Final Report to the Department for International Development

A Study of Shrimp Defence Mechanisms and Immunomodulation to Enhance Sustainability and Reduce Antibiotic Usage in Shrimp Culture (Shrimp Defence Mechanisms and Immunomodulation)

(R 6426)

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Contents

1. From Project Memorandum

1.1. Project purpose

The project will help to reduce the non-sustainable exploitation of the coastal tropical environment. It will address an important aspect of sustainable shrimp culture, reducing the reliance on antibiotics and improving understanding of the relationship between management, environment and disease, consequently reducing production losses due to disease.

1.2. Project Activities

1.2.1. Research into shrimp defence mechanisms and effect of immunomodulators.

- Refinement of appropriate techniques for defence mechanism assays including phagocytosis, bactericidal, bacterial clearing and phenoloxidase activity.
- Study the effect of common environmental stressors on shrimp defence mechanisms.
- Study the effects of immunomodulators on shrimp defence mechanisms.
- Study long term effects of potential immunomodulators on shrimp.

1.2.2. Conduct trials of immunomodulators under farm conditions.

1.2.3. Information dissemination through workshops and other routes

1.2.4. Actively pursue collaboration with other complimentary projects.

1.3. Project Outputs

1.3.1. Information allowing a better understanding of disease pathogenisis and prevention in shrimp ponds.

1.3.2. Evaluate the efficacy and methods of administration of immunostimulants.

2. Contributing personnel

Academic staff

Dr. Kamonporn Tonguthai, Director of AAHRI

Dr. Pornlerd Chanratchakool, AAHRI staff

Dr. Supranee Chinabut, AAHRI staff

Dr. James F Turnbull, IAS Research Lecturer

Dr. Valerie Inglis, IAS Senior Lecturer

Supporting staff

Dr. Pikul Jiravanichpaisarn, ODA funded full-time Research Assistant

Mr. M Srithep, ODA funded full-time Research Assistant

Ms. S Maneerat, ODA funded part-time Research Assistant

Dr. R Campbell, IAS Research Assistant

3. Background

3.1. Shrimp farming

Shrimp farming is a large important industry with wide-ranging environmental, economic and social effects throughout the tropics. The industry is also responsible for destruction and pollution of coastal ecosystems, including depletion of mangroves. These effects have implications not only for fisheries but also other resources.

Contrary to common opinion the majority of shrimp farms in East Asia are small units managed or owned by single families. In many cases the families have obtained loans to develop farms on the their own land. If such farms fail, usually due to poor management, the family is left with no land and some cases large debts. An example of such a shrimp farming system is a Vietnamese culture system that combines rice during the wet season and shrimp during the dry season. During the dry season, local farmers have very little if any alternative to extensive shrimp rearing and a very high proportion of the population depends on this source of income, over 75% of the households in some areas (Local department of Fisheries data). These already impoverished communities have suffered extreme hardship as a result of failures of crops over the last three years (Unpublished data, Corsin, Morgan and Turnbull).

A proportion of shrimp farms are owned or financed by more wealthy organisations or individuals. If production on these farms collapses then the investors often develop farms in other areas. This leaves the original land derelict and utilises still more coastal resources. Collapse of the shrimp farms also has an effect on local employment. In

addition to the staff employed on the farms there are significant numbers of women from under privileged households employed in processing plants. Since processing plants have to be close to the production sites, the collapse of shrimp farming may leave the communities without land or employment, forcing migration, frequently to large cities.

3.2. Current methods for controlling shrimp disease

Successful management of the environment is essential for shrimp health. In the vast majority of cases shrimp disease results from a primary environmental stress and secondary opportunist pathogens. There is growing evidence that this is even the case with the highly pathogenic viruses currently affecting the industry (Yellow head virus and White Spot Disease Virus [1]). Although the relationship between the environment and disease is widely acknowledged, the specific mechanisms are still poorly understood. At present management is directed towards maintaining a healthy plankton bloom within the pond and avoiding toxic levels of nutrients and other substances (e.g. ammonia and hydrogen sulphide). Unfortunately current management systems do not necessarily prevent diseases problems.

