INVESTIGATING HEALTH MANAGEMENT STRATEGIES IN THAI SHRIMP HATCHERIES

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BY

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DECLARATION

I hereby declare that this thesis has been composed entirely by me and has not been submitted for any other degree or qualification. The work presented in this thesis, except where specifically acknowledged, is the result of my own investigation which have been conducted by me independently.

Siriwan Nooseng

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ABSTRACT

Marine shrimp like Penaeid are one of the most important farmed species which provide an economically valuable seafood product in Thailand. However, infectious disease outbreaks continue to be a serious issue that results in production losses. To support the grow-out farmers, who rely on the good quality of hatchery reared seed, the shrimp larvae supplies from the hatcheries must be healthy and pathogen free to ensure good growth rate and a high value product in the grow-out section. Health management is an important aspect of ensuring that the sector remains buoyant and can produce high quality of post larvae (pl).

One of the findings from the hatchery survey data of this study found that control of temperature in larger tanks gave a statistically significant survival rate in the pl shrimp ($P \leq 0.05$) compared with those without temperature control and using small scale tanks. Controlling temperature with larger tanks as well as probiotic supplementation are recommended for the Thai hatchery section. Furthermore, from the survey data, there was a high level of use of probiotics by many of the Thai shrimp hatcheries. A study was performed to investigate the effect of probiotic on the health of the marine shrimp. The results of this experimental study found that administration of a single probiotic substance containing the Gram positive *Bacillus licheniformis* gave a statistically significant higher level of survival ($P \leq 0.05$) compared with shrimp in the control group. The probiotic was fed to the shrimp via live artemia at a concentration of 10^6 cfu per ml. While no other statistically significant changes were identified between the shrimp fed the probiotic and the control group, it was important to note that administration of the probiotic did not cause any negative side effects.

A further experimental study was performed to evaluate if the probiotic fed shrimp were less susceptible to pathogenic strains of *Vibrio parahaemolyticus* which caused Acute Hepatopancreas Necrosis Diseases (AHPND). Two strains of *V. parahaemolyticus* were included in the study and these were administered to the shrimp by static bath. The results of the bacterial challenge study showed that for

both *V. parahaemolyticus* strains less mortalities occurred in the shrimp groups administered the probiotic before challenge. There appeared to be a bacterial concentration effect of the *V. parahaemolyticus* strains as the highest cumulative mortality was found in the shrimp group receiving the highest bacterial concentration. Overall the experimental bacterial challenge studies suggested that there was a trend for the shrimp receiving the probiotic to be associated with less AHPND.

In conclusion, this study used a mixture of methods in order to improve our understanding of health management strategies in Thai marine shrimp hatcheries. The data provided evidence that temperature control in larger size tanks gave improved survival of the shrimp. Under experimental conditions, administration of probiotics could be beneficial to reduce bacterial infection from AHPND-causing *V. parahaemolyticus*, as well as improving of survival rate. The research performed has generated new knowledge on improvements in health management in Thai shrimp hatcheries and has provided the foundation for future studies to explore the mechanistic effect of probiotics within these systems.

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LIST OF ABBREVIATIONS

%	Percentage
§	near to significant but weak association
µl	Microlitre
AHPND	Acute Hepatopancreatic Necrosis Disease
AHPNS	Acute Hepatopancreatic Necrosis Syndrome
AIC	Akaike Information Criterion
ASDD	Abdominal Segment Deformity Disease
ATM	Aggregated, Transformed Microvilli
B or Br	Broodstock
BL	*Bacillus licheniformis*
BP	Baculovirus penaei
Bps	Base pairs
cfu	colony-forming unit
CHROME	Chromogenic
Cm	Centimeter
CMNV	Covert Mortality Nodavirus
CoC	Code of Conduct
DCHT	Degeneration of Central Hepatopancreatic Tubules
DOF	The Department of Fisheries
EHP	*Enterocytozoon hepatopenaei*
EMS	Early Mortality Syndrome
EU	European Union
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FMD	Fry Movement Document
G	Gram
GAP	Good Aquaculture Practice
GIH	Gonad Inhibiting Hormone
H&E	Hematoxylin and Eosin
HPH	Hepatopancreatic Haplosporidiosis
HPV	Hepatopancreatic parvo-like virus
ICE	a novel integrase-containing element
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
IMNV	Infectious Myonecrosis Virus
Ind	Individual
Kg	Kilogram
Km	Kilometer
L	Litre

LSNV	Laem Singh Virus
M	Metre
m^2	square metre
m^3	Cubic metre
MD	Movement Document
Ml	Milliliter
mm	Millimeter
MM	Minimal Media
MSGS	Monodon Slow Growth Syndrome
n or No.	Number
N or Nur	Nursery
OD	Optical Density
PCR	Polymerase Chain Reaction
pl or pls	post larvae
R	Replicate
R^2	Correlation co-efficiency
RDS	Runt Deformity Syndrome
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SPF	Specific Pathogen Free
sq.m	Square metre
ST	Surat Thani
T	t-test
T	Treatment
TCBS	Thiosulfate Citrate Bile Salt Sucrose Agar
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSV	Taura Syndrome virus
TVBC	Total Viable Bacteria Count
TVC	Total Vibrio Count
UK	The United Kingdom
USA	The United States of America
USD	US Dollar
VP	*Vibrio parahaemolyticus*
W	Wilcoxan test
WSSV	White Spot Syndrome Virus
WTD	White Tail Disease
Y/N	Yes/No
YHV	Yellow Head Virus

CHAPTER 1

General introduction and literature review

1.1 Rationale and Study Aim

Marine shrimp is an economically valuable seafood product in Thailand which is one of main shrimp exporter in worldwide market. However, between 2013 - 2019 Thai shrimp exports decreased due at least in part to infectious disease outbreaks (Sriurairatana *et al*., 2014 ; Piamsomboon *et al*., 2015 ; Putth and Polchana, 2016). Other issues included competition for market share problem and a drop of shrimp prices in world markets (Piamsomboon *et al.*, 2015 ; Panichpattanakit and Siriburananon, 2018 ; Gnews, 2019). Disease epidemics particularly Acute Hepatopancreatic Necrosis Disease (AHPND) occurred in Thailand since 2012 (Flegel, 2012 ; Thitamadee *et al.*, 2016) and it was a serious issue at the time of my PhD study causing Thai shrimp production to plummet and reducing Thai shrimp production share of the global market from 27% in 2012 to 14% in 2016. Consequently, it was a trade opportunity for other countries including India, Ecuador, Indonesia and Vietnam to increase export markets share (Wanasuk and Siriburananon, 2017 ; Panichpattanakit and Siriburananon, 2018).

When considering the disease problems, health management in both hatchery and grow-out farming was not optimal, with poor quality of post larvae (pl) and subsequent production. However, this project focused on hatchery sites. At the time the Thai grow-out farmers were of the opinion that infectious disease problem in their farms was associated with pathogens in unhealthy pl. They were of the opinion that healthy pathogen-free pl would certainly reduce the impact of infectious disease problems (Moss *et al*., 2012 ; Tumnong, Pers. Comm., 2014 ; Wyban, 2019). Therefore, this study focused on understanding the existing health management strategies employed in Thai shrimp hatcheries and identified limitations which might enhance vulnerability in the hatchery stocks to disease outbreaks. To achieve this, a combination of theoretical with applied knowledge will be used to provide realistic strategies to improve the current health management approaches within Thai marine shrimp hatchery systems. The main aims of each chapter in this studies are as followed:

- To survey in Thai shrimp hatcheries to characterize and describe current practices and look for associations between any differences in practices and productivity or health of the post larvae (pl).
- To evaluate the effect of probiotics *Bacillus licheniformis* administration to shrimp larvae as an alternative strategy to promote improved animal health.
- To investigate the effect of the probiotic *Bacillus licheniformis* against a bacterial infection from pathogenic strains of AHPND *V. parahaemolyticus* in shrimp.

1.2 What is Marine Shrimp?

Shrimp or prawn is an aquatic animal, belonging to a group of invertebrates called crustaceans and they have hard external shells with jointed legs to help them walk. They belong to the order called Decapoda, which are described as scavenging organisms. Shrimp and prawns make up a large proportion of the Decapoda and members can be found in both fresh and salt water environments. Members of 2 superfamilies are considered as commercially viable for farming: Penaeidae and Caridea (Marin, 2014). There are 5 families of Panaeideans; which are Solenoceridae, Aristaeidae, Penaeidae, Sicyonidae and Sergestidae, and 3 families

of Carideans; Palaemonidae, Pandalidae and Crangonidae, where members of these families contribute towards the commercially available species. Information on their habitats and behaviours are described in Table 1.1 (Marin, 2014 ; Wikipedia, 2015a ; Holthuis, 1980).

Table 1.1. Information on marine shrimp produced commercially

Family	Habitat	Behaviour
Solenoceridae	- exclusively marine	No information
Aristaeidae *Aristeus antennatus*	- exclusively marine	- pelagic
Penaeidae *Penaeus merguiensis* *Penaeus semisulcatus* *Penaeus monodon* *Penaeus vannamei*	- coastal areas/shallow or moderately deep water (commercial species) - tropical/subtropical/warm temperate waters - marine/estuarine - depth 10-45 m/muddy bottom - marine/estuarine - depth 2-130 m/muddy bottom - marine/estuarine - depth 0-110 m/muddy bottom - marine/estuarine - depth 0-72 m/muddy bottom	- amphibiotic (migration) - gregarious - gregarious/nocturnal - nocturnal
Sicyonidae *Sicyonia brevirostri*	- exclusively marine	- nocturnal
Sergestidae	- exclusively marine	- pelagic
Palaemonidae *Palaemon serratus*	- coastal/brackish waters - tropical to temperate zones - seaweed&seagrass areas	- seasonal inshore-offshore Migration
Pandalidae *Pandalus borealis*	- deep shrimp; depth 500-800 m - temperate and cold sea - marine species; depth 20-1380 m	- pelagic
Crangonidae *Crangon crangon*	- coastal species - soft bottom (sand&mud) - temperate zone - estuarine	- buries itself during low tide

One of the biological characteristics of the Penaeid shrimp is that the spawners directly lay their eggs into the water, whereas when the Carideas release the eggs, they remain attached to the abdominal appendages until ready to hatch. Female Penaeid shrimp reach sexual maturity at less than 1 year old, with the egg fecundity between 100,000 and 1,000,000 eggs per spawner (Marin, 2014). Penaeid shrimp is a migratory shrimp and the natural lifecycle includes a juvenile period found in estuaries or mangrove with brackish waters. It is only after reaching the adult stage that these animals move toward deeper marine waters to reproduce (Marin, 2014). Of all of these species, members of the Penaeidae have contributed more of the farmed marine shrimp species produced for human consumption.

The marine Penaeid shrimp life-span is divided into 4 stages with different characteristics separating them into Nauplius, Protozoea, Mysis and Post Larvae (pl). The life cycle depicted in Figure 1.1 shows that post-hatching, the eggs reach the Nauplius stage, then larvae develop through the Protozoea and Mysis stages before metamorphosing to pl. This takes approximately three weeks in total to complete the lifecycle to pl. In the first stage, Nauplius, Protozoea and Mysis are planktonic while pl is more similar to the adult shrimp (Figure 1.1) (Boyd and Clay, 1998 ; Marin, 2014).

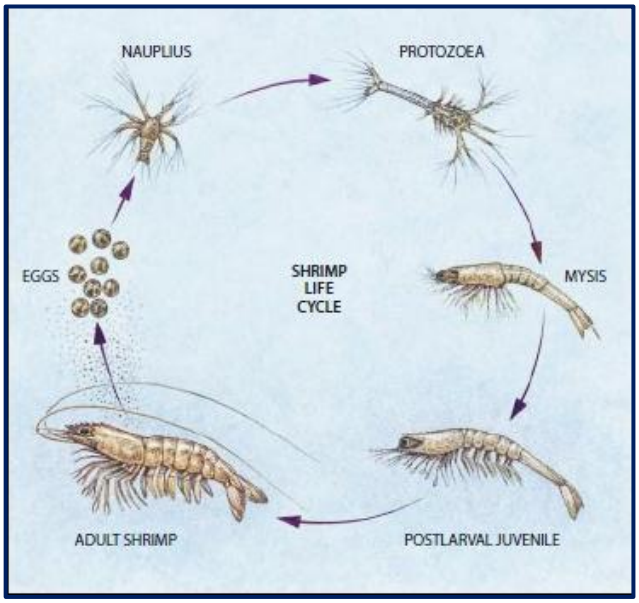

Figure 1.1. Life cycle of shrimp (Source: Boyd and Clay, 1998)

1.3 What is the Global Situation for Farmed Shrimp?

1.3.1 Global Marine Shrimp Species and their Distribution

Marine shrimp species are located widespread around the globe, naturally distributed from Polar to tropical regions (Marin, 2014 ; Wikipedia, 2015a). Concern has been raised about over-exploitation of the varied shrimp stocks from capture fisheries and one way to sustain supply and support the number of wild stocks, is through shrimp farming. The potential of global commercially produced shrimp belonging to the family Penaeidae, has been successful where *Penaeus vannamei* Boone, 1931 and *Penaeus monodon* Fabricius, 1798 are the most commonly farmed shrimp and account for approximate 80% of the total shrimp production globally (Wikipedia, 2015b). The taxonomy of both species has changed and variations are used depending on the source of information, so to add clarity for the purposes of this thesis the taxonomy of *Penaeus* will be used following FAO, 2014b ; FAO, 2014c and UniProt, 2018.

Shrimp culture occurs in brackish and fresh water throughout the Eastern Pacific Ocean, the West and Eastern Atlantic, the Western Indian Ocean, and the Indo-Pacific which *P. monodon, P. merguiensis, P. vannamei* and *Macrobrachium rosenbergii* are the more popularly cultured species (Leung and Sharma, 2001).

Penaeus merguiensis, common name is the Banana shrimp and these animals are distributed in the Indo-West Pacific from the Persian Gulf to Thailand, Indonesia, New Guinea, New Caledonia, North Australia, Hong Kong and Philippines (Figure 1.2).

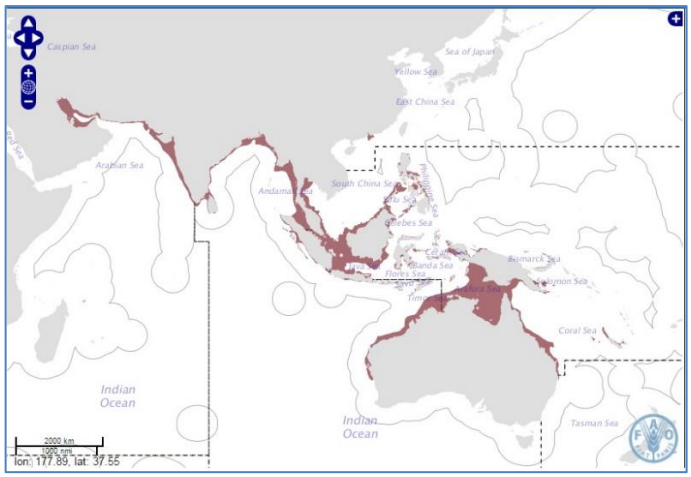

Figure 1.2. Banana shrimp global distribution, as highlighted in red coloured areas (Source: FAO Fisheries and Aquaculture Department, 2014a)

The *Penaeus monodon*, commonly called giant tiger prawn, lives throughout the coastline of South Asia, South East Asia, East Africa and Australia where the main producting countries are shown in orange in Figure 1.3 (FAO Fisheries and Aquaculture Department, 2014b). Whereas *P. vannamei*, commonly named whiteleg shrimp, is an endemic species found off the eastern Pacific ocean, distributed from the Sonora, Mexico to Peru (Holthuis, 1980). Ecuador, Mexico, Peru and Brazil are the countries that culture this species more intensively (Figure 1.4).

