle of frog was initiated by closing the wound gap by tensile force of the proliferated fibroblasts at the wound edges followed by the regeneration of muscle to replace the damaged muscle. In the case of hybrid catfish, subcutaneous wound repair occurred by the replacement of regenerated muscle in the damaged area and the new muscle bundles expanded until they filled up the wound gap. After the wound healing and repair were complete, the newly formed fascia held between the hypodermis and subcutaneous fascia of frog. This newly formed fascia can be an indicator of the complete wound healing in frog, but this type of new fascia did not occur in complete wound healing of hybrid catfish. The time required for the completion of wound repair in the hybrid catfish and the frog were 28 days and 43 days after incision respectively. On completion of the healing processes no scar was apparent on the skin of both kind of animals.

APPENDIX 8

Programme for Workshop on the Health Profile of Farmed Aquatic Species at AAHRI from 25 September-4 October1996

25 Sept.

Sampling fish for bacteriology.

Simulated case history.

Pipetting-safety, accuracy and asepsis.

Bacteriological media preparation.

26 Sept.

Separation of mixed cultrue of bacteria.

Viable counts and standard curves of optical density and colony forming units,

Culture preservation.

27 Sept.

Primary identification tests.

API^{TM} and BIOLOG^{TM} identification systems.

Immunological methods and molecular biology techniques in identification-tutorial.

DNA extraction.

28 Sept.

DNA measurement.

Antibiotic sensitivity - disk tests and minimum inhibitory concentration (MIC) determination.

29 Sept.

Bioassay of concentration of active antibiotic in tissue or environmental sample.

Read MIC results.

Final discussion session.

30 Sept.

Frog dissection-demonstration.

Removing spleen aseptically and separating macrophages using Percoll gradient.

Preparing coverslip cultures of macrophages.

1 Oct.

Staining cover slip cultures.

Preparing solutions for cell separation.

Bleeding frog.

Making macrophage monolayers.

2 Oct.

Counting cells adherent in cultures.

Preparing solutions and do assay of intracellular respiratory burst (NBT).

Separating serum from blood.

Examining stained macrophage coverslip cultures.

3 Oct.

Lysozyme assay (spectrophotometric method).

Collecting blood to:

      measure haematocrit

      do total white cell counts

      prepare smears for differential blood cell counts.

      start phagocytosis assay

      measure intracellular respiratory burst in blood phagocytes.

4 Oct.

Staining and exmining blood smears and phagocytosis assays.

Demonstration of assay of macrophage killing potential.

Recapitulation and talk session about possible participant projects.