Shrimp are stressed by environmental conditions before clinically toxic levels are reached and therefore the objective of good management must be to maintain and optimal environment, not merely to avoid clinically toxic conditions. Unfortunately there is still little information available regarding the effects of sub-lethal but stressful levels of many environmental parameters. In most cases very crude measurements have been used to evaluate the effect of stressors, including growth rate and mortality. Therefore, it is difficult to identify and control the most stressful parameters in a pond environment.

In addition to husbandry measures vaccines and antibiotics are used in an attempt to control shrimp disease. The evidence for the efficacy of vaccine in shrimp is still controversial[2]. In addition to the equivocal nature of the scientific results there is no obvious mechanism by which a vaccine might work in animals without an adaptive immune system[3]. The other method of controlling infectious disease has traditionally been the use of chemotherapeutants. However, such compounds are unsuitable for the

treatment of many disease problems. Many of the most serious infections of shrimp are caused by viruses for which there is no chemical treatment available. The use of some drugs, especially antibiotics, may also be associated with the development of resistant bacteria and hazardous residues in food for human consumption[4]. In addition, since most of the infectious diseases are secondary to environmental problems, killing the pathogen with drugs does not necessarily address the initial cause.

It has also been suggested that disease resistant strains of shrimp might be bred or developed by genetic engineering[5]. There are valid arguments against the release of genetically altered animals into the wild and there is no doubt that shrimp would escape from any commercial culture facility. The breeding of animals resistant to specific pathogens has been attempted in several species of vertebrate including swine, cattle and poultry. In most cases this has been an unsuccessful method of disease control.

It is clear that alternative health management strategies are required to sustain productivity in shrimp farms.

3.3. Shrimp defence mechanisms

Compared to higher vertebrates, phylogenetically less advanced invertebrates rely to a greater extent on the non-specific defence mechanisms. Shrimps do not have a specific immune system although they do have a degree of specificity and there is some controversial evidence for a limited cellular immune memory[6].

The specificity is related to the activity of pattern recognition proteins and other receptors with only limited specificity. In a comprehensive review of invertebrate immunology Smith (1991) stated "....there are few convincing examples of either-true adaptive immune reactions in the invertebrates or of primitive antibody precursors/molecules in invertebrate body fluids."[3]. It is possible, therefore, that the so-called vaccines are working as immunomodulators rather than true vaccines.

In crustaceans the main non-specific defences include physical barriers, phagocytosis, encapsulation and the prophenoloxidase cascade. Recent publications have provided

substantial information regarding the mechanisms of the shrimp defence systems (e.g.[7,8,9]). As a result there are a very a wide range of assays which can be used to determine the activity of the shrimp defence system. However, these assays have not yet been used to examine the effect of environmental stressors on disease susceptibility. The relationship between stress and disease resistance is complex and mediated by a communication between the immune and neuroendocrine systems. The effects of stress on disease resistance depend not only on the type of stressor but also the duration and frequency of application. In addition the effects of stress on the immune system are not invariably negative. There is evidence for an increase in the bacteriocidal activity of salmonids serum associated with stress[10].

A range of tests was selected for this study on the basis that they should have provided a useful practical representation of the ability of shrimp to resist infection.

3.4. Immunomodulators

Immunomodulators are substances or conditions that alter the defence response. They can either be used in association with vaccines or in isolation. An increasingly wide range of immunomodulators has been used in mammals over the last 70 years. In human medicine there has been a trend towards targeting specific aspects of the immune system rather than non-specific stimulation. Although, their use in fish is a relatively recent development, a wide range of substances has been investigated[11]. A positive effect following injection of immunomodulators has been conclusively demonstrated in shrimp under laboratory conditions[12,13].

Scientific evidence for the activity of immunomodulators is mostly restricted to *in vitro* studies[14,15,16]. A small number of recent studies have suggested that orally administered immunomodulators may also have an effect in shrimp [17,18].

Large quantities of immunomodulators are sold to shrimp farmers throughout South East Asia. In Thailand alone there are more than 20 commercial brands of immunostimulants available. Despite their commercial use there is very little published evidence that immunomodulators alleviate the effects of stress or to define the optimum method of administration under field conditions. The farmers have no

reliable information on whether immunomodulators will improve their productivity or just add to their production costs with no significant return.