Figure 1.3. Giant tiger shrimp main producer countries shown in orange (Source: FAO Fisheries and Aquaculture Department, 2014b)

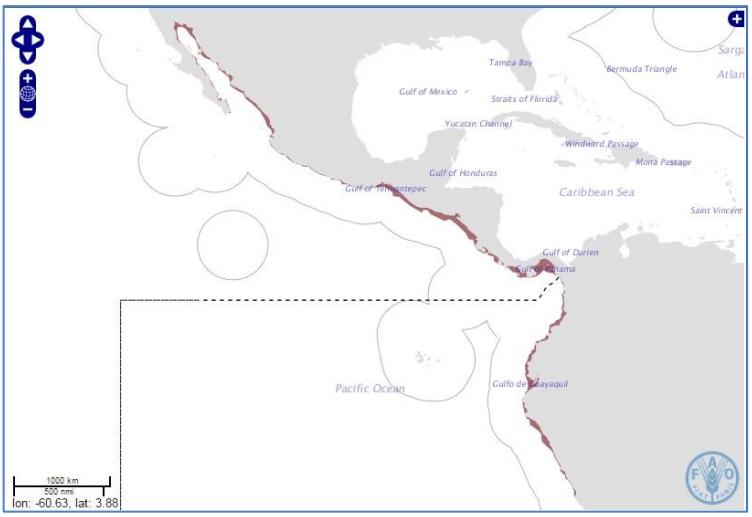

Figure 1.4. The red colour area showed whiteleg shrimp distribution in South American Countries (Source: FAO Fisheries and Aquaculture Department, 2014c)

1.3.2 Global Shrimp Markets

The global production of the shrimp farming sector has expanded over time as the production has continued to intensify. In 2010, the biggest contribution in terms of production volume, came from farmed whiteleg shrimp reaching 2.75 million tonnes followed by giant tiger prawn at 0.75 million tonnes (Figure 1.5, FAO, 2012).

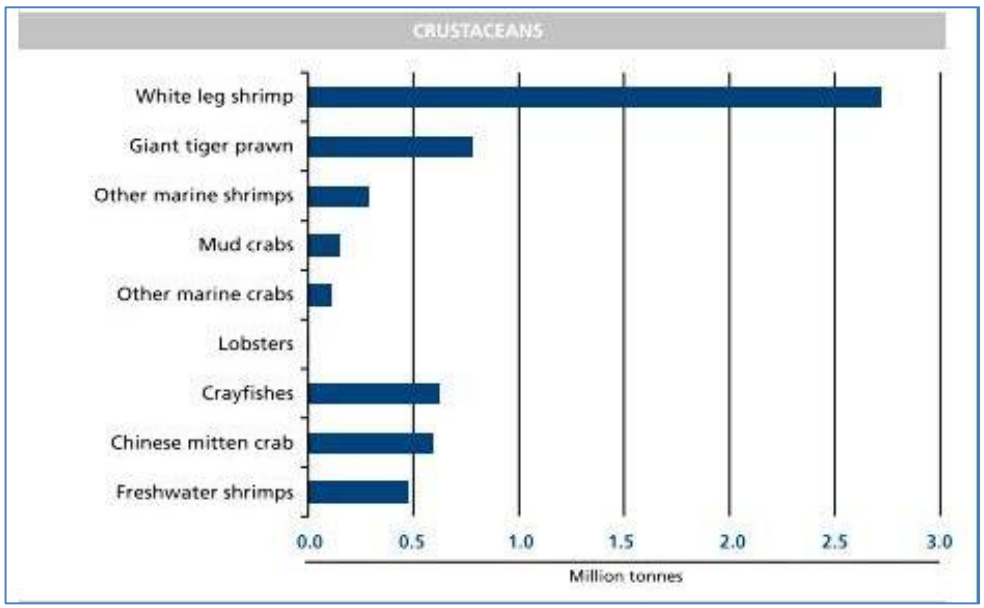

Figure 1.5. Volume of different crustacean group produced from aquaculture in 2010 (Source :FAO Fisheries and Aquaculture Department, 2012)

From 2010 - 2016, this trend to higher proportion of farmed whiteleg shrimp production continued. Whiteleg shrimp remains the dominant species of crustacean production and biggest source of financial value in Asian and Latin American countries. Whiteleg shrimp contributed 54% of the total crustacean production and giant tiger prawn provided about 10% of total crustacean production (Figure 1.6, FAO, 2018).

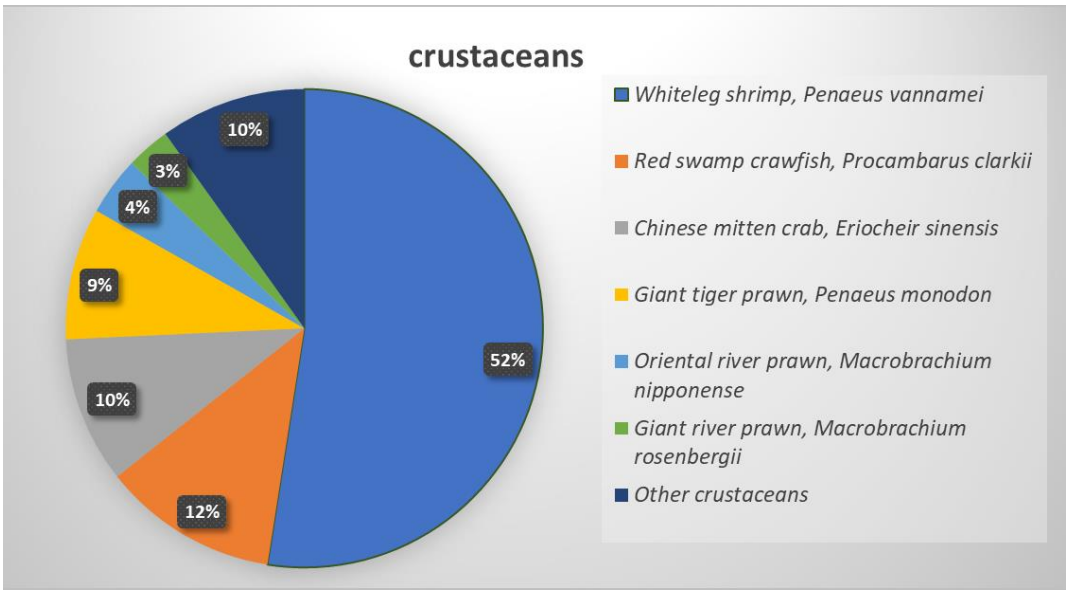

Figure 1.6. Percentage of crustacean produced by major species of world aquaculture in 2016 (Source :FAO Fisheries and Aquaculture Department, 2018)

Josupeit (2004) presented that production of global cultured shrimp had steadily been increasing from the 1990s up to 2002, but many of the key shrimp producing countries have suffered production losses over the years due to disease outbreaks. As seen in Figure 1.7, there was a slight decrease in shrimp production volumes identified in China in 1993, Thailand in 1996 and 1997, and Ecuador in 1999. All of these declines were from disease outbreaks.

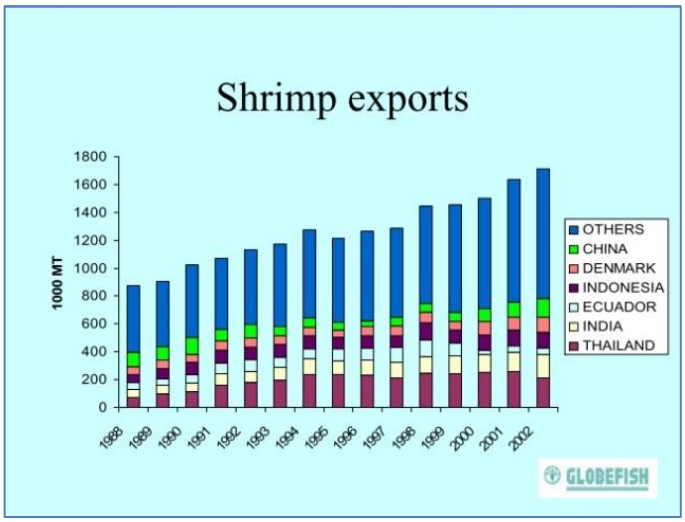

Figure 1.7. Volume of farmed shrimp exports (Source: From Josupeit, 2004)

Leung and Sharma (2001) said that the largest proportion of global shrimp aquaculture was supplied from Latin America and Asia. In the Asian region Thailand, China, Ecuador and the Philippines, were the major producers, providing about 78.6 and 83.3% of total production in 1984 and 1994, respectively. Furthermore in 1998, Thailand was the top producer country providing 28% of all Asian marine shrimp production globally and sold for human consumption. Boonmeechot (2011) expressed that the global shrimp market continues to do well, as the demand for shrimp from customers has not changed and the market price continues to increase.

However, FAO (2019a) reported that in 2017 - 2018, the largest shrimp exporter in the world was India followed by Ecuador, Viet Nam, China, Indonesia and Argentina. While, Thailand dropped down to the seventh position of global shrimp exports. The percentage of Thai shrimp produced for export dropped by 20% from 2017 to 2018 as a result of disease outbreaks and decreasing market prices.

1.4. What is the Thai Situation for Farmed Shrimp?

1.4.1 Marine Shrimp Species and Distribution in Thailand

In Thailand, wild shrimp are widely distributed along the coastline, both the Gulf of Thailand and the Andaman Sea (Petchsri, 2009 ; Nilwanich, 1999 ; Wiboonkit, 2002 ; Chantawong, 1992 ; FAO Fisheries and Aquaculture Department, 2014d). Penaeid

shrimp farming in Thailand is a commercially valuable aquaculture sector where the exported shrimp consistently remain in the top 10 highly valued seafood products globally. The production sector in general consists of seed supplied from the hatchery to the grow out systems in earthen based pond production units, which are located in coastal areas of 23 provinces in Thailand. These are distributed along the Gulf of Thailand and the Andaman Sea coastline and inland in 13 provinces (Figure 1.8) (Kung Thai Newspaper, 2012 ; Marine Shrimp Culture Research and Development Institute, 2015)

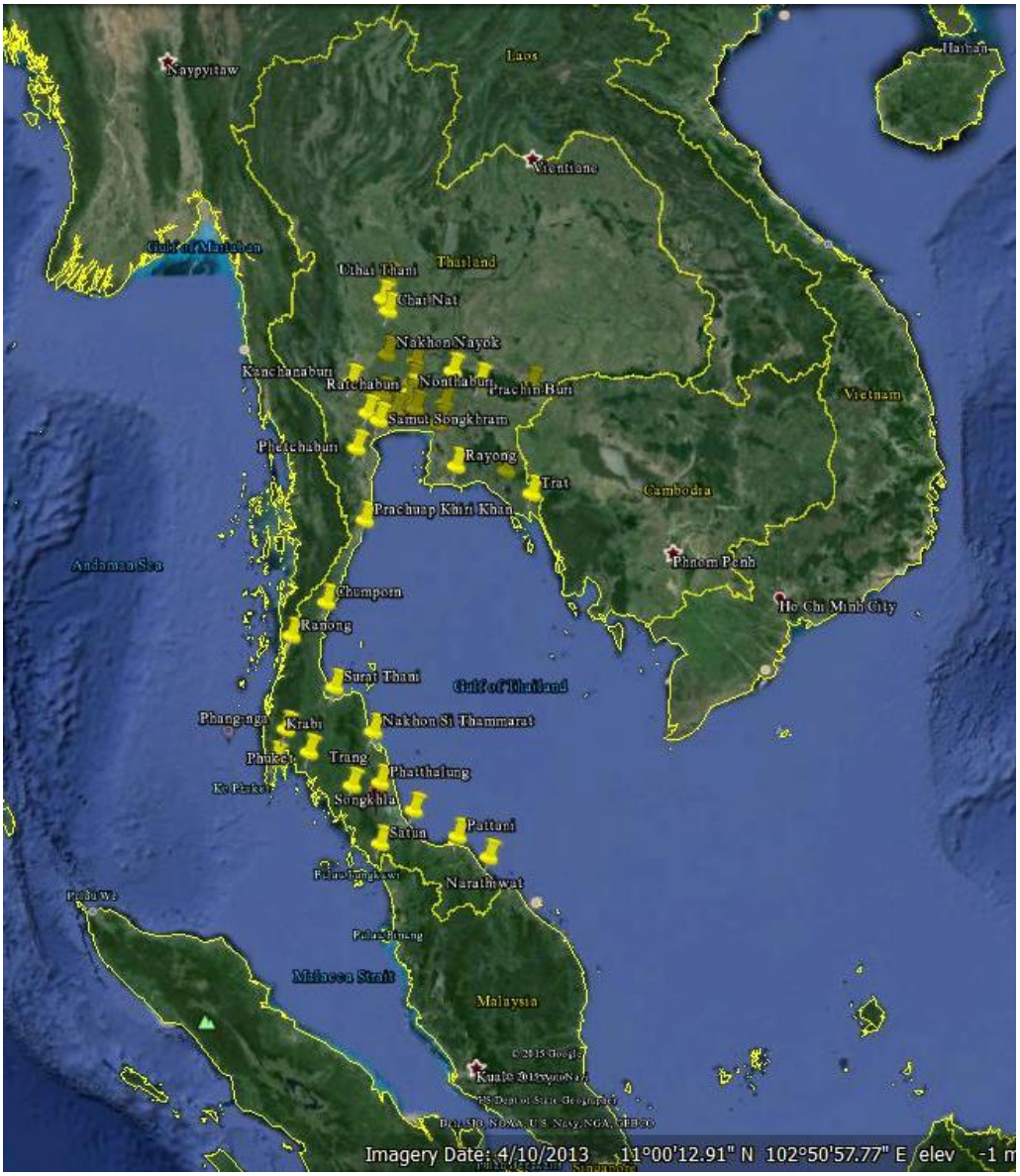

Figure 1.8. Distribution of marine shrimp culture areas in Thailand (source: Marine Shrimp Culture Research and Development Insitute, 2015)

These production systems are principally intensive farms producing for the global seafood market with the 2 species, giant tiger prawn and whiteleg shrimp dominating. The seed supply for the grow out sector comes from the hatcheries which produce primarily giant tiger prawn and whiteleg shrimp for the intensive systems, but these hatcheries are also the main suppliers of the banana shrimp which are produced primarily for restocking purposes, which is a routine responsibility of government hatchery sections (Table 1.2).

Table 1.2. Information on the 3 main shrimp species farmed in Thailand

Characteristics	***P. monodon***	***P. vannamei***	***P. merguiensis***
size	Biggest	*P. vannamei* and *P. merguiensis* is similar	*P. vannamei* and *P. merguiensis* is similar
source	endemic species	non-native species	endemic species
habitat	marine-adult, juvenile in brackish water	marine-adult, juvenile in brackish water	marine-adult, juvenile in brackish water
farmed production	the 2nd level	largest portions	rare
farmed price	Highest	lower	no information
culture period	Longer	shorter	no information

Giant tiger prawn (Plate1.1) is the biggest size species of the Penaeidae family. It lives naturally in tropical marine regions and adults are generally found in marine zone in sandy bottoms at depth of 20-50 m or over muddy sand. However, in larval stage as juvenile and sub-adults, they generally live in brackish water area, in which these stages can tolerate low salinity as 1-2 ppt. They display nocturnal feeding behaviour and are considered more predatory than other penaeid shrimp.

In Thailand, giant tiger prawn is normally caught in offshore areas. Mature female fecundity is about 500,000 to 750,000 eggs/female and it can spawn all year round (ARDA, 2014 ; FAO Fisheries and Aquaculture Department, 2014b). Both eyestalk ablation and non-ablated female broodstock have been used in Thailand where eyestalk ablation is practiced to stimulate the female shrimp to develop mature ovaries and spawn. The current understanding is that eyestalk ablation aims to reduce gonad inhibiting hormone (GIH) level that is produced by the X-organ and

sinus gland complex in order to induce the ovarian maturation of female broodstock (Treerattrakool *et al*., 2014). Primavera and Posadas (1981) reported that the highest egg hatching rate came from unablated wild stock, while ablated cultured broodstock had the lowest hatching rate. Zacarias *et al*., (2019) supported that non-ablated broodstock got lower mortality in the females and higher egg fecundity than eyestalk ablated broodstock.

In Thailand, the Phuket Fisheries Station which belongs to the Department of Fisheries was the first to successfully produce giant tiger prawn pl in 1972. At that time the production systems were mostly extensive and semi-intensive commercial shrimp farms which are recorded in 1972 and 1974, respectively (FAO Fisheries and Aquaculture Department, 2014b). Thus, giant tiger prawn was originally the main cultured species in Thailand, however at present (2019), almost all production has shifted to whiteleg shrimp. The reason for the change in species is most likely due to the faster growth rates combined with higher yield from more intensive production systems (Limsuwan, 2010). In addition, there was a general shift in shrimp species production in the intensive farming systems from giant tiger prawn to white leg shrimp in Asia as the whiteleg shrimp were considered more robust against viral disease outbreaks (Flegel, 2009).

Plate 1.1. Image of *P. monodon* Fabricius, 1798

Whiteleg shrimp (Plate 1.2) lives naturally in the estuarine zone as juveniles and moves to marine condition as adults. The water depth range from 0 to 72 m with a muddy bottom is preferred (Holthuis, 1980 ; FAO Fisheries and Aquaculture Department, 2014c). Although, this species is not endemic shrimp species in Thailand, it can tolerate a wide range of environmental conditions and has been readily accepted into the Thai production systems. Therefore, it is currently being an important commercial shrimp replacing giant tiger prawn for Thailand, even though the price of giant tiger prawn was much higher than whiteleg shrimp (FAO Fisheries and Aquaculture Department, 2014d).

Plate 1.2. Image of *P. vannamei* Boone, 1931

Banana shrimp (Plate 1.3), in the early stage juveniles inhabit estuarine waters, whilst they are found mostly in marine waters when in the adult stage (KGT, 2014). Robertson (1988) studied the feed and predators of juvenile banana shrimp in the east coast of Australia, and stated that the mangrove habitat possibly provides shelter for banana shrimp to avoid predation, so this is a reason why its preferred feeding areas are in the mangroves. KGT (2014) showed that the qualities of fine and firm meat from this species of shrimp resulted in high demand from Japanese importers and consumers. In Thailand, this species is cultured in the earthen pond (Shigueno, 1975).

Plate 1.3. Image of *P. merguiensis* De Man, 1888

Generally in Thailand, nauplii are initially stocked in hatchery tanks made from concrete or fiberglass, then nursed until the larvae metamorphose to pl identified as pl10 - 12. At this stage, they will be directly transferred to the earthen ponds and cultured until reaching marketable size. A nursing step can be introduced where the pl10 - 12 would be transferred from the hatchery to a dedicated nursery area at the pond sites until the pl become bigger at approximately 7 - 30 days before they are stocked into earthen pond and cultured to market size.

1.4.2 Markets, Threats and Opportunities for the Farmed Thai Shrimp

1.4.2.1 Status of Thai Shrimp, Markets and Exports

Josupeit (2004) stated that worldwide shrimp exports have constantly been increasing from 1990 to 2002 which the majority of shrimp exported originated from Thailand. However, the Thai shrimp farming sector suffers from a range of threats to the sustainable production including fluctuating market prices and disease outbreaks. The Thai sector increased production well initially, and in 1997 was the major exporter of edible shrimp. However, it has suffered from a range of disease issues affecting production and then again suffered from an EU ban on the exported products due to the high antibiotic residues in 1998, consequently Thai exports also declined.

Significant changes to farming species, systems and practices as well as changes to regulations have been applied over the years in Thailand. Fisheries Statistics Analysis and Research Group, Department of Fisheries; Thailand (2015) reported

that from 1993 to 2000, Thai marine shrimp production was increasing and in 2001 the production decreased slightly, due to a change in site farming regulation. From 2009 to 2012, the marine shrimp aquaculture production increased from 575,098 tonnes in 2009 and 609,552 tonnes in 2012. However, since 2013 the Thai farmed shrimp production plummeted into 325,395 tonnes due to an emerging disease called Acute Hepatopancreatic Necrosis Syndrome (AHPNS) (Fisheries Statistics Analysis and Research Group, Department of Fisheries; Thailand, 2015 ; Flegel, 2012).

The changes to farming species, systems and practices have supported Thailand to be the world top 6 and 7 aquaculture producers of fishes, crustaceans, molluscs, amphibians, reptiles (excluding crocodiles) and other aquatic animals for human consumption in 2010 and 2011, respectively (FAO Fisheries and Aquaculture Department, 2013a). In 2010, the production volume was 1,286,122 tonnes, while it had slightly decreased to 1,008,049 tonnes in 2011 (FAO Fisheries and Aquaculture Department, 2013a). Thailand shares a major position in world fisheries and was one of the main farmed seafood exporting countries (FAO Fisheries and Aquaculture Department, 2012). In 2015, Thai marine shrimp production from aquaculture was 294,740 tonnes of which 95% came from whiteleg shrimp and 5% from giant tiger prawn.

Boonmeechot (2011) stated that in 2009 farmed shrimp at 50 individuals/kg (ind/kg) the farm gate price was about 3.6 USD[1], while at the same size it was 4.7 – 5.02 USD in 2010 even though price of raw materials increased around 30 – 50%. On the other hand, in 2010 the exporter faced loss of income due to the lower value of the Thai Baht currency from 34 to 30-31 baht/1USD. The qualities of Thai shrimp are widely known around the globe and the products sustain a high standard of quality when compared with other country competitors. The good quality standard must be maintained if the Thai product is to remain within the top 10 seafood commodoties, for example, farmers have to avoid using forbidden residues or chemicals. Thai shrimp products are sold internationally, where the proportion of the market share is provided in Figure 1.9.

[1] 1US$ = 31.90 Thai Baht in 2019

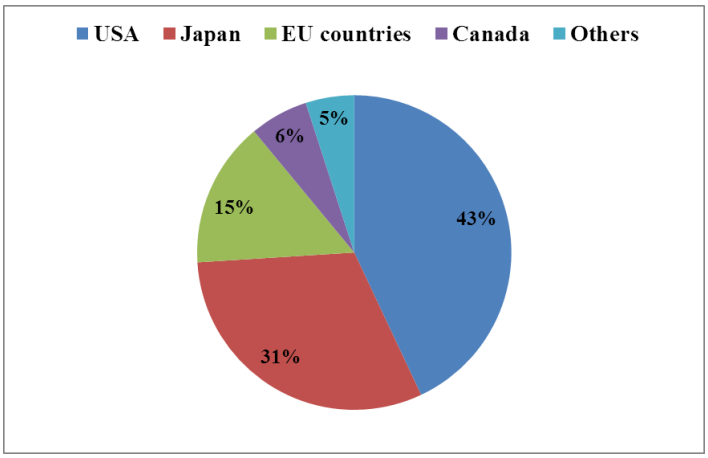

Figure 1.9. The export market share of Thai shrimp products in 2010 (Information from Boonmeechot, 2011)

Pratruangkrai (2013) published the interview with the president of Thai Shrimp Association in the Thai English-speaking newspaper The Nation, said that in 2013 Thai shrimp production had plummeted, so Thailand lost the position of the world's main exporter to Ecuador and India. He illustrated that the volume of Thai shrimp had decreased, but the value remained high as the overall global production had also declined, thus stabilising the market value of shrimp. Currently, Thai shrimp hatcheries practice rigorous hygiene and shrimp farmers are constantly improving their culture techniques and bio-secure management. As a consequence of these improvements, the Association expected that Thailand could be the world's leading exporter again in 2015.

Yuwabenjapol (2014a) identified that the United States, Japan and European Union were the main market outlets for Thai shrimp farmed products. The biggest was in the European region where 700,000 tonnes/year of farmed Thai shrimp was imported in 2012. Yuwabenjapol (2014a) identified that high quality standards and hygiene was the most important issues that producers and exporters should be concerned about to ensure their product reached the appropriate markets.

TFFA; Thai Frozen Foods Association (2018) reported that the five main markets for Thai shrimp export production in 2018 were the United States, Japan, China,

Australia and South Korea. The largest importer of Thai shrimp was the United States accounting for 31.7% (Figure 1.10.).

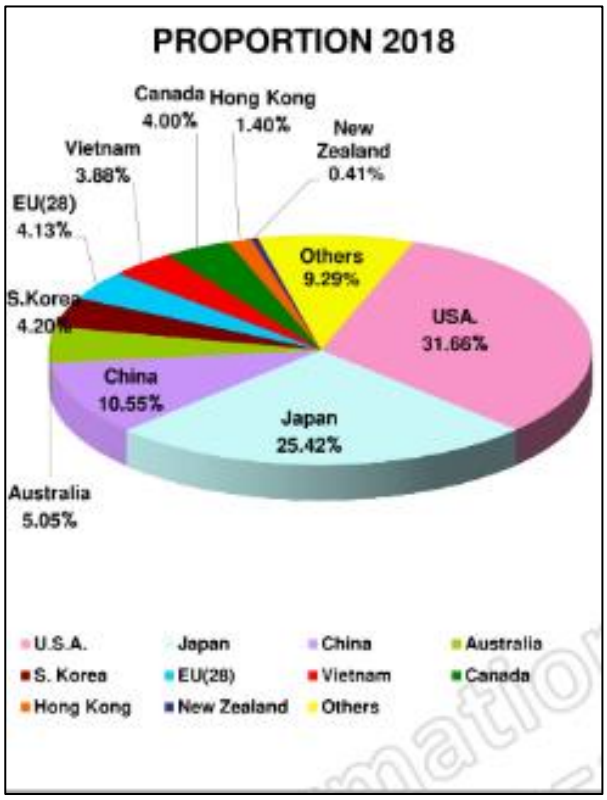

Figure 1.10. The export market share of Thai shrimp products in 2018
(Source : modified from Thai Frozen Foods Association; TFFA, 2018)

FAO (2019b) reported that in 2017, Thai shrimp production was lower than expected and this was caused by animal health and weather problems as well as constraints in the supply chain. Thailand was faced with EU supplies that the preferential tariff was withdrawn and deficient of shrimp raw material on exports to the USA.

1.4.2.2 Factors/Sustainable competitiveness of Thai shrimp section

Production of farmed shrimp has been increased rapidly since 1980. Thailand was one of the main shrimp producers in Asia. The Thai shrimp production in 2004 reported that *P. vannamei* was accounted for 66% of production, followed by *P. monodon*, 26% and 8% was from other shrimp species (Lebel *et al*., 2010). Since 2013, Thai shrimp production has been declined with many contributory factors, particularly disease epidemics. Future sustainability will depend on all stakeholders

addressing the issues. Sustainability of shrimp aquaculture, Thai government's policy, the farm level with concerning on food safety has been successfully contributed to shrimp industry. (Giap *et al*., 2010 ; Lebel *et al.*, 2016).

NNT, National News Bureau of Thailand (12 December, 2019) reported that the Thai Shrimp Association President (Dr.Somsak Paneetatayasai) said that global shrimp prices had declined which affected national prices and competitiveness of Thai shrimp in worldwide markets. However, he said that due to the advantages of Thai shrimp seed, which in his opinion, was the best quality shrimp seed in the world, fast growing, strong, disease-free, consequently Thailand had a great deal of potential to produce large size shrimp. He continued that the direction of Thai shrimp production will not focus on quantity but on producing good quality large shrimp, which are safe, free from residues, traceable and environmentally friendly. All of these opportunities of Thailand could be possible to be returned to the top position of shrimp producers again.

Although, Thai shrimp industry encountered disease outbreaks which impacted production and export losses causing the decline of the competitiveness in global market. However, Thailand has strengths over competitors including aquaculture technology, skills of labour, well supported industry and good quality of products, but cost of shrimp production such as raw materials, high minimum wage are still disadvantages in competitive trade (Panichpattanakit and Siriburananon, 2018).

In term of government support for Thai shrimp in long term competitiveness, the Department of Fisheries (DOF), Thailand had been giving priority to helping the Thai shrimp industry meet international standards with high quality products including sustainability, food safety, eco-friendly, social responsibility and traceability to support the Thai shrimp industry. Aquaculture zoning, culture control process, monitoring of aquatic animal diseases and chemical used by checking lot-by-lot as well as Good Labour Practice (GLP) certified, These have implemented under the Royal Ordinance on Fisheries 2015 and its amendment in order to push up Thailand's shrimp culture towards sustainability (Gnews, 2019).

Boonmeechot (2011) suggested that there are several advantages for customers buying Thai shrimp products compared with products from other countries. The main advantages included : 1) Thai farmers produce products of high quality in agreement with the varied certification standards e.g. CoC, GAP ; 2) Thai farmers follow the Fry Movement Document (FMD) and Movement Document (MD) to improve traceability of the product from hatchery until processing plant ; 3) Thailand has a sustained high production volume, hence it can fulfill the market demand from a range of importers ; 4) Thai shrimp are available as a range of variable product types e.g. frozen, fresh and can be included into the added value chain.

In order to maintain being the main of shrimp producer. It would say that improving qualities of shrimp production should be concerned for customers, not only from on grow farming but also looking back to the hatchery production. As high quality products in the hatchery can support Thai shrimp to be a more sustainable product.

1.4.3 Marine Shrimp Hatchery Practiced in Thailand

1.4.3.1 Hatchery System

Leung and Sharma (2001) classified shrimp hatcheries into 3 scales which were described as small, medium and large size. Basically, backyard hatcheries or small-scale was a low operating costs and low construction, seed supply depended on wild source with low stocking densities using small tanks. Medium-scale hatcheries were described as low stocking densities using large tanks and low water exchange. Whereas, in large-scale systems the facilities and technologies included seed produced all year round and high levels of water exchange using filtered water.

Hatchery systems have also changed over the years in Thailand, initially using the small-tank system implemented from the USA and then applying the large-tank systems from Japan as the sector grew (Kungvankij, 1985). Both of these systems are still used in Thailand, where small hatcheries include family-operated system run by the owners. These are often considered as more cost-effective than the large size due to no need to hire labour (Kongkeo and Davy, 2010).

In 2014 – 2018 (during period of this study), shrimp farms in Thailand faced disease problems associated with V*ibrio parahaemolyticus* (AHPND). The disease resulted

in mass mortalities in early stage of 15-35 days post stocking of the pl. Farmers assumed that larger pl (>pl 15) might be more tolerant to pathogens than smaller pl (pl12-15). The farmers have managed and altered their operations by using a nursery phase (both indoor and outdoor facilities) before introduction to the grow-out ponds. For indoor nursery production, farmers built the nursery building beside the ponds, allowing them to grow the pl to a larger size before stocking into ponds.

1.4.3.2 Broodstock source/Domestication of Thai Marine Farmed Shrimp

Previously, more and more areas were suffering from a lack of wild-caught broodstock (Browdy, 1998) and in the Andaman Sea, overfishing was considered as one of the main causes of broodstock reduction in wild populations. Particularly as locations of ripe female shrimp were difficult to find at this time. In 1983, black tiger prawn and banana shrimp pl were successfully produced by Department of Fisheries (DOF), Thailand (Anantanasuwong, 2001) as these methods provided a source of shrimp seed for production. Furthermore, poor stock health management has been an increasing concern globally and to support this sector, there was a need to develop reared broodstock to reduce over reliance on wild broodstock supplies.

Research on technological advances to support domestication, has shown that male broodstock with eye ablation did not produce more sperm quality or spermatopore size than non-ablation (Pratoomchat *et al*., 1993). Pond-reared gravid female broodstock were found to have a lower quality and quantities of eggs than wild broodstock. The importance of egg quality and quantity is critical for the sustainable development of the sector, as Menasveta *et al.* (1994) pointed out that egg qualities characterised by hatching rate, percentages of fertilisation and metamorphosis, will impact the success of the grow out stage.

To support the sustainability of the broodstock sector, domestication/selective breeding programme was being considered. Some researchers who studied on broodstock domestication factor (Wyban, 2007 ; Pongtippatee *et al.*, 2018 ; Wyban, 2019). Wyban (2007) demonstrated that the progression of shrimp domestication breeding programme have has contributed to the shrimp industry growth. In 1998 -

2006 the production of shrimp have been expanded from 10% to 75% ,and at that period the *P. vannamei* has come to Asia.

In Thailand, shrimp domestication of whiteleg shrimp broodstock for the traits of disease resistance and fast growth have been conducted in association with the use of SPF broodstock. Domesticated shrimp broodstock are already commercially available in Thailand, both whiteleg shrimp and giant tiger prawn, however wild giant tiger prawn broodstock were still being used in some small hatcheries.

Regarding the shrimp genetic improvement programmes, they are costly but cost effective. There are several companies in Thailand which conducted the domestication/SPF breeding programmes. The Charoen Pokphand (CP), for example, is one of companies that has developed techniques with bio-secure to produce healthy SPF domesticated stocks, in which the genetic breeding programme could be prevention of disease with fast growth (McIntosh, Charoen Pokphand Foods Public Co., Ltd. Pers. Comm.).

To be sustainable, further research is required regarding genetics of cultured broodstock because in the long-term the shrimp industry cannot remain successful by using wild seed or broodstocks alone.

1.4.3.3 Hatchery Husbandry Management in Marine Shrimp

Achieving an optimal stocking density for the hatchery system is not easy as high stocking densities can produce low animal survival rates. A study by Anantanasuwong (2001) identified that high stocking density increased population numbers, the shrimp larvae produced high levels of waste products resulting in increased stress responses in the animals as shown by greater susceptibility to disease. Previously, about 10-20 pl/L was initially considered to be a suitable stocking density in the hatchery, whereas recently, this has increased to approximately 50-100 pl/L (Tumnong, Pers. Comm., 2019).

After the eggs hatch, the nauplius are quickly transferred to nursing tanks and during nursing stages, the larvae are fed on microalgae, before moving onto artemia nauplii and then eventually artificial diets. The feeding strategies have been developed over

many years and applied to support the growth of the animals between the different development stages, and if successful then after 3 weeks the pl can be released into ponds (Boyd and Clay, 1998).