In this study it was decided to concentrate on a single immunostimulant, that was a β-1,3,-1,6 D-glucan. The substance selected has been shown to have an effect over a wider range of doses than some of the other substances available[19]. In addition this substance is sold commercially as Macroguard® (Trouw UK) in a relatively pure form and was therefore easily available. Some of the other preparations sold commercially are relatively unrefined and may have introduced another uncontrolled variable into the experiments.

3.5. Immunomodulators as part of a health management strategy

With an improved understanding of the relationship between environmental conditions and disease it should be possible to focus management system on the most important environmental parameters. However, even with the most efficient management systems there will always be some occasions when the shrimp are subject to stress, for example during transfer between the hatchery and the pond. Shrimp may also be stressed as a result of the climatic or unforeseen problems with the inlet water. At present if the shrimp are stressed, the farmers reduce feeding and may even administer antibiotics to reduce opportunist bacterial infections. It would be preferable to optimise the shrimps own defence mechanisms thereby giving the animals the best chance of resisting opportunist infections.

In order to be effective, any method of disease control has to be part of an integrated health management system. Some systems are available[19] but they require further development if they are to be truly ecologically and financially sustainable. It is possible that immunomodulators may play a role in such systems.

4. Summary of the project activities

4.1. Refinement of techniques to determine the activity of shrimp defence mechanisms.

- 4.1.1. Phagocytosis activity.
- 4.1.2. Bactericidal activity.
- 4.1.3. Phenoloxidase activity.
- 4.1.4. Bacterial clearance ability.

4.2. Studies on the defence mechanisms of normal shrimp.

4.3. Physiological effect determined by haemolymph glucose of a range of stressors.

4.4. Effects of stressors on the defence mechanisms of shrimp.

4.5. Effects of an immunomostimulant on the defence mechanisms of shrimp

4.6. Effects of combined stress and immunomostimulant on the defence mechanisms of shrimp.

4.7. The long term effects of an immunomostimulant on the growth and survival of shrimp.

4.7. Dissemination of results.

5. Comments

During the project there were some problems which occurred, these interfered with some of the objectives.

In Thailand although it was possible to buy animals from commercial facilities they were not always available at the appropriate time. In order to obtain suitable animals, healthy populations had to be identified and shrimp transported back to AAHRI. They then required a period of acclimation (Appendix 3). Obtaining suitable animals was difficult at some times due to a severe epidemic of White Spot Disease on Thai farms during the project. The supply of suitable experimental animals was a serious problem in Stirling. The Normal supplier was no longer able to supply animals to Stirling. This problem was overcome to some extent since Dr Campbell who was conducting the work agreed to suspend her employment until the animals became available.

The project suffered from the departure of some staff. Dr. Pikul Jiravanichpaisarn left to take up a permanent post and Dr Inglis retired during the project. Both of these departures were unavoidable and unanticipated and resulted in some delay in to the project.

The most serious problem encountered during this project was access to the farms for field trials. It proved impossible to arrange farm based field trials with the resources available and due to logistic problems at the time. The major problem was the serious White Spot Disease outbreak that made many of the farms unsuitable for field trial work. A field trial was initiated but he farmer failed to comply with the experimental protocol after just one week and therefore the trial had to be terminated.

6. Recommendations

6.1. Environmental stressors

A reduction in temperature produced a significant physiological stress response as measured by heamolymph glucose (9.3.4.), however, there was no significnat reduction in the activity of the defence mechanisms (9.4.1.).

An increase in pH or slainity (9.3.2. and 9.3.3.) resulted in a physiological stress response in some experiments but the effect was not reproducible. With these two stressors the only significant reduction in the defence parameters was in the phenoloxidase activity associated with increased salinity (9.4.2. and 9.4.3.).

Studies in the field of stress physiology and health frequently suffer from the inability to define or obtain a stress free population. The experiments conducted in this study took place under laboratory conditions in partial re-circulating systems. Although such conditions are not necessarily ideal for shrimp, they were the most practical compromise to allow the sampling and analyses to be conducted efficiently. Despite the relatively consistent conditions under which the shrimp were maintained there was still a great deal of variability between individuals within treatments. Every effort was made to reduce such variability by selecting animals of a similar size from similar husbandry systems. However, it is clear from the variability between animals that there are aspects of the response to stress in shrimp that we still do not understand. There is

the need for further more detailed work on the fundamental aspects of the crustacean response to stressors.

6.2. Value of Immunostimulants

The immunostimulant (β-1,3,-1,6 D-glucan) did not affect the defence parameters when fed at 5g/kg but increased phenoloxidase, phagocytosis and bacteriocidal activity when fed at 10g/kg feed (9.5.). When the interaction between stress and the effect of the glucan was examined the bacteriocidal activity of the non-stressed shrimp and those exposed to salinity stress were enhanced (9.6.1.). The remainder of the results were equivocal.

In the long term feeding trail, there was no adverese effect associated witht the feeding of the glucan and those fed at 10g/kg were actually larger at the end of the experiment.

There is evidence that the β-1,3,-1,6 D-glucan can induce changes in both the cellular and humoral components of the immune system in shrimp. However, the evidence for increased resistance to infection or amelioration of the negative effects of stress is more difficult to interprate. There is a lack of published information concerning the necessary dose and duration of immunostimulants application for optimal effect. In this study it would seem that the glucan had to be fed at 10g/kg in order to produce a significnat result but there was no adverese effects associated with prolonged administration.

6.3. Requirement for Field trials

In order to evaluate the potential benefit of immunostimulants, as part of an integrated husbandry strategy, there is a need for further laboratory studies on the application of immunostimulants and such studies will have to be supported by rigorous field trials. Laboratory results cannot be generalised to the farm for a number of reasons relating to the condition of individual animals and the dynamics of large populations. The only method by which an effect on real farms can be evaluated is through carefully planned and executed field trials. Such trials are often expensive and difficult to conduct, even in terrestrial animal systems. In shrimp culture such trials are even

more problematic, since the experimental unit cannot be smaller than the pond and there is large variability between ponds. Laboratory studies provide essential information in the development of any health management strategy but without field trials it is impossible to predict the benefits and costs for the farmer.

In this study the potential expense and difficulty of arranging field trials was underestimated. Despite the obsticles to conducting valid field trials they are still essential when developing treatment regimes or husbandry strategies. At the conclusion of this project there were no published reports of valid field trials in shrimp. Commercial companies should conduct valid field trials before selling commercial products to farmers. However, in practice products can be sold without such information and therefore there is little commercial incentive for companies to conduct filed trials.

6.4. Requirement for impartial information.

The lack of field data for products sold to shrimp farmers has resulted in companies developing large and lucrative markets for ineffective products at the expense of often poor farmers. Many farmers not only those farming shrimp have a desperate need for impartial information, this has been confirmed by many of the workshops organised through the SEAADCP. Many of the potential sources of information available to farmers are commercially biased. There has been an ongoing effort to provide information to shrimp farmers through the book Health Management in Shrimp Farms[20]. The experience gained during this project was included in the 3rd edition of that book in a section on evaluation of products or management strategies.

7. Interactions and networking

There has been collaboration with the following workers during the project.

- Dr. Hao, RIA 2, Ho Chi Minh, Vietnam.
- Mr. M. Atmomarsono, Research Institute for Coastal Fisheries (RICF), Indonesia.
- Mr. Rolando C. Miranda, BFAR, Philippines.
- Dr CV Mohan, Manglaore Fisheries College, India.

- Dr Bruno GomezGil-R and Dr Ana Roque - Department of Pathology, CIAD, Mazatlán, Sin. México

7. Dissemination of findings

The scientific findings and the experience gained from this project have been disseminated through a variety of routes.

- Fifth Asian Fisheries Forum at Chiang Mai, Thailand, 11-14 November 1998 (Appendix 1).
- International Foundation for Science - Shrimp Immunity and Disease Control: an integrated approach - IFS. Chiang Mai, Thailand 8-9 November 1998 (Appendix 1).
- 6th Kasetsart University Annual Conference between 3-5 February 1998.
- Abstracts of all publications have been included in AAHRI Newsletter, which can also be accessed through the AAHRI website (http://www.agri-aqua.ait.ac.th/aahri).
- Health Management in Shrimp Ponds 3rd Edition[20].
- Shrimp Health Management Workshop - for participants from the Asia Pacific region in May 1998.
- Technical seminar for shrimp farmers within Thailand and organised by various companies and the Thai Government.
- MSc courses in Aquatic Veterinary Studies, Aquatic Pathobiology and Aquaculture at the Institute of Aquaculture, University of Stirling.