Typical feeding strategies applied during the protozoea stage (until pl4 - 5), include feeding phytoplankton such as *Skeletonema* sp., *Chaetoceros* spp. or *Tetraselmis* sp. at density of 30,000 - 50,000 cells per ml. Moreover, microencapsulated feeds can also be supplied to the larvae. From the mysis stage, 50 g of cysts of *Artemia* nauplii are given to 100,000 larvae (FAO, 2014b). To reduce operating cost, *artemia* flakes can also be used as a supplement as well as *Artemia* nauplii. Artificial feeds are commonly fed in order to control water quality, which can degrade more quickly whenever using fresh feed from pl4 to pl15. It takes about 26 days for the nauplius to reach pl15. Varadharajan and Pushparajan (2013) studied the gut content in whiteleg shrimp and found that phytoplankton is the highest percentage of dietary composition in both male and female shrimp which are less than 120 mm size. In contrast shrimp size from 130 mm up the maximum of food item is supplementary feeds.

FAO Fisheries and Aquaculture Department (2014b) pointed that black plastic cloth or roof tiles should be used to cover nursery tanks in order to control fluctuated temperature of water and to shade light intensity. Without appropriate cover, these can negatively influence the growth and survival of the pl.

Kongkeo and Davy (2010) said providing good quality shrimp seed combined with efficient culture technologies were keys indicators of success in shrimp production. By incorporating the support from Thai Government at the hatchery stage, all these factors would improve the sustainability of Thai shrimp farms. They also identified adoption of certification standards in hatcheries would be beneficial to the development of high quality Thai shrimp products. Therefore, uptake of farm and hatchery registration has been one of the activities supported by the DOF, Thailand resulting in higher numbers of hatcheries being registered for Code of Conduct (CoC) and Good Aquaculture Practice (GAP) certificates since 2003.

1.4.3.4 Number and Location of Shrimp Hatcheries in Thailand

In 2014, there were approximately 823 hatcheries in Thailand, mostly located in Provinces near the seashore. Only a few provinces that farm shrimp are far from the sea, so the seawater supply must be transferred from seawater source. (Table 1.3; Source: Department of Fisheries, Thailand, Pers. Comm., 2014)

Table 1.3. List of marine shrimp hatchery in Thailand.

Province	Total (unit)	Broodstock (unit)	Broodstock +Nursery (unit)	Nursery (unit)	Species/No. of hatchery
Chanthaburi	6	1	4	1	black tiger/4, whiteleg/2
Chachoengsao	239	4	33	202	black tiger/14, whiteleg/ 221, macrobrachium/4
Chonburi	134	6	38	90	black tiger/53, whiteleg/80, macrobrachium/1
Chumphon	6	0	5	1	black tiger/1, whiteleg/4, banana shrimp/1
Trang	10	0	6	4	black tiger/6, whiteleg/4
Krabi	8	0	8	0	black tiger/4, whiteleg/3, banana shrimp/1
Trat	9	0	5	4	Whiteleg/9
Nakhon Pathom	36	3	20	13	Whiteleg/36
Nakhon Si Thammarat	54	8	34	12	black tiger/14, whiteleg/39, green tiger/1
Narathiwat	1	0	1	0	black tiger/1
Prachuap Khiri Khan	21	3	4	14	black tiger/2, whiteleg/19
Pattani	2	0	2	0	black tiger/1, whiteleg/1
Phang Nga	32	3	23	6	black tiger/11, whiteleg/21
Phetchabri	5	1	4	0	black tiger/2, whiteleg/3
Phuket	94	10	45	39	black tiger/21, whiteleg/73
Ranong	1	0	1	0	banana shrimp/1
Rayong	12	3	6	3	black tiger/3, whiteleg/9
Ratchaburi	3	1	2	0	white leg/3
Song Khla	108	14	57	37	black tiger/15, whiteleg/93
Satun	17	0	11	6	black tiger/5, whiteleg/12
Samut Prakan	1	0	1	0	whiteleg/1
Samut Songkhram	6	0	5	1	black tiger/3, whiteleg/2, other shrimp/1
Samut Sakhon	5	3	2		black tiger/1, whiteleg/4
Suphanburi	9	2	7	0	Whiteleg/9
Surat Thani	4	1	3	0	black tiger/2, whiteleg/2
	823	**63**	**327**	**433**	

Note : Broodstock = produce nauplii only , Broodstock+Nursery = produce nauplii and pl , Nursery = produce pl only

1.4.4 Marine Shrimp Culture Practiced in Thailand

1.4.4.1 System of Thai Shrimp Farms

Many references describe the varied farming practices. The farmed shrimp system can be classified broadly using the data provided in Table 1.4.

Table 1.4. Description of the Thai farmed shrimp systems

Type of system	Catagories/Descriptions	References
extensive	Low number of seed stocks, no water pumping, mostly wild seed stock with stocking density not more than 2/m², natural foods are fed regularly.	FAO (2014b)
semi-intensive	Medium number of stocking densities, reared seed stocks with densities of 5 to 20 pl/m². Water exchange required. Natural food in the pond as well as artificial diets provided.	
intensive	Only high stocking densities are used, water supply by pumping and drained and dried system can be completed before each crop. Hatchery-produced seeds purchased only with stocking density of 20-60 pl/m², aeration provided, artificial feed given about 4-5 times/day with feed tray checking. Main parameters of water quality regularly inspected.	
traditional, extensive, semi-intensive, intensive and ultra-intensive	Stocking densities and management varied the type of system.	Ronnback(n.d.)
extensive, semi-intensive and intensive	These were separated based on technology inputs and economic incomes.	Leung and Sharma (2001)
extensive, semi-intensive, intensive and super-intensive	Those can be catagorised by stocking densities and pond area. He pointed that a few shrimp farms only can conduct super-intensive farms due to input requirements of very high technologies and high financial inputs.	Tookwinas (1996)

1.4.4.2 Current Status of Marine Shrimp Culture in Thailand

In Thailand, right now, all farms and hatcheries should have certification from the Thai Good Aquaculture Practice (GAP). This is a certification standard applied from the Thai DOF Government. The number, area and production of Thai marine shrimp

aquaculture from 2011 to 2015 is shown in Table 1.5 (Fisheries Statistics Analysis and Research Group, DOF, 2015). Clearly the number of farms and production area by rai has reduced over the 5 years but the proportion of shrimp production remains highest from the farmed sector.

Table 1.5. Marine shrimp culture in Thailand from 2011 to 2015

	2011	2012	2013	2014	2015
No. of farms (unit)	23,675	23,832	21,668	21,071	21,082
Area (rai)	362,645	367,624	311,589	295,568	299,844
Total production (tonnes)	653,428	605,107	362,308	316,683	328,071
- Shrimp culture	611,194	609,552	325,395	279,907	294,740
- Capture	42,234	40,555	36,913	36,776	33,331

Note: 1 hactares equa to 6.25 rai

Fisheries Statistics Analysis and Research Group, Department of Fisheries; Thailand (2018) reported that the dominant species farmed was whiteleg shrimp.

1.5 Environmental Issues/Shrimp Policy and Regulation in Thailand

From 1972, Thai shrimp aquaculture was encouraged by Thai Government by offering financial assistance to those wishing to participate (Goss *et al*., 2000). Promotion of shrimp farms by the DOF, Thailand during the 1970s supported the investment in the shrimp industry by the Government Board of Investment. The support provided when prices of shrimp were reducing were part of the Government policies and regulations on the Shrimp Farming industry in Thailand (Patmasiriwat *et al.*, 1996). This support helped to establish the production systems in practice currently, however as with many farming systems there were some concerns regarding impact on the environment.

In the early to mid 1990's destruction of mangrove areas to accommodate shrimp farming was a particular concern especially as this time was aligned with the rapid development of the farming sector (Boromthanarat, 1996). Tookwinas (1996)

expressed that Thailand had a long coastal area about 2,600 km. So, by farming along the coastal area, this can avoid unnecessary destruction of mangrove area and reduce environmental impact due to shrimp farming. The DOF, Thailand supported the policy of removing shrimp farms out of the mangrove area to reduce negative impacts. These policy changes helped to support the further development of the Thai shrimp farming sector.

1.6 Disease Issues

1.6.1 Shrimp Disease Outbreaks

Over the last 20 years, several reports have occurred describing the cause and effect of infectious diseases in farmed shrimp species. The existing and emerging disease are listed in Table 1.6 where several of these, particularly the viruses, have significantly impacted the sustainable development of the Thai shrimp farming sector.

Table 1.6. Examples of the most common and emerging diseases causing outbreaks in farmed shrimp

Aetiological Agent/Disease	Reference
White Spot Syndrome Virus (WSSV)	Lo *et al*., 2012
Yellow Head Virus (YHV)	Cowley *et al*., 2012
Acute Hepatopancreatic Necrosis Syndrome (AHPNS)	Tran *et al*., 2013
Runt Deformity Syndrome (RDS) from Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV)	Lightner, 1996
Taura Syndrome virus (TSV)	Lightner *et al*., 1995
Baculovirus penaei (BP)	Wang *et al*., 1996
Covert Mortality Nodavirus (CMNV)	Zhang *et al*., 2014
Hepatopancreatic microsporidiosis caused by *Enterocytozoon hepatopenaei* (EHP)	Chayaburakul *et al*., 2004; Tangprasittipap *et al*., 2013; Tourtip, 2005
Aggregated, transformed microvilli (ATM) in the tubule lumens	Sriurairatana *et al*., 2014
Infectious Myonecrosis Virus (IMNV)	Poulos *et al*., 2006
Abdominal Segment Deformity Disease (ASDD)	Sakaew *et al*., 2008
White Tail Disease (WTD) reported from *P. monodon*, *P.indicus* White Tail Disease (WTD) reported from *P. vannamei*	Ravi *et al*., 2009 Senapin *et al*., 2012; Senapin *et al*., 2013
Monodon Slow Growth Syndrome(MSGS) associated with Laem Singh Virus (LSNV)	Pratoomthai *et al*., 2008; Sritunyalucksana *et al*., 2006
a novel integrase-containing element (ICE)	Panphut *et al*., 2011
Hepatopancreatic Haplosporidiosis (HPH)	Utari *et al*., 2012

Flegel (1997) studied disease of *P. monodon* in Thailand and reported that viruses causing economic loss in giant tiger prawn farms included white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvo-like virus (HPV),

infectious hypodermal and hematopoietic necrosis virus and monodon baculovirus. Almost 10 years later Flegel (2006a) reported that viral diseases remained one of the biggest issues in Thai shrimp farming where the highest to lowest economic losses, at that time, arose from infections from WSSV, YHV, HPV, and monodon baculovirus, respectively. Flegel (2009) said that the reason why the species of shrimp culture was shifted from giant tiger prawn to whiteleg shrimp was due to disease susceptibility.

Since 2002, whiteleg shrimp has become the more dominant species produced in Thailand, as they are thought to less susceptible to diseases compared with black tiger shrimp. However, it must be noted that both species are mostly susceptible to the same diseases. In order to avoid the spread or introduction of diseases, all transported shrimp stocks movement should be strictly inspected for quarantine measurement. Lack of robust quarantine and inadequate processes for transboundry health checks would threaten biosecurity practices and support the transference and survival of viral pathogens in the long-term (Flegel, 2006b ; Flegel, 2007).

Thitamadee (2016) has reviewed the current disease problem of farmed penaeid shrimp in Asia and found that many diseases described above exist but there is an increase in emerging disease, included acute hepatopancreatic necrosis disease (AHPND). To reduce the risk of increase disease outbreaks, improvements must be made to the existing biosecurity practices. Back in 1998, Bowdy reported that by using specific pathogen free stocks, combined with stricter hygiene and sanitation practices and better quality feed would all produced more healthy postlarvae while reducing cost and improving reliability of shrimp production.

1.6.2 Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND)

An emerging disease condition affected the global farmed marine shrimp sector and emerged in Thailand since 2011 (FAO, 2013b) and 2012 (Flegel, 2012 ; Lightner *et al*., 2012). This condition has spread to all intensive shrimp producing countries within Southeast Asia, reported in China (2010), Vietnam (2010), Malaysia (2011) (Flegel, 2012 ; Lightner *et al*., 2012), and more recently in Mexico (2013) (Nunan *et*

al., 2014). FAO (2013b) reported that in Thailand, in 2011 the area of shrimp farm in eastern Gulf of Thailand was first affected by EMS/AHPND, which the disease infested the early stage of 15-35 days of pl whiteleg shrimp released to earthen pond. The result of this emerging disease outbreak was high mortality with approximately 100% occurrence reported. The condition then continued to spread throughout Thailand reaching the shrimp farms on the east coast of the Gulf of Thailand in 2012.

This disease was originally called Early Mortality Syndrome (EMS) as the mortalities affected the early pl, at 30 days post stocking into the grow-out ponds (FAO, 2013b ; Hong *et al*., 2016), hence the term early mortalities. Many factors can cause EMS, not all of them pathogens or infectious agents and in the affected shrimp the clinical signs showed abnormal hepatopancreas with shrunken or atrophied, pale coloration, lethargy and anorexia and soft or loose shell (NACA, 2014). Given the possible number of aetiological agents combined with the early mortalities, identification of a single causative agent was problematic. Initial experimental studies performed in Vietnam did confirm a bacterial aetiology and confirmed that the pathology observed initially and described as emerging EMS was caused by a specific strain of bacteria called *Vibrio parahaemolyticus* (Tran *et al*., 2013). The disease was then called Acute Hepatopancrease Necrosis Disease or AHPND based on the pathology findings. Several studies were performed on the shrimp-specific *V. parahaemolyticus* to understand the pathogenesis and eventually, the AHPND-strains of *V. parahaemolyticus* could be differentiated from other non-pathogenic strains as the shrimp strains all contain a plasmid with a Photorhabdus insect-related (Pir) toxin. Recovery of the bacteria from naturally infected shrimp was difficult and not robust and so diagnosis of AHPND relies on histopathology to identify the cause and confirm cellular changes indicative of AHPND. However, given the ubiquitous nature of the *V. parahaemolyticus* bacteria in the farmed shrimp sector there was a need for a more rapid screening tool to confirm the presence of the AHPND-bacteria in affected animals. This led to several versions of a PCR assay to detect the presence of the toxins located on the plasmid of the bacteria.

As the transmission route and source of the bacteria is not well established, in order to reduce the impact of this emerging disease, the biosecurity in hatchery and farm

is needed to be improved as well as pl seed needs to be inspected. Yuwabenjapol (2014b) suggested that pl must be inspected before stocking to the ponds in order to prevent AHPND in the grow out systems, and stated that the following criteria are practised:

- Observing the pl under the microscope to check that there is a normal hepatopancreas without bacteria and plenty of completed lipid cell in the shrimp.
- Stress test is also done to inspect the health of the pl.
- Age of pl should not less than pl10.
- The optimal pH of water is not less than 8

1.6.3 *Vibrio harveyi* in Thai shrimp hatchery

Vibrio harveyi is halophilic bacterium which was identified as Gram-negative, rod shaped (Mirbakhsh *et al*., 2014). It is associated with luminescent bacterial disease which causes mass mortalities in shrimp hatcheries. In Thailand's hatcheries, previously, there have been serious disease problems from which *V. harveyi* was isolated by Ruangpan and Kitao (1991). However, at the time of my PhD surveys (2014-2015) the interviewees pointed out that this bacterial disease was less serious issues than previously. The reduction in the incidence and severity of this condition may have been due to farmers learning from experience. Any improvements must have been due to health management since antibiotics are not very effective.

1.7 Probiotic and Biofloc Use in Aquaculture Systems

Studies have shown an increased interest in the use of alternatives to antibiotics in global aquaculture systems (Farzanfar, 2006 ; Ninawe and Selvin, 2009 ; Vinoj *et al*., 2013 ; Thammasorn *et al.*, 2017; Wang *et al*., 2019 ; Chien *et al*., 2020), which has promoted a greater use of probiotics. Probiotics are usually bacteria claimed to reduce effects of pathogenic bacterial species and have been used in shrimp aquaculture systems for many years (Chiu *et al.*, 2007; Ajitha *et al*., 2004; Vieira *et al.*, 2007; Castex *et al.*, 2008 ; Wang *et al*., 2019 ; Amoah *et al.*, 2020).

The mode-of-action of many probiotic bacteria is not well understood. Although *in vitro* laboratory based tests can be a useful screening tool to identify if the probiotic

strains can impair bacterial growth from potential pathogens, the gold-standard test remains an *in vivo* application. Probiotics are often administered in the feed and then animals are exposed to the bacterial pathogen by experimental challenge and the morbidity/mortality levels measured against a control group of animals not fed the probiotic but exposed to the bacteria. This method has been used by a wide range of researchers and a summary of the varied probiotics, and pathogens is provided in Table 1.7. A reproducible challenge model or feed study of the probiotics does not currently exist and instead a range of probiotic/pathogen concentrations have been used.