9. Details of work conducted.

A description of the acclimation process for experimental animals and the husbandry systems used is contained in Appendix 3.

9.1. Refinement of techniques to determine the activity of shrimp defence mechanisms.

The assays for determining the assay of shrimp were refined at both AAHRI and Stirling, however, due to lack of experimental animals at Stirling the majority of the refinement was conducted at AAHRI. Four different aspects of the shrimp non-defence mechanisms were used in this study; including a phagocytic index, phenoloxidase enzyme activity, bacteriocidal activity and ability to eliminate the bacteria from haemolymph. A variety of techniques were examined but eventually the techniques contained in Appendix 3 were used for the subsequent studies. During the preliminary trials the bacteria were cleared from the haemolymph within 3 days but for logistic reasons this test was not used further.

9.2. Studies on the defence mechanisms of normal shrimp.

Once the assays had been refined it was necessary to determine how many assays could be conducted on individual shrimp and how many shrimp could be sampled on a single occasion. Apparently normal *P.monodon* (10 to 15gms) were brought from a commercial farm, acclimatised and used for the experiment. From the results, based on 20 animals it was clear that there was considerable variation between individuals. The results are presented in Table 1

Table 1

Mean and range of assays, n = 20

Assay	Mean	Range
% phagocytosis	27.47 %	(19.25 - 39.5 %)
Phagocytic index	11.36	(5.52 - 19.39)
Phenoloxidase enzyme activity	4.87	(2.63 - 8.0)
Bacteriocidal activity	46.98	(26.56 - 71.43)

9.3. Physiological effect determined by haemolymph glucose of a range of stressors.

One of the objectives of this study was to determine the effect of environmental stressors on the defence mechanisms of shrimp. In order to do this it was necessary to examine a range of stressors to determine which induced the greatest physiological stress response and therefore was likely to have the greatest effect on the defence mechanisms. For this purpose haemolymph glucose was measured after exposure to a range of potential stressors[21].

It was anticipated that evaluating the effect of environmental stressors on the physiological stress response of shrimp would be a relatively simple task. In practice this component of the project took a considerable amount of time at AAHRI and Stirling. There was a chronic shortage of experimental animals in Stirling and although the supply was better at AAHRI the supply was still limited. In addition it was necessary to use animals at a similar stage of the moult cycle which further reduced the number of experimental animals. It was difficult to obtain reproducible haemolymph glucose levels in response to environmental stressors. Although significant results were produced in some experiments a considerable amount of work produced non-significant trends. In all the experiments there was considerable variation between individuals. The source of this variability was not identified but may have been associated with the unavoidable stress associated with keeping animals under laboratory conditions. Considerable effort was directed towards reducing or minimising this variability, however, since the source of variability was not identified it may have compromised the results of subsequent experiments.

The stressors examined included handling, changes in pH, salinity and temperature. The pilot studies to reduce variability are not reported here.

9.3.1. Handling : When shrimp were exposed to severe handling stress the peak of the stress associated hyperglycaemic response was 90 minutes after exposure to the stressor. Four groups of eight randomly selected and individually housed shrimp were sampled 0, 60, 90 and 180 minutes after exposure to a severe handling stress. The glucose levels were significantly higher 90 minutes after exposure to the stressor (Kruskal-Wallis One Way Analysis of Variance on Ranks, H = 16.4 with 3 degrees of

freedom, P = 0.0009 and Dunnett's multiple comparisons test P< 0.05). In the subsequent experiments the stressor was cumulative and hence the maximum haemolymph glucose levels were observed longer after the onset of the stressful conditions.

9.3.2. pH : In one experiment using three groups of six randomly selected shrimp exposed to ambient pH of 7.5, pH6 or pH10, those exposed to the highest pH demonstrated a significantly higher haemolymph glucose levels (Kruskal-Wallis One Way Analysis of Variance on Ranks, H = 6.17 with 2 degrees of freedom, P = 0.0457 and Dunnett's multiple comparisons test P< 0.05).

9.3.3. Salinity : A non-significant increase in haemolymph glucose was detected when shrimp were exposed to salinity increased from 15 to 30 ppt (n=5, One Way Analysis of Variance, P= 0.119). When the salinity was reduced from 15 to 0 ppt there was a significant increase in haemolymph glucose 180 and 240 minutes after the change in salinity (Kruskal-Wallis One Way Analysis of Variance on Ranks, H = 18.8 with 4 degrees of freedom, P = 0.0009 and Dunnett's multiple comparisons test P< 0.05).

9.3.4. Temperature : When shrimp were exposed to decreased temperature from 26 to 15°C there was a significant increase in blood glucose (n = 5, Kruskal-Wallis One Way Analysis of Variance on Ranks, H = 11.0 with 4 degrees of freedom, P = 0.0261 and Dunnett's multiple comparisons test P> 0.05). This result was replicated in a subsequent experiment (n = 5, One Way Analysis of Variance, P = 0.00000446 and Dunnett's multiple comparisons test P> 0.05).

9.4. The effect of stressors on the defence mechanisms of shrimp

Different stressors including temperature, pH and salinity were used as the stressful condition for these experiments. On the basis of the initial experiments, groups of normal shrimp were subjected to changes of the parameters before being sampled for analysis. The number of animals sampled was limited by the time taken to conduct the assays.

9.4.1. Temperature

Two groups of three shrimp were subjected to temperature shock (18°C for 3 hours) and then sampling for analysis. Although there was a reduction in all the defence parameters measured the only significant difference was in the phenoloxidase activity (t-test, t = 10.3 with 4.00 degrees of freedom, P = 0.0005). The results are summarised in Table 2.

Table 2.

Parameter	Mean control	Mean stressed	% inhibition
% phagocytosis	25.02	17.22	31.2
phagocytic index	9.92	6.89	30.5
phenoloxidase enzyme	5.51	1.38	75.0*
bacteriocidal activity	18.98	9.17	28.9

* denotes significant difference between control and stressed animals.

9.4.2. pH

Shrimp were subjected to high water pH (pH 10 for 2 hours) and then sampled for analysis. Although there was a reduction in all the defence parameter measured there were no significant differences. The results are summarised in Table 3.

Table 3.

Parameter	Mean control	Mean stressed	% inhibition
% phagocytosis	30.91	25.11	18.8
phagocytic index	18.45	11.06	40.0
phenoloxidase enzyme	3.71	2.56	30.8
bacteriocidal activity	53.04	25.90	51.2

9.4.3. Salinity

Shrimp were subjected to increased salinity (40 ppt for 12 hours.) and then sampled for analysis. The results are shown below. Although there was a reduction in all the defence parameter measured, the only significant difference was in the phenoloxidase activity (t-test, t = 3.32 with 4.00 degrees of freedom, P = 0.0293). The results are summarised in Table 4

Table 4.

Parameter	Mean control	Mean stressed	% inhibition
% phagocytosis	23.41	20.24	13.5
phagocytic index	9.93	7.27	26.8
phenoloxidase enzyme	3.85	2.53	34.1*

* denotes significant difference between control and stressed animals.

9.5. Effects of immunostimulant on the defence mechanisms of shrimp

The effects of β-1,3,-1,6 D-glucan on the defence mechanisms of shrimp were examined. Shrimp from a commercial hatchery were brought to the laboratory and acclimatised. They were then split into three groups of 20 all fed on a commercial shrimp feed. The control group was fed with untreated feed, one group's was feed was top dressed with 5g/kg glucan and the last group's feed was top dressed at 10g/kg. The phenoloxidase, phagocytosis and bacteriocidal activities were assayed in all three groups 1, 2 and 3 days from first administration of the top dressed feed. At a rate of 5 g/kg no significant differences were detected in the defence mechanisms, however, at 10 g/kg feed there was a significant improvement in phagocytosis and bacteriocidal activity (Student t test: Phenoloxidase, $t = -2.20$ with 4.00 degrees of freedom, $P = 0.0932$; bacteriocidal, $t = -17.2$ with 4.00 degrees of freedom, $P = <0.0001$).