Table 1.7. List of *in vivo* probiotic studies applied for shrimp species globally

Animals	Probiotic Species/Strain	Bacterial / viral challenge species	References
P. vannamei	*V. alginolyticus*, *B. subtilis*, *Roseobacter gallaeciensis*, *Pseudomonas aestumarina*	*V. parahaemolyticus*	Balcazar *et al*. (2007)
P. vannamei	*V. alginolyticus*	*V. parahaemolyticus*	Garriques and Areval (1995)
P. vannamei	*B. licheniformis*	*V. harveyi*	Hong *et al*., (2005)
P .monodon	*B. subtilis*	*V. harveyi*	Vaseeharan and Ramasamy (2003)
P .monodon	*Bacillus* S11	*V. harveyi*	Rengpipat *et al.* (1998)
P. vannamei	*L. plantarum*	*V. parahaemolyticus* Yellow Head Virus	Thammasorn *et al.* (2017)
P. vannamei	*L. plantarum*	*V. harveyi*	Vieira *et al.* (2010)
P. vannamei	*L. plantarum*	*V. harveyi*	Kongnum and Hongpattarakere(2012)

Many researchers have attempted to find the ways to solve the problems of massive mortality of shrimp by using biofloc technology or aerated mixed systems to encourage growth of bacteria and other organism on suspended particulate matter (Avnimelech, 2012 ; Kim *et al*., 2014 ; Pamanna *et al*., 2017 ; Promthale *et al*., 2019 ; Ferreira *et al*., 2020). Biofloc is a technique which has been widely used in shrimp aquaculture. Use of biofloc technique has been used to minimise the water exchange to reduce cost and pathogens incoming water (Avnimelech, 2012 ; Bossier and Ekasari, 2017). By adding carbon sources into the water system in order to balance the carbon and nitrogen ratio this can help to control water quality. Published claims for its efficacacy include: improving water quality, immune response and survival (Crab *et al*., 2012 ; Kim *et al*., 2014 ; Yun *et al*., 2016 ; Promthale *et al*., 2019). Pamanna *et al*., (2017) studied the efficacy of utilization of different carbon source for establishing biofloc in *L. vannamei* rearing and found that the survival of *L. vannamei* was higher than control group when adding carbohydrate (wheat flour, tapioca flour and molasses) for biofloc and wheat flour souce was the highest survival rate of 73.36%. Currently, use of fishmeal as an ingredient in formulated diets trends not to satisfy the needs of the shrimp, and Promthale *et al*. (2019) found that substitution of fishmeal by bioflocs could enhance the immunity of shrimp that can prevent *V. parahaemolyticus* infection as well as improving of survival rate.

1.8 Current Shrimp Health Management Practises in Thailand

1.8.1 What is Health Management?/Why Health Management Need to Be Improved?

Shrimp health management is just as important as any other farmed animal or crop destined for the food market. As with other industries it plays an important role but is can often be confused with disease diagnosis, when they are separate entities. There were various considerations to determine shrimp health; these are often based on measurable outcomes which include survival rates, mortality rates, growth rates, size variation, Feed Conversion Ratio (FCR), appearance of shrimp, effect of environment on health e.g. gill examination, gut content examination or stress test (Main and Laramore ,1999).

Shariff (1995) supported that health management practices were important in aquaculture. He pointed that the criteria applied to maintain good health should be based on the interaction of the farm environment, the host and potential pathogens, but it should also consider the diet of the animals as well as the genetic stocks of the farmed species. It is much more comprehensive than simple disease or not disease, which is why disease diagnosis can be part of the overall health criteria applied but should not replace health criteria. In the case of health management in the hatchery, the criteria applied to determine good health status of the stocks will include ensuring that there is optimal water quality, good sanitary measures and quarantine facilities and will incorporate routine health management checks with proper procedures included feeding regimes, aeration and water exchange, stocking densities and temperature should be concerned. The aim is to optimise all aspects to promote good health of the farmed shrimp at each stage of the production cycle, but obviously this is crucial at the hatchery stage as compromised health status will quickly show when the animals are stocked in the ponds.

1.8.2 Biosecurity

Prevention of illhealth, particularly through infectious disease outbreaks is high on most farmers agenda and shrimp farmers are no exception, hence the need for high levels of biosecurity. The gold standard of farm management includes optimal biosecurity and ensuring disease prevention. Healthy seed from specific pathogen free (SPF) stocks would certainly support in preventing infectious disease problems and this has been applied for viral pathogens in marine shrimp species. However, infectious and non-infectious causes can result in illhealth or poor seed quality leading to infectious disease outbreaks in the grow out sector, therefore the stocks should be inspected and if necessary quarantined to check for potential pathogens when buying (Main and Laramore, 1999).

Identification of relevant risks in shrimp farm should be considered to make appropriate biosecurity. Site selection, operating facility standard, water treatment, SPF shrimp stocks are all important aspects of biosecurity (Lightner, 2005). A report from NACA (2012) identified that biosecurity and hygiene practices which included pl screening and sanitary improvement in the hatchery as well as well management of shrimp farm, could reduce the effect of disease outbreaks. Flegel (2009)

suggested that rearing shrimp without proper biosecurity can cause disease problems.

In 2013, the DOF, Thailand established “STOP EMS Programme” to control the disease outbreak in shrimp hatchery and nursing sections from EMS/AHPND. Whilst the aetiology of AHPND is now known, many still refered to this emerging disease as EMS, hence the use of the term EMS/AHPND here. Moreover, more rigorous disease surveillance and monitoring were also implemented by the DOF, which shrimp aquaculturist and DOF officers can communicate regarding disease situation including diagnostic services providing, improved farm management and practicing of shrimp health management.

FAO (2014b) reported that the regulations of worldwide market of shrimp exports must be to maintain the highest levels of hygiene and promote food safety standards. To implement this methods are required to screen for the uses of chemicals and medications including antibiotics and chemical residues.

1.8.3 Shrimp Health Assessment

In Thailand, the Government established criteria for assessing the shrimp quality and health management in order to improve the quality of pl seed in the hatcheries. The current shrimp health management strategies within Thai hatchery products are provided in Appendix I and Appendix II. These are a comprehensive set of criteria applied widely throughout the Thai shrimp hatcheries and are followed to promote the quality of the pl.

1.8.4 Standards and Certification Schemes for Thai Shrimp Hatchery Operation

Thailand has paid attention to safety of shrimp products and trade responsibility in order to achieve the competitiveness in worldwide markets. To improve the Thai shrimp quality so the DOF, Thailand established the standard and certificate of Good Aquaculture Practice (GAP) and Code of Conduct (CoC) in 1999 to develop shrimp culture to be sustainable. Moreover, “TraceShrimp”; a trial computerized traceability programme was produced and implemented by the DOF to determine residue limits/issues throughout the value chain products such a trace, feed mills information, suppliers, processing plants (Yamprayoon and Sukhumparnich, 2010).

A summary of the different types of standards and certification schemes for Thai shrimp hatcheries is shown in Appendix III. In 2018, the Thai Agricultural Standard (TAS) 7422 - 2018 for marine shrimp hatchery and nursery was also established to certify farm standards to achieve good quality shrimp production. This certificate including hatcheries, grow-out sections, harvesting, and processing practice.

1.9 References

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CHAPTER 2

Husbandry and health management in Thai shrimp hatcheries

2.1 Introduction

2.1.1 Marine Shrimp Hatchery Development and Status

Farmed marine shrimp are a commercially valuable food-species in Thailand, where in 2013 marine shrimp shared almost 60% of the total coastal aquaculture production which equated to 325,400 tonnes of produce (Information and Communication Technology Centre; DOF Thailand, 2015). Whilst there are many species available for aquaculture the major commercial shrimp species farmed include the whiteleg shrimp, (*Penaeus vannamei,* Boone, 1931) and giant tiger prawn, (*Penaeus monodon* Fabricius, 1798) (Fisheries Statistics Analysis and Research Group, Department of Fisheries; Thailand, 2015). This Thai seafood sector has continued to grow since the first breeding success of *P. monodon* in 1972 by Phuket Coastal Aquaculture Station, Department of Fisheries (DOF) (FAO Fisheries and Aquaculture Department, 2014). Between 1985 to 2002, the growth of this aquaculture sector was primarily from *P. monodon* production. However, this is no longer the case as the whiteleg shrimp *P. vannamei* now has a much larger share reaching 97% of the production sector globally including Thailand, (Marine Shrimp Culture Research and Development Institute; DOF, 2015). The switch in species farmed was in part due to the perceived reduced susceptibility to viral and other infectious diseases in *P. vannamei* compared with *P. monodon* (Flegel, 2009). *P. vannamei* also has faster growth rates combined with higher yield from more

intensive production systems (Limsuwan, 2010). Further investment in the production systems, disease diagnosis and the introduction of farm and processing plant certification systems have all significantly influenced the development of this sector not only in Thailand, but globally.

Whilst there are many players in the aquaculture chain, ultimately farmers rely on the hatcheries providing them with high quality seed, which are then stocked onto the farm sites. Several certification systems have been adopted over the years at both the hatchery and farm sites to support the development of robust and healthy shrimp stocks. Examples include the Thai shrimp farm certification scheme, Good Aquaculture Practice (GAP) and Code of Conduct (CoC) which has been developed to set aquaculture standards since 1999 (Yamprayoon and Sukhumparnich, 2010) and in 2002 the standards were officially launched (Tookwinas, 2002).

Traceability of stocks plays an important role in the development of a strong food supply chain and in Thailand the Fry Movement Document (FMD) and the Movement Document (MD) were both developed as early as 2002 (Yamprayoon and Sukhumparnich, 2010). The FMD records the movement of shrimp from hatcheries to the farm sites whereas the MD is the movement of market size shrimp to the processing plants. Within the Thai shrimp hatcheries there are several issues considered important to ensure the health status of the stocks. As a result hatcheries mostly have husbandry practices which will include health screening of the broodstock and post-larvae shrimp. Specific Pathogen Free (SPF) broodstock from abroad are also permitted entry into Thailand by DOF since 2002 (Tookwinas *et al*., 2005).

2.1.2 Broodstock Health Management

Biosecurity is a very important issue for shrimp aquaculture industry, and Flegel (2009) summarized the disease problems that can result from a lack of biosecurity in shrimp culture. Importing of broodstock without checking the pathogens can cause the disease outbreak and producing bad quality larvae. The Thai Government allowed the import of domesticated *P. vannamei* SPF broodstock to Thailand in 2002 (Briggs *et al*., 2005). Broodstock with SPF status should be inspected in order to quarantined the pathogens to prevent disease (Lightner, 2005) when bringing the

broodstock to the hatchery. SPF shrimp are produced with freedom from several important pathogens. Broodstock are sampled using a variety of pathogen detection methods but primarily including PCR test for White Spot Syndrome Virus (WSSV), TSV (Taura Syndrome Virus), Yellow Head Virus (YHV), and Infectious Hepatopancratic Hemopoietic Necrosis (IHHNV). The shrimp are then cultured under biosecure conditions (http://www.shrimpaqua.com, 2014). Live feed is a major biosecurity risk for shrimp broodstock. The Thai Shrimp Association (2013) pointed that broodstock should not be fed with fresh feed such as fresh Polychaetes (blood worms, sand worms), molluscs or squid because they might be a source of infection.

2.1.3 Aims of this Part of the Study

While there were references to health management in shrimp hatcheries there had been no recent systematic survey of Thai shrimp hatcheries to characterise current practices. Therefore, a systematic nationwide survey was planned to describe current practices and look for associations between any differences in practices and productivity or health of the post larvae (pl).

2.2 Materials and Methods

2.2.1 Survey

Cross-sectional survey was conducted, and the primary raw data were collected using questionnaire-based interviews performed in Thailand between December 2014 to April 2015. The period for which the data was requested, was the previous year's production. The hatcheries included in the survey were randomly sampled from the list provided by the Thai DOF (section 1.4.3.4, Table 1.3) and included small, medium and large-scale hatcheries and those producing all of the main farmed marine shrimp species such as *P. vannamei*, *P. monodon* and *P. merguiensis*.

Hatcheries were visited and the questionnaire completed by the authors during face-to-face interviews. Secondary data were collected from a wide range of peer review and non-peer review information to help inform the development of the

questionnaire, to detect trends in the health care of the hatcheries in Thailand as well as to confirm the primary data results.

2.2.2 Hatchery Site Surveys

The study was carried out in marine shrimp hatcheries which represented the 9 provinces with the largest number of shrimp hatcheries in Thailand (Figure 2.1). The hatcheries visited in each province were randomly selected from hatchery registration data provided by Department of Fisheries (DOF; Thailand, Pers. Comm., 2014) (Appendix IV). The design selected was random proportional to size, resulting in a similar proportion of the hatcheries in each province and district being selected randomly. The provinces included were divided into three different areas, Central and East (Chonburi, Chachoengsao, Nakhon Pathom provinces), the Andaman sea (Phuket, Phang Nga, Satun provinces) and the Gulf of Thailand (Nakhon Si Thammarat, Songkhla, Prachuap Khiri Khun provinces) (Figure 2.1). A total of 10% of the hatcheries located in these 3 areas were included in the survey (n_{total} = 78) which was broken down further into Government (n = 10) and Private (n = 68) hatcheries. The distribution of the hatcheries in the 3 areas are presented in Table 2.1. The hatcheries visited were described as nursery only, broodstock only or nursing and broodstock combined.

Table 2.1. The distribution of the hatcheries included in the study

	Provinces	Districts	Nursery	Broodstock	Broodstock&Nursery	Total
Central & East	3	6	37	1	5	43
The Andaman sea	3	5	3	2	9	14
The Gulf of Thailand	3	8	13	3	5	21
Total	9	19	53	6	19	78

Since there were 19 sites which had both nursery and broodstock activities, there were effectively two sites operated by one owner or manager and in the same location. The data were collected, summarized and analysed as nursery (n=72) or broodstock (n=25).

2.2.3 Questionnaire Design

Questionnaire was designed to enable primary data to be collected regarding the current practises in a wide range of marine shrimp hatcheries in Thailand. The questions were grouped under various topics: background of person interviewed, hatchery profile, husbandry, feed and water management, disease problems, health management, hygiene and biosecurity, market and production. The questionnaire included both opened and closed questions. Separate questionnaires were designed for broodstock and nursery sites and both were used on sites with nursery and broodstock combined. The final English questionnaires are included as Appendix V.

The initial questionnaires were pilot tested prior to use in the field with a fisheries biologist who was in charge of a shrimp hatchery at Prachuap Khiri Khun Coastal Fisheries Research and Development Centre, DOF, Thailand. The pilot test was conducted by phone from the UK to Thailand. Following the pilot test the questionnaire was edited in consultation with the project supervisors. The pilot questionnaire was judged to take too long for most farmers, therefore minor changes were applied and the questionnaire was adjusted and grouped in order to reduce time when interviewing. The final English questionnaires were translated into Thai before being used for the face to face interviews. Answers were written directly onto individual questionnaire sheets during the interviews.

2.2.4 Data Collection/Data Analysis

The raw data from surveys were transcribed into an Excel spreadsheet, and the answers from closed question interviews such as sex of person interviewed or other Y/N questions were transformed into numeric values. Data entry was validated by a backwards check on each questionnaire.

All survey data were summarised and analysed using Microsoft Excel 2010™ (Microsoft, USA) and JMP statistical software (JMP®, Version 14.0, SAS Institute Inc., Cary, NC, 1989-2019). The data were examined to determine which variables had enough data and variability to be analysed. Then all those exposure (independent) and outcome (dependent) variables suitable for analysis were examined for normality using a Normal Quartiles Plot. If normally distributed they

were analysed using univariate t-test, ANOVA or regression. If they were not normally distributed they were either transformed or analysed using non-parametric analysis mostly Wilcoxon/Kruskal-Wallis tests. Those exposure variables (e.g. tanks size, temperature control, etc.) that were significantly associated with the outcome variables (e.g. mean survival) were further analysed in a stepwise series of multiple regression models to explore the potential interactions and confounding. P=0.05 was used as the conventional threshold of significance but in the initial univariate analyses, any association approaching normality P<0.07 was included in the multivariable models.

2.2.5 Ethical Issues

The study was approved by the University of Stirling, Institute of Aquaculture Ethics committee (October 2014) and all data was treated in compliance with the UK Data protection Act 2018 and the EU General Data Protection Regulations. The interviews were arranged by a phone call and the potential interviewee, was informed about the purpose of the study, that the data would be treated anonymously and confidentiality would be rigorously maintained.