9.6. Effects of combined stress and immunomostimulant on the defence mechanisms of shrimp.

Two pilot studies were conducted to investigate the effects of β-1,3,-1,6 D-glucan and first increased salinity on shrimp. Then in a second experiment the effects of the glucan and increased pH. In both experiments 10 shrimp were maintained in each of 4 separate tanks. The tanks received the following treatments :

A) Unchanged management (Control).

B) Exposure to 33ppt salinity or increased pH from 7.8 to 10 for three hours (Stressed).

C) A diet top dressed with 1% β-1,3,-1,6 D-glucan as a top coat (Glucan).

D) Exposure to increased salinity or pH and the glucan diet (Stressed/Glucan).

The glucan was administered for 3 days prior to the salinity stress and three days after. Three shrimp were sampled for humoural bactericidal activity and phenoloxidase activity from each of the tanks, 1, 2 and 3 days after the salinity stress. The experimental design is represented diagrammatically in Table 5.

Table 5

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Glucan feed	■	■	■	■	■	■
Stress			■			
Samples				■	■	■

9.6.1. Salinity stress

The bactericidal activity was significantly increased in the Glucan and Stressed/Glucan shrimp on the third sample day (Day 6) (One Way Analyses of Variance, $P=0.0124$, Student-Newman-Keuls multiple comparison Method, $P<0.05$).

The phenoloxidase activity was significantly higher in the Glucan compared to the Stressed/Glucan shrimp on the first sample day (Day 4) (One Way Analyses of Varience, $P=0.0238$, Student-Newman-Keuls multiple comparison Method, $P<0.05$)

9.6.2. pH stress

The bactericidal activity in the Glucan treatment was significantly higher than other treatments on the third sample day (Day 6) (One Way Analyses of Varience, $P=0.0178$, Student-Newman-Keuls multiple comparison Method, $P<0.05$). No significant differences were detected in the phenyloxidase activity between treatments.

9.7. The long term effects of immunostimulant on the growth and survival of shrimp.

Shrimp post larvae were obtained from a commercial hatchery and reared in fifteen tanks each containing 20 shrimp at AAHRI. The tanks were randomly allocated one of three treatments. The control group was fed with untreated commercial feed, one group's feed was top dressed with 5g/kg β-1,3,-1,6 D-glucan and the last group's feed was top dressed at 10g/kg. The experiment was continued for 50 days. The mean body weight was measured in each tank every 7 days and the cumulative survival calculated at the end of the experiment.

After feeding the post larvae for 3 weeks both the 5g/kg and the 10g/kg shrimp were significantly heavier than the controls ($n = 5$, One Way Analysis of Variance, $P = 0.00610$, Student-Newman-Keuls Multiple Comparison $P<0.05$).

After feeding the post larvae for 7 weeks those fed on the 10g/kg were significantly heavier than the controls or those fed on the 5g/kg diet (One Way Analysis of Varience, $P = 0.00777$, Student-Newman-Keuls Multiple Comparison $P<0.05$).

There were no other significant differences in the growth rate or the survival associated with the treatments.

Appendix 1

Publications derived from project

Chanratchakool, P., Sukrakanchana, N. and Kongyangyurn, P. (1998). The effects of β (1→3) (1→6) D-glucan on the immune response of the Black Tiger Shrimp (*Penaeus monodon* Fabricius). 6th Kasetsart University Annual Conference between 3-5 February 1998.

Chanratchakool, P., Sukrakanchana, N. and Pancharatana, S. (1998). The effects of environemtnal change on the immune response of the shrimp (Penaeus monodon Fabricius). 6th Kasetsart University Annual Conference between 3-5 February 1998.

Turnbull, J.F. & Chanratchakool, P. (1998) Use of Immunostimulants in Shrimp Culture. Shrimp Immunity and Disease Control: an integrated approach - IFS. Chiang Mai, Thailand 8-9 November 1998.