2.3 Results

2.3.1 Overview

There were three types of marine shrimp hatchery identified during this survey, broodstock alone, combined broodstock/nursery, and nursery only. Originally there were 73 hatcheries included in the survey, including 8 Broodstock only, 40 nursery only and 25 combined broodstock/nursery hatcheries. However, during the visits the nature of the sites did not always match the DOF records, for example, the combined nursery/broodstock was changed into nursery only. Therefore, the number and type of hatcheries were slightly different than those originally included. Finally, 78 hatcheries were included in the survey conducted which represented 6 broodstock only, 53 nursery only and 19 combined broodstock/nursery (Figure 2.1 and Table 2.2). The 73 hatcheries represented 10% of the total 735 in operation at that time according to DOF data.

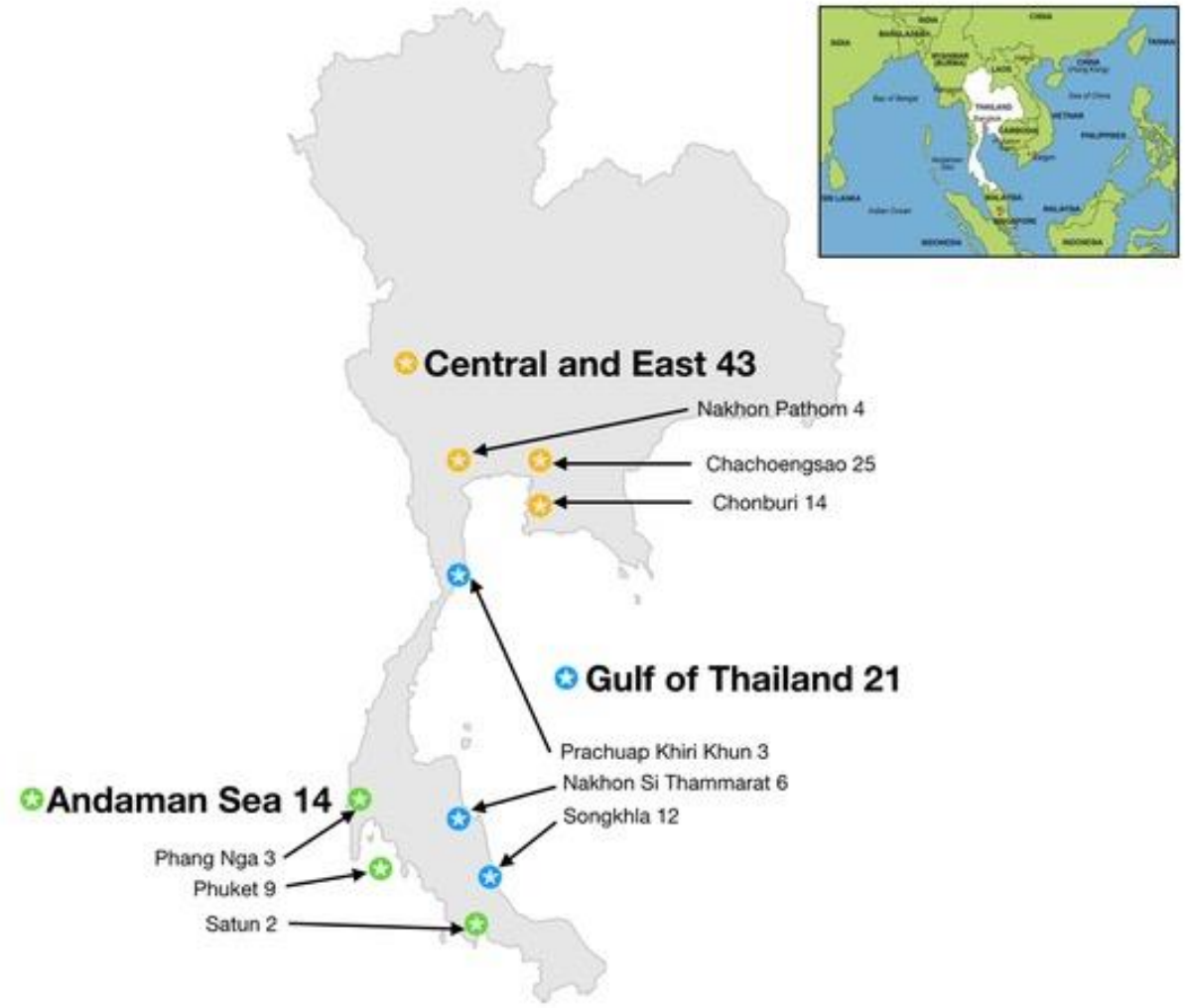

Figure 2.1. The three areas, 9 provinces and the number of hatcheries visited in each

Table 2.2. Details of number and type of hatcheries visited per district and province in Thailand

Area	Province	District	No. of hatcheries	Type		
				Nursery	Broodstock	Combined
Central and East	Chachoengsao		Total 25			
		Mueang	2	2		
		Bang Pakong	6	5		1
		Bang Po	17	17		
	Chonburi		Total 14			
		Mueang	11	8	1	2
		Sriracha	3	2		1
	Nakhon Pathom		Total 4			
		Kumpangsan	4	3		1
Gulf of Thailand	Nakhon Si Thammarat		Total 6			
		Sichol	4	1	1	2
		Tasala	1	1		
		Pak Panang	1	1		
	Songkhla		Total 12			
		Satingpra	10	7	1	2
		Ranode	1	1		
		Mueang	1			1
	Prachuap Khiri Khun		Total 3			
		Mueang	2	1	1	
		Gui Buri	1	1		
Andaman sea	Phang Nga		Total 3			
		Takua Thung	2		1	1
		Taai Muang	1			1
	Phuket		Total 9			
		Mueang	6	2	1	3
		Ta Lang	3	1		2
	Satun		Total 2			
		La Hoo	2			2
	Total		**78**	**53**	**6**	**19**

The sites visited fell into three categories, broodstock or nursery or broodstock and nursery combined. Since, broodstock and nursery processes are quite different, much of the data had to be summarised and analysed separately. Therefore, there were effectively 72 nursery sites, including 53 nursery only sites and 19 from combined sites. There were 25 broodstock sites, including 6 broodstock only sites and 19 from combined sites. In the results, it is stated where the data are presented as broodstock and nursery or broodstock, nursery and combined. The results of the survey are summarized under 3 main headings:

Description of Hatchery Sector,

Health Management Practices and

Health Outcomes.

2.3.2 Description of Hatchery Sector

Education. Level of education was classified by highest level attained; these were in order: No formal education, Primary School, Secondary School, High School, Diploma, Undergraduate or Postgraduate. Those interviewed were 45% owners, 27% managers, 3% workers and 3% family members. Their level of education is summarized in Table 2.3. The level of education by province is summarized in Table 2.4 and the level of education by type of hatchery in Table 2.5.

Table 2.3. Those interviewed by role and level of education

People interviewed	Educated above diploma (%)
Owner	58
Manager	93
Worker	67
Family member	67

Table 2.4. Area, province and level of education

Area	Province	Educated above diploma (%)
Central & East	Chachoengsao	60
	Chonburi	50
	Nakhon Pathom	50
Gulf of Thailand	Nakhon Si Thammarat	83
	Songkhla	92
	Prachuap Khiri Khun	67
Andaman sea	Phang Nga	100
	Phuket	100
	Satun	100

Table 2.5. Type of hatchery and level of education of those interviewed

Type of hatchery	Educated above diploma (%)
Nursery	68
Broodstock	67
Combined	79

Duration of operation. The length of time that the site had been in operation is represented for broodstock sites and nurseries in Figure 2.2. Although the mean length of operation was similar, the majority of broodstock sites had been in operation for more than 6 years (58% at 6 years or more, Figure 2.2) and the majority of nurseries for more than 11 years.

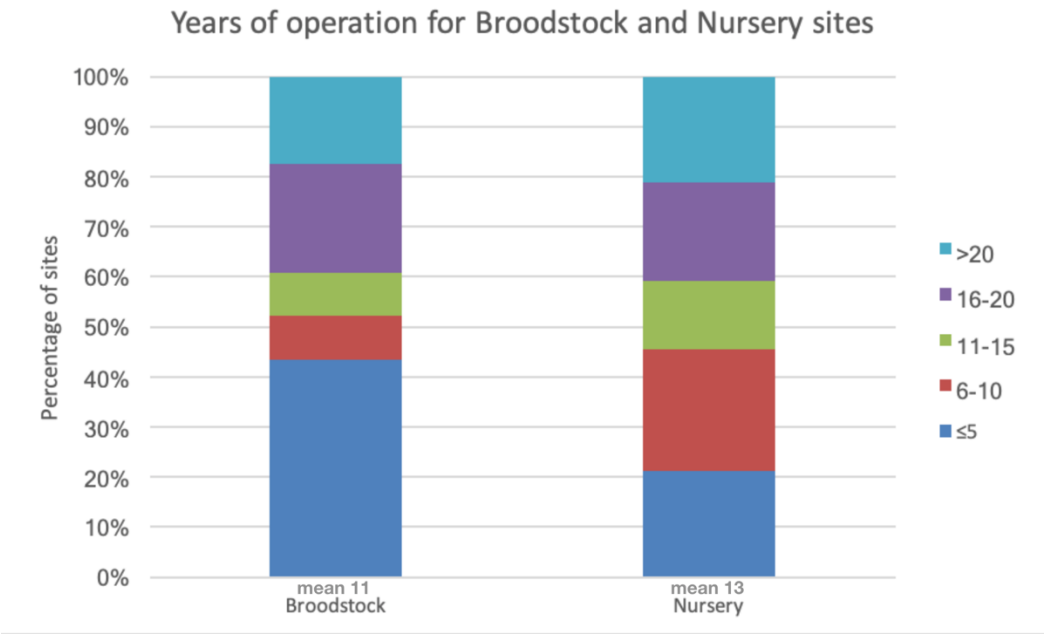

Figure 2.2. Years of operation for Broodstock or Nursery sites with mean for each

Species of shrimp. The species of shrimp grown were primarily *P. vannamei*, with some *P. monodon*, some other species including *P. merguiensis* and mixtures of the 3 species. There were similar proportions of these species in the three areas (Figure 2.3). The broodstock only and nursery only sites were dominated by *P. vannamei* with more mixed species in the sites with both broodstock and nursery operations (Figure 2.4).

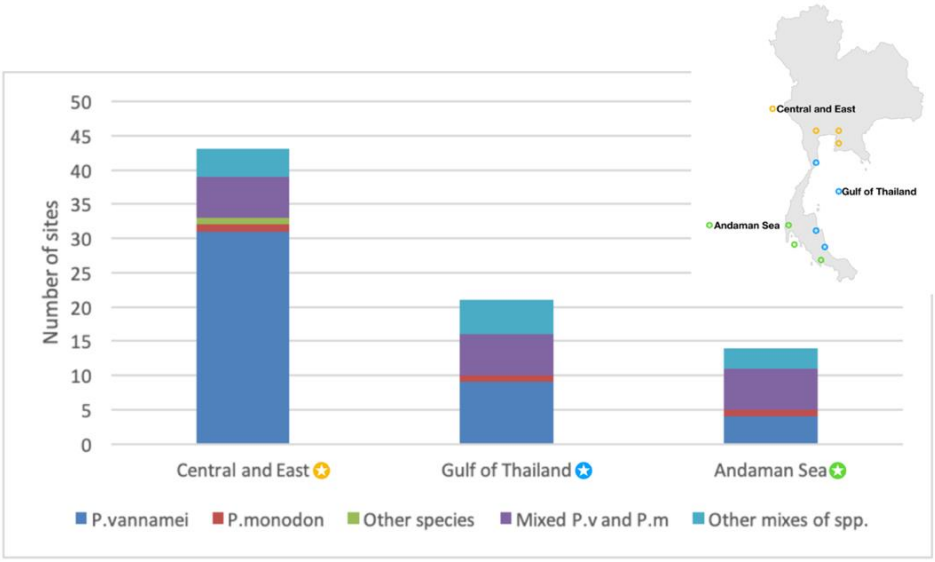

Figure 2.3. Distribution of shrimp species across the three areas

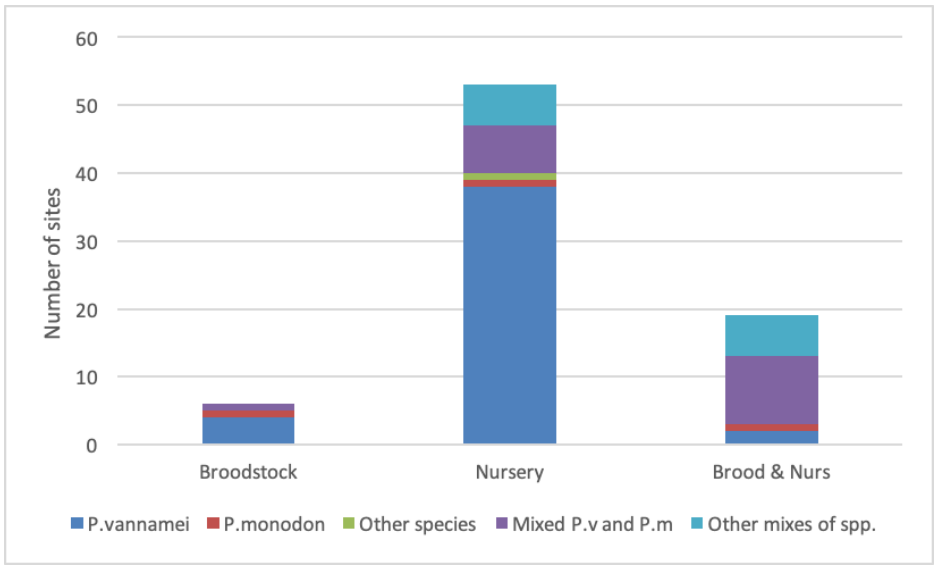

Figure 2.4. Distribution of species by type of hatchery

Source of water. All of the hatcheries in both the Andaman Sea area and the Gulf of Thailand took their water from the sea. In the Central and East area, all of the farms in Nakhon Pathom used concentrated saline diluted with local fresh water, in Chachoengsao only one farm used sea water the rest used the diluted concentrate. In Chonburi there were 5 using sea water, 3 using diluted concentrate and 6 using both.

Volume of tanks. There was considerable variability in the volume of the tanks (Table 2.6).

Table 2.6. Summary of the volume of tanks on the sites, total volume of the tanks on the site, average volume of tanks on site, with minimum, maximum and mean over the whole survey

Volume (m^3)	Minimum	Mean	Maximum
Total volume of Broodstock tanks	6	188	1,711
Average volume of Broodstock tanks	0.5	13.2	32.0
Total volume of Nursery tanks	40	302	2,000
Average volume of Nursery tanks	3.0	7.5	20

The distribution of combined tank volume on the sites was over-dispersed with the majority below the mean and a few very large tanks. The distribution of average tank volume was skewed to small tanks but less dispersed. The pattern was similar in broodstock and nursery sites, but for clarity only the nursery data is presented in Figure 2.5a and 2.5b as an example.