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Appendix 2

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Appendix 3
Experimental Methods

1. Husbandry systems

For the all the experiment at AAHRI (with the exception of 9.7.) shrimp of 3 to 7 g were obtained from commercial farm and acclimatised in 500 litres fiber glass tank, containing diluted sea water at 15 ppt. The shrimp were fed with commercial pellet (Chareon Pokpand Group), 3 time per day at approximately 3 % body weight a day. Approximately 20% of the water was changed every 3 days. Acclimatisation period depended on the apparent health of the animals, usually 7 to 10 days before being randomly selected for each treatment. The glass aquaria were (40 x 23 x 27 cm.) containing 15 litres of water were used for the experiments.

2. Phagocytosis activity

Haemolymph (0.5ml) was drawn from the ventral sinus of the shrimp into a 5 ml. syringe containing K-199 medium supplemented with 2.5 % L-cysteine as an anticoagulant. The haemocytes were collected and washed twice with K-199 by centrifugation at 1,000 rpm, 4°C for 5 minutes. Haemocytes (1 x 10^5 cells/ml) were mixed with fluorescent labelled latex beads (Polysciences, Inc. Warrington, PA.) (1 x 10^7 beads/ml) in K -199 on a glass disc placed in a well plate. After incubation at 25°C for 1 hr, fixed the haemocytes with 20% glutaraldehyde. Non-phagocytised latex beads were removed by washing with 0.15 M NaCI and distilled water. Phagocyte monolayer was stained with Giemsa stain, rinsed with distilled water and air-dried. The number of cells which had ingested bead and the total number of beads ingested were counted from 200 cells observed under fluorescent microscope. The following parameters were calculated :

Percent phagocytosis =

$$\frac{\text{Number of cells ingesting beads}}{\text{Number of cells observed}} \times 100$$

Phagocytic index =

$$\frac{\text{Number of cells ingesting beads}}{\text{Number of cells observed}} \times \frac{\text{Number of beads ingested}}{\text{Number of cells observed}} \times 100$$

3. Bactericidal activity

Vibrio harveyi was cultured in tryptic soy broth with 1.5% NaCI overnight at 25°C. Bacteria were collected by centrifugation and washed once in 2% sterile saline then diluted with saline to obtain the bacteria suspension at optical density of 0.1 (540 nm). The haemolymph was prepared by centrifugation at 9,700 rpm for 20 minutes with 2.5% L-cysteine (as anticoagulant). Then 100 µl of bacterial suspension was incubated with 100 µl of cell free haemolymph. Samples were incubated in sterile microtube for 3 hours at 25°C. Aliquots of 100 µl were taken from each microtube and spread onto thiosulphate citrate bile salts agar (TCBS) plates in order to count the colony forming units (cfu) (Adams, 1991)[22]. Positive controls were bacteria suspended in saline incubated in K-199 with 2.5% L-cysteine.

Percentage inhibition =

$$\frac{\text{+ve control cfu} - \text{sample cfu}}{\frac{\text{+ve control cfu}}{100}}$$

4. Phenoloxidase activity

From the ventral sinus 0.5ml of haemolymph was taken into a syringe. Haemolymph was immediately transfered to microtube and haemocytes were broken by sonication for 10 seconds. Phenol oxidase activity in haemolymph samples was determined using L-dihydroxyphenylalanine (L-DOPA) as a substrate. Tris buffered saline (TBS) at 30 µl was added to the cuvette containing 30µl of haemolymph sample. Then, 60µl L-DOPA solution (1.6mg/ml in TBS) was added and mixed. Next, 200µl TBS was added as diluent and enzyme activity was determined by measuring the absorbance of dopachrome at 490nm against a blank containing 200µl TBS and 60µl L-DOPA. The absorbance value was recorded between 1 to 10 min after addition of the diluent. Enzyme activity was expressed as enzyme units, with one unit being defined as the amount of enzyme giving an increase in absorbance of 0.001 per minute at 490nm.

5. Bacterial clearance ability

Following injection challenge with 1 x 10^5 cfu *Vibrio* spp. per shrimp, 100µl haemolymph samples were taken from ventral sinus of shrimps. Then, the samples were immediately added to 1.9 ml of ice cold sterile Van Harrevald's salt solution

(VHS) (Van Harrevald, 1936). Haemolymph (100µl) in VHS were spread onto TCBS agar plates for enumeration of cfu. The numbers of total *Vibrio* spp. in haemolymph on TCBS plates were counted after incubation time for 18 hours.