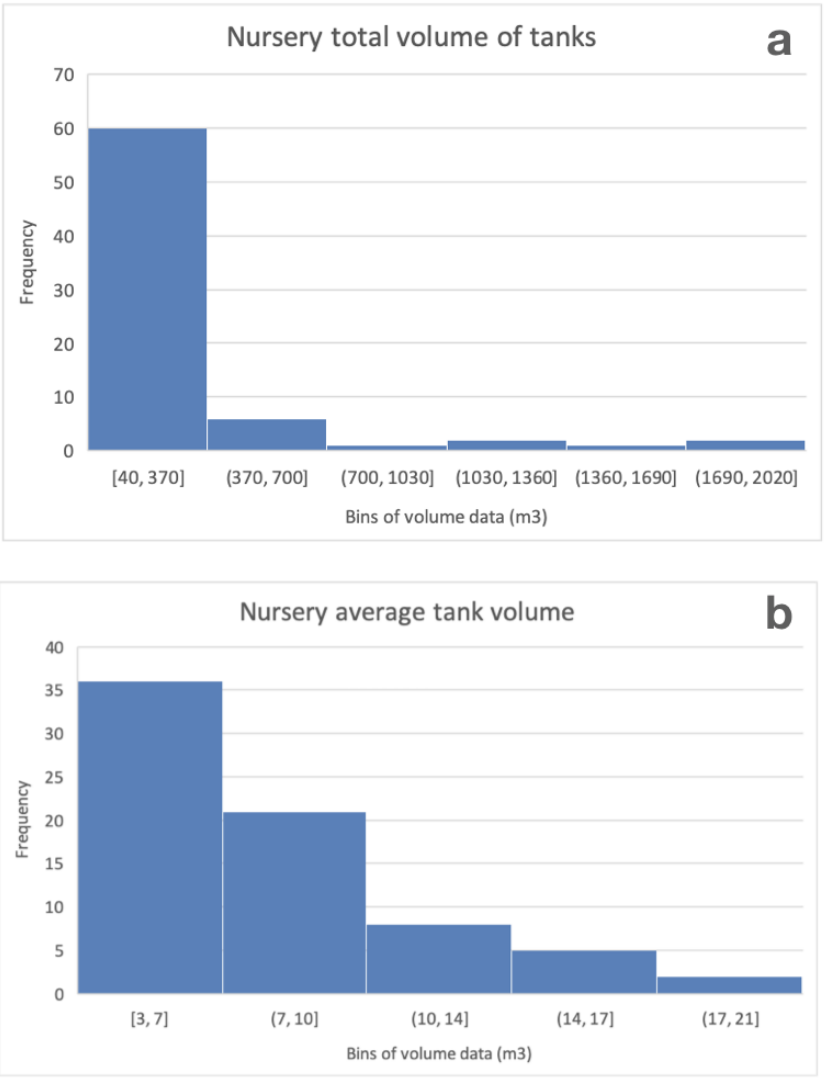

Figure 2.5. An example of the distribution of the total (a) and average (b) tank volume data for Nursery sites

Temperature control. The small and medium sized hatcheries in Thailand are normally operated by family owner. Almost of these hatcheries in the survey showed there was a similar pattern and materials that was used for shading and controlling temperature. Most of the small and medium sized hatcheries in survey were outdoor and shading during rearing operations was with black PE or tiles to cover the rearing tanks (Plate 2.1a, 2.1b) and on the big farms (based on interviews), the hatcheries were inside (Plate 2.2). However, the results of the survey data analysis showed there was no significant association with type of farm, size of farm or location of farm.

a

b

Plate 2.1. Black PE or tiles covering the rearing tanks

Plate 2.2. The hatchery has built as a building in big farm.

2.3.3 Health Management Practices

Below, the health management practices have been summarized (Tables 2.7 - 2.14). Hatcheries use a variety of chemicals and products for hygiene, prophylaxis and treatment. Nurseries and broodstock sites used a variety of substances for biosecurity, hygiene and treatment (Table 2.7). Chlorine is used as powdered calcium hypochlorite at around 5 to 10 ppm chlorine. A probiotic was used but was not easy to define exactly what the composition was on many sites. Some hatcheries used a commercial powdered probiotic and a smaller number used the DOF liquid probiotic but some hatcheries were not prepared to divulge the brand or source of the probiotic.

Table 2.7. Use of substances in health control on Broodstock and Nursery sites

	Substance					
Purpose	Detergent	Povidone iodine	Chlorine	Formalin	Probiotic	Oxytetracycline
Cleaning tanks	B/N	B/N	N			
After water exchange		B/N			B/N	
Control water quality		B			B	
Prophylactically		B/N	B/N	N	B/N	
Treatment		N		N	N	N

B= Used on broodstock sites
N= Used on nursery sites
Prophylactically= To prevent disease before it has started
Treatment= In response to disease outbreak

Table 2.8. Use of substances to clean tanks prior to filling. Chlorine was not used in broodstock sites.

Area	**Province**			**Detergent**	**Povidone Iodine**	**Chlorine**
			(n)	%	%	%
Central and East	Chachoengsao	Nur	(25)	48	48	24
		Br	(1)	0	0	
	Chonburi	Nur	(13)	23	0	31
		Br	(4)	50	0	
	Nakhon Pathom	Nur	(4)	0	75	100
		Br	(1)	0	100	
Gulf of Thailand	Nakhon Si Tham'	Nur	(5)	80	80	20
		Br	(3)	100	100	
	Songkhla	Nur	(11)	82	91	9
		Br	(4)	75	50	
	Prachuap K' Khun	Nur	(2)	50	0	50
		Br	(1)	0	0	
Andaman sea	Phang Nga	Nur	(2)	50	50	100
		Br	(3)	33	0	
	Phuket	Nur	(8)	88	88	13
		Br	(6)	83	67	
	Satun	Nur	(2)	100	0	0
		Br	(2)	100	0	

(n)= total number of hatcheries sampled in Province.
Nur=Nursery site; Br=Broodstock site.

Table 2.9. The proportion of hatcheries using povidone iodine or probiotics following water exchange

Area	Province		(n)	Povidone Iodine %	Probiotics %
Central and East	Chachoengsao	Nur	(25)	16	32
		Br	(1)	0	0
	Chonburi	Nur	(13)	8	69
		Br	(4)	0	0
	Nakhon Pathom	Nur	(4)	0	75
		Br	(1)	0	0
Gulf of Thailand	Nakhon Si Thammarat	Nur	(5)	100	60
		Br	(3)	0	0
	Songkhla	Nur	(11)	45	45
		Br	(4)	0	0
	Prachuap Khiri Khun	Nur	(2)	50	0
		Br	(1)	0	0
Andaman sea	Phang Nga	Nur	(2)	50	0
		Br	(3)	0	0
	Phuket	Nur	(8)	50	88
		Br	(6)	0	67
	Satun	Nur	(2)	100	50
		Br	(2)	0	0

(n)= total number of hatcheries sampled in Province.
Nur=Nursery site; Br=Broodstock site.

Table 2.10. The proportion of broodstock sites using substances to improve water quality during production

Area	Province	(n)	Proidone Iodine %	Probiotics %
Central and East	Chachoengsao	(1)	0	0
	Chonburi	(4)	0	0
	Nakhon Pathom	(1)	0	0
Gulf of Thailand	Nakhon Si Thammarat	(3)	33	33
	Songkhla	(4)	25	0
	Prachuap Khiri Khun	(1)	100	0
Andaman sea	Phang Nga	(3)	0	100
	Phuket	(6)	0	67
	Satun	(2)	50	50

(n)= total number of Broodstock sites sampled in Province.

Table 2.11. The proportion of hatcheries using povidone iodine, chlorine or formalin prophylactically. Formalin was not used prophylactically in broodstock sites.

Area	Province			Povidone Iodine	Chlorine	Formalin
			(n)	%	%	%
Central and East	Chachoengsao	Nur	(25)	36	100	20
		Br	(1)	0	100	
	Chonburi	Nur	(13)	77	69	0
		Br	(4)	50	50	
	Nakhon Pathom	Nur	(4)	75	100	50
		Br	(1)	0	100	
Gulf of Thailand	Nakhon Si Tham'	Nur	(5)	80	100	60
		Br	(3)	33	100	
	Songkhla	Nur	(11)	91	100	18
		Br	(4)	25	75	
	Prachuap K' Khun	Nur	(2)	50	100	0
		Br	(1)	100	100	
Andaman sea	Phang Nga	Nur	(2)	50	100	50
		Br	(3)	67	33	
	Phuket	Nur	(8)	88	88	50
		Br	(6)	67	50	
	Satun	Nur	(2)	100	100	100
		Br	(2)	50	50	

(n)= total number of hatcheries sampled in Province.
Nur=Nursery site; Br=Broodstock site.

Table 2.12. The proportion of hatcheries using probiotic prophylactically

Area	Province			Probiotic
			(n)	%
Central and East	Chachoengsao	Nur	(25)	36
		Br	(1)	0
	Chonburi	Nur	(13)	77
		Br	(4)	50
	Nakhon Pathom	Nur	(4)	75
		Br	(1)	0
Gulf of Thailand	Nakhon Si Tham'	Nur	(5)	80
		Br	(3)	33
	Songkhla	Nur	(11)	91
		Br	(4)	25
	Prachuap K' Khun	Nur	(2)	0
		Br	(1)	0
Andaman sea	Phang Nga	Nur	(2)	50
		Br	(3)	100
	Phuket	Nur	(8)	88
		Br	(6)	83
	Satun	Nur	(2)	100
		Br	(2)	50

(n)= total number of hatcheries sampled in Province.
Nur=Nursery site; Br=Broodstock site.

Table 2.13. The proportion of nurseries using povidone iodine or formalin as treatment

Area	Province	(n)	Povidone Iodine %	Formalin %
Central and East	Chachoengsao	(25)	64	8
	Chonburi	(13)	23	0
	Nakhon Pathom	(4)	50	0
Gulf of Thailand	Nakhon Si Tham'	(5)	100	40
	Songkhla	(11)	100	36
	Prachuap K' Khun	(2)	0	50
Andaman sea	Phang Nga	(2)	50	50
	Phuket	(8)	38	25
	Satun	(2)	0	50

(n)= total number of Nurseries sampled in Province.

Table 2.14. The proportion of nurseries using probiotics or oxytetracycline as a treatment

Area	Province	(n)	Probiotics %	Oxytetracycline %
Central and East	Chachoengsao	(25)	0	12
	Chonburi	(13)	15	8
	Nakhon Pathom	(4)	0	50
Gulf of Thailand	Nakhon Si Thammarat	(5)	0	20
	Songkhla	(11)	9	9
	Prachuap Khiri Khun	(2)	0	50
Andaman sea	Phang Nga	(2)	0	50
	Phuket	(8)	38	13
	Satun	(2)	50	50

(n)= total number of Nurseries sampled in Province.

2.3.4. Health Outcomes

It proved difficult to get reliable information on health outcomes, either because the data were not available or because the farmers considered the data to be sensitive and were not prepared to share it. Those health outcomes that were available are summarised below.

For broodstock sites the only meaningful outcome was hatching rate of eggs, this is summarised for the provinces in Figure 2.6. In the Andaman Sea provinces, there was less variation between hatcheries in the mean % hatching rate (Figure 2.6).

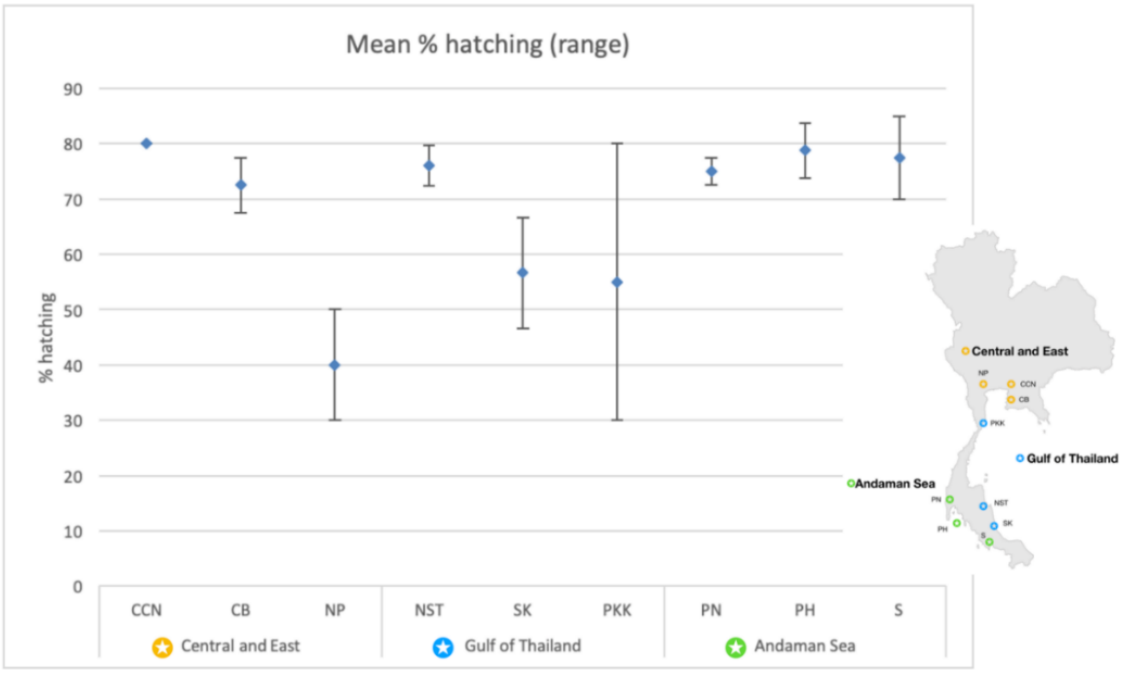

Figure 2.6. Hatching rate of eggs in Broodstock sites, with mean, minimum and maximum for each province

In the nursery sites health problems were recorded as number of “mortality events” per year (Figure 2.7) and mean survival (Figure2.8). Farmers used the term “mortality event”, to describe the sudden loss of a substantial proportion of the stock, exceeding expected losses. This type of term is commonly used rather than any quantifiable data. In practice a “mortality event” is when the farmer looks into the tank and sees a “lot” of dead shrimp. Small numbers of dead shrimp are common and may or may not be observed. Although gradually increasing mortalities may occur, farmers expressed the opinion that health problems were often a threshold event. That is, very few dead shrimp for a few days then suddenly very obvious large numbers of dead that are easily detectable. Therefore, they were of the opinion that there was a clear distinction between routine losses and a “mortality event”. Definitions of a “mortality event”, varied from farm to farm but were in the range of losses of greater than 50 to 70% of the stock within 24 to 48 hours.

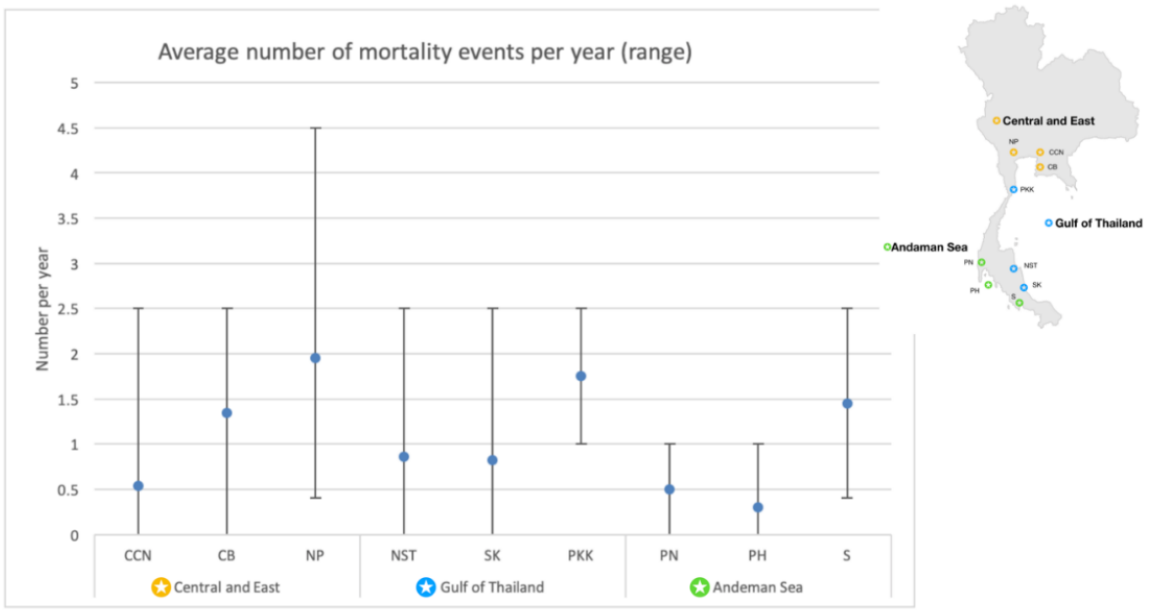

Figure 2.7. Mortality events per year, with mean, minimum and maximum for each province

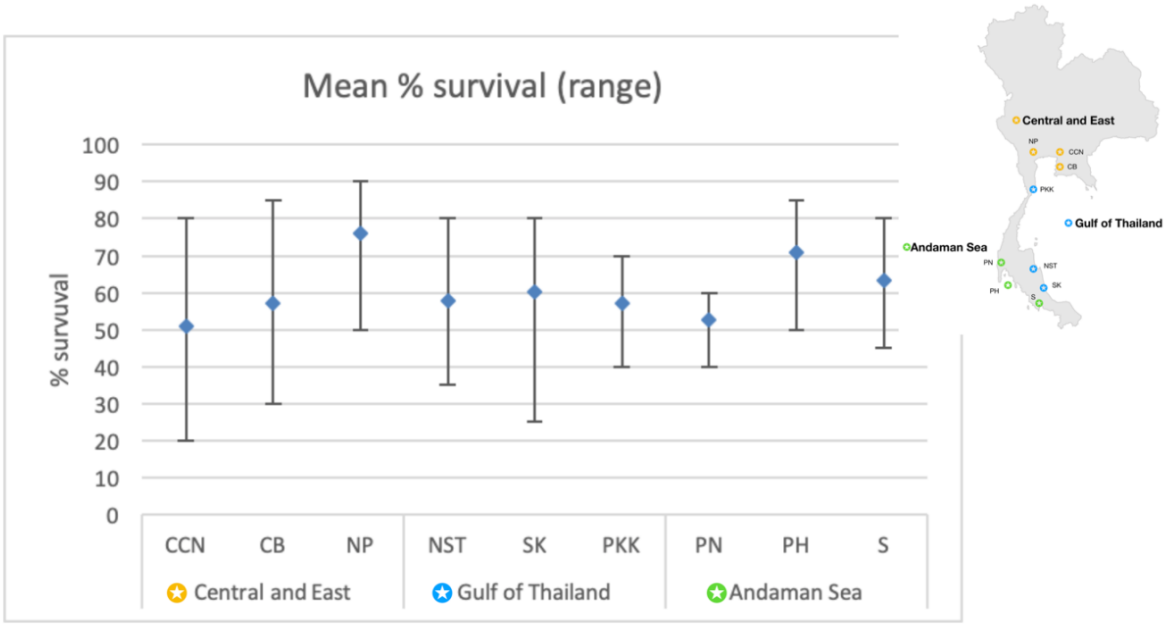

Figure 2.8. Mean % survival, with minimum and maximum, by province

There was no significant difference between the survival in the different seasons (March to June – Hot; July to October – Rainy; November to February – Cooler), see Figure 2.9, however, there was a slight trend towards higher survival in hot season compared with the cooler season. Although the farms considered this to be an important factor.

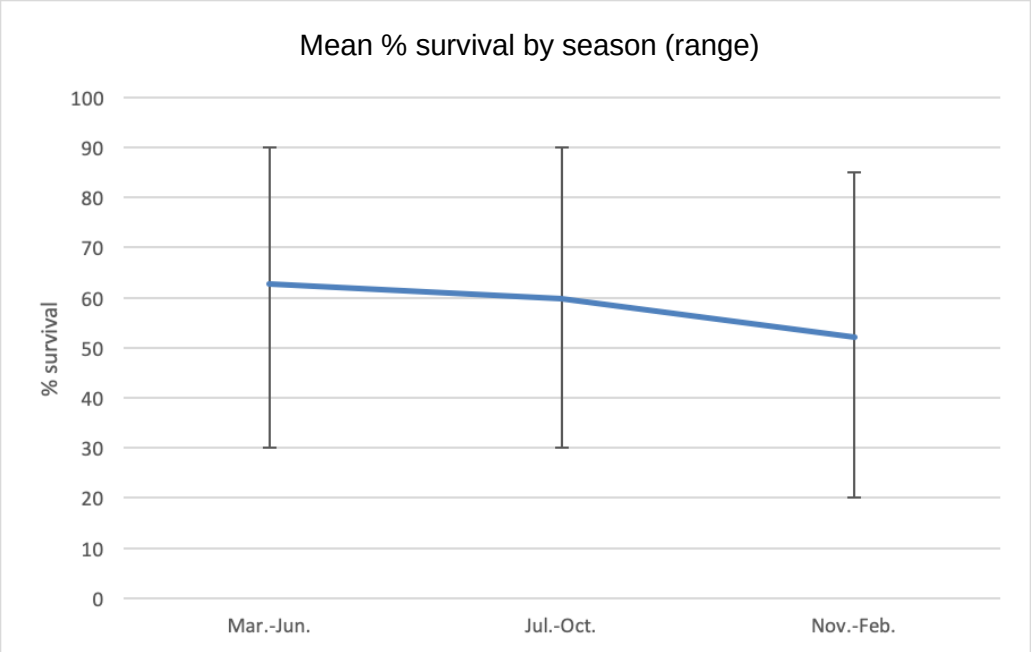

Figure 2.9. Mean % survival, with minimum and maximum by season

Nursery sites were infested by non-specified disease but also by three identified conditions, i.e. *Vibrio harveyi* infection, unidentified bacterial infections and *Zoothamnium* spp. The *V. harveyi* was the most prevalent condition reported affecting 55% of sites (Table 2.15), unidentified bacteria affected 20% (Table 2.16) and *Zoothamnium* spp. 35% (Table 2.17).

Table 2.15. The proportion of nurseries affected by disease where *Vibrio harveyi* was identified as the cause

Area	**Province**	(n)	**Infested by *Vibrio harveyi*** %
Central and East	Chachoengsao	(25)	12
	Chonburi	(13)	23
	Nakhon Pathom	(4)	25
Gulf of Thailand	Nakhon Si Tham'	(5)	80
	Songkhla	(11)	82
	Prachuap K' Khun	(2)	50
Andaman sea	Phang Nga	(2)	50
	Phuket	(8)	75
	Satun	(2)	100
Mean			**55**

(n)= total number of Nurseries sampled in Province.

Table 2.16. The proportion of nurseries affected by disease suspected as bacterial infections

Area	Province		Infested by bacterial spp.
		(n)	%
Central and East	Chachoengsao	(25)	12
	Chonburi	(13)	46
	Nakhon Pathom	(4)	50
Gulf of Thailand	Nakhon Si Tham'	(5)	0
	Songkhla	(11)	18
	Prachuap K' Khun	(2)	50
Andaman sea	Phang Nga	(2)	0
	Phuket	(8)	0
	Satun	(2)	0
Mean			**20**

(n)= total number of Nurseries sampled in Province.

Table 2.17. The proportion of nurseries affected by disease related to *Zoothamnium* spp. infestation

Area	Province		Infested by *Zoothamnium* spp.
		(n)	%
Central and East	Chachoengsao	(25)	40
	Chonburi	(13)	31
	Nakhon Pathom	(4)	0
Gulf of Thailand	Nakhon Si Tham'	(5)	20
	Songkhla	(11)	45
	Prachuap K' Khun	(2)	50
Andaman sea	Phang Nga	(2)	50
	Phuket	(8)	25
	Satun	(2)	50
Mean			**35**

(n)= total number of Nurseries sampled in Province.

The farmers were also asked about reports of EMS (AHPND) following stocking of their pls into growout sites. While it was considered useful to ask this question the answers were not reliable with many farmers claiming they were uncertain.

2.3.5 Analysis for Risk Factors.

Only those analyses which produced significant (P=0.05) or near significant results were considered further. The non-significant results are presented in Table 2.18.

Table 2.18. P values for non-significant results from analysis of questionnaire data.

	Broodstock	Nursery			
	Hatching rate	Minimum survival	Mean survival	Maximum survival	Mortality events/year
Probiotic use	0.5148(T)				
Mean water exchange frequency					0.06916 ($R^2$0.0141)
Mean water exchange %			0.0638§ ($R^2$0.0346)		0.1108 ($R^2$0.04121)
Average tank size					0.1942 ($R^2$0.0339)
Average stocking density (larvae per litre)			0.0552§ ($R^2$0.038)	0.0678§ ($R^2$0.033)	
Frequency of cleaning		0.6275 (R^2-0.01085)	0.7577 (R^2-0.0129)	0.8362 (R^2-0.01366)	0.0702 ($R^2$0.0469)
Probiotic post water exchange				0.0957(T)	0.5132(W)
Probiotic as prophylactic		0.1975(T)	0.1947(T)	0.3496(T)	
Probiotic as treatment		0.1065(T)		0.0739(T)	0.9920(W)
Povidone iodine post water exchange		0.5070(T)	0.5582(T)	0.8322(T)	0.5261(W)
Povidone Iodine as prophylactic			0.628(T)	0.0972(T)	
Povidone as treatment		0.839(T)	0.1532(T)	0.5756(T)	0.8986(W)
Formalin as prophylactic		0.2095(T)	0.1800(T)	0.4637(T)	
Chlorine as prophylactic		0.0814(T)			
Oxytetracycline as a treatment		0.2760(T)	0.1749(T)	0.2008(T)	
Presence of *V.harveyi*		0.0898(W)			0.0663(W)
Presence of *Zoothamnium* spp.		(0.4770 W)	0.5980(W)	0.9002(W)	
Presence of unidentified bacteria		0.9427(W)	0.9432(W)	0.7850(W)	
EMS identified in growout		0.5143(W)	0.1030(W)		0.2502(T)

T=t-test for normally distributed data
W=Wilcoxan test for non-normally distributed data
R^2=Adjusted R^2 from regression linear fit
§near to significant but weak association (i.e. low R^2)

In the broodstock only one significant association was identified. Hatching rate was better in the broodstock tanks that were cleaned with detergent (t-test, mean with cleaning 77.8 mean without 64.2, 21df, P=0.0243).

The results of the nursery data are summarised in Table 2.19. Some of the nurseries (47%) controlled the temperature in their tanks between 28 to 32 ^{o}C, by covering the tanks with black plastic or keeping the tanks in a building with the capacity to ventilate or keep closed to increase the temperature.

Table 2.19. P values from significant results from analysis of questionnaire data, with p values and means or medians.

	Nursery			
	Minimum survival	Mean survival	Maximum survival	Mortality events/year
Control of temperature ***Use=better survival***	0.0109(T) Use 53.5 Not use 45.2	0.0088(T) Use 62.3 Not use 54.4	0.0510(T) Use 69.6 Not use 63.3	
Water exchange frequency ***More better***		0.0415 ($R^2$0.0446)		
Average stocking density (larvae per litre) ***Higher density lower survival***	0.0118 ($R^2$0.074)			
Average tank size (m^3) ***Larger tanks better survival***		<0.0001 ($R^2$0.1930)		
Probiotic post water exchange ***Use=better survival***	0.0325(T) Use 52.5 Not use 45.8	0.0437(T) Use 61.1 Not use 55.2		
Probiotic as treatment ***Use=better survival***		(T)0.0559 Use 66.2 Not use 57.3		
Formalin as prophylactic ***Use=worse survival***		0.0081(T) Use 72.2 Not use 64.1	0.0494(T) Use 65.3 Not use 79.5	
Formalin as treatment ***Use=more mortality events***				0.0171(W) Use 1.4 Not use 0.7
Chlorine as prophylactic ***Use=worse survival***		0.368(T) Use 57.3 Not use 69.5	0.0016(T) Use 65.3 Not use 79.5	
Oxytetracycline as a treatment ***Use=more mortality events***				0.0037(W) Use 1.8 Not use 0.7
Presence of *V.harveyi* ***Presence= better survival***		0.0131(T) With 62.3 Without 55.2	0.0221(T) With 70.4 Without 63.3	
Presence of *Zoothamnium* spp. **Presence=more mortality events**				0.0172(W) With 1.19 Without 0.68
Presence of unidentified bacteria **Presence=more mortality events**				0.0006(W) With 1.64 Without 0.67
EMS identified in growout ***Presence= better survival***			0.0115(T) With 70.8 Without 63.0	

T=t-test for normally distributed data
W=Wilcoxan test for non-normally distributed data
R^2=Adjusted R^2 from regression linear fit

Multivariable analysis

A multiple regression model was constructed stepwise with the outcome (dependent) mean survival and exposure (independent) variables:

Use of probiotics post water exchange

Control of temperature

Average Density

Water exchange frequency

Average Tanks size

Area (Central and East; Gulf of Thailand; Andaman Sea)

The fit of the models was assessed on the R^2 or the amount of variability explained and the loss of information Akaike Information Criterion (AIC). AIC is a measure of the loss of information and allows an evaluation of the goodness of fit verses the simplicity of the model. The lower the AIC the less information lost.

From 33 models constructed there were two models with a similar fit. The first is summarised in Table 2.20.

Table 2.20. Probability for individual variable from stepwise multiple regression first model

Parameter	P
Use of probiotics post water exchange (yes/no)	0.09494
Control of temperature (yes/no)	0.00976
Average tank size (m^3)	0.01914
Average stocking density (larvae per litre)	0.22782

R^2=0.3111; AIC=552.575

The second is summarised in Table 2.21.

Table 2.21. Probability for individual variable from stepwise multiple regression second model

Parameter	P
Use of probiotics post water exchange (yes/no)	0.10107
Control of temperature (yes/no)	0.00962
Water exchange frequency	0.57253
Average tank size (m^3)	0.05474
Average stocking density (larvae per litre)	0.2125

R^2=0.3145; AIC=554.683

The R^2 for the second model was marginally higher (more information explained) but the AIC was also marginally higher (more information lost). Both models indicate that the most significant variables associated with mean survival were the control of temperature (control = better survival) and average tank size (larger = better survival). The other variables were not significant when adjusted for interactions and confounding in the two models.

2.4 Discussion

We could not find any published reference to a previous systematic survey of the Thai shrimp hatchery sector. Given that we had access to the entire list of operating hatcheries and were able to construct a systematic random-proportional-to-size survey, we feel this is the first robust representation of the sector. In the majority of cases we were able to contact the farmers prior to visiting and found no reluctance to participate. The majority were very happy to share information, however, some farmers were reluctant to share information on specific production figures or use of certain chemicals or treatments. This may have been due to concerns about practices meeting DOF regulations, however, there is no evidence to support this hypothesis.

There appeared to be higher level of education in the Andaman Sea area than the other two areas and higher in the Gulf of Thailand compared with Central and East. This was probably due to the role of the people interviewed with managers generally having a higher level of education and may reflect a difference in the management

structure of the farms in the different areas although the data did not allow us to test this.

The difference in species produced was largely due to both the availability of broodstock and demand of pls of grow-out farmers. However, from anecdotal evidence appeared to be strategic (a choice) rather than opportunistic (what was available). the data provided in this study confirmed that the dominant species farmed was *P*. *vannamei* which supported the data of the main Thai marine shrimp production, was *P*. *vannamei* in 2014 (Fisheries Statistics Analysis and Research Group, Department of Fisheries; Thailand, 2018).

The differences in hatching rate between provinces would suggest that it was lower in Nakhon Prathom and Songkhla, with the rates in Prachuap Khiri Khun being most variable. While these might be explained, for example, by poor access to sea water in Nakhon Prathom. These data were estimated by the farmers and based on a small samples size and therefore not necessarily reliable. However, cleaning the broodstock tanks with detergent did appear to be significantly associated with better hatching rates. The alternative method was to clean just with water. This survey data supported that sanitation and hygiene must be concerned in the hatchery to avoid the disease which could cause the low hatching rate. This finding warrants further investigation and if substantiated by further investigation and/or experiments may lead to useful advice for broodstock site management. Many researchers have investigated the hygiene and sanitation practises for marine shrimp hatcheries; Moullac *et al*. (2003) reported that in Tahiti and New Caledonia, domestication of broodstock could be reared as specific pathogen free due to the isolation of the location and disease testing.

Some of the variables tested in this study were associated with the health outcomes e.g. survival (significance just exceeding P=0.05) when correlated with mean water exchange and average stocking density (Table 2.18). Although these relationships approached significance, they were not a strong association. They had R^2 values of around 0.03, or only 3% of the variability in survival was accounted for by the average stocking density. Associations have two main components, the strength of association or how well the independent variable predicts the dependent variable

and the statistical significance. Even a very weak association can be statistically significant if there is a sufficiently large sample size. However, such weak associations are unlikely to be biologically useful and therefore they were therefore not included in the subsequent multivariable models.

Several treatments and prophylactics were associated with poorer survival or more mortality events (Table 2.19). This is probably because these therapies are mostly used on sites that have problems and therefore the increased use of the therapy is correlated with more health problems but not as a cause, more as an effect. It could also be the type of products and/or the combination of treatments that the farmers used more than 1 treatment at 1 time and there may be an antagonistic effect in the animals. However, the data of using combination of treatment and prophylactics are not mentioned in the survey. The further investigation of side effect of treatment needs to be evaluated.

The presence of *Zoothamnium* spp. or unidentified bacteria were associated with more mortality events but not poorer survival. In general, there was not a significant association between mortality events per year and the mean survival. This is not surprising since you can have both occasional very serious mortality events or regular minor events which do not have a dramatic effect on overall survival.

The presence of *V. harveyi* and EMS (referred to as AHPND in subsequent chapters) reports from growout farmers were both associated with better survival. The EMS data were unreliable because this is feedback from the grow-out section when they bought the pl from hatcheries and although this might warrant further investigation little can be concluded from this association. The association between the presence of *V. harveyi* and improved survival is harder to explain. It may just be due to chance, since the data were screened with multiple univariate analyses and the probability of obtaining a significant result by chance increases with the number of analyses performed. However, there may also be a real association here that would warrant further investigation, could *V. harveyi* be protective in some way? There were some *V. harveyi* report studies of Vaseeharan and Ramasamy (2003) found using the strain probiotic *B. subtilis* BT23 could reduce 90% cumulated mortality of juvenile *P. monodon* when exposed to *V. harveyi*. Rengpipat *et al.*

(1998) found that probiotic strain Bacillus S11 could resisted *V. harveyi* in *P. monodon* pl. In the hatcheries where the presence of *V. harveyi* could be high, they might use some prophylactics or treatments that are not mentioned in the survey. This is just a hypothesis, and further investigation needs to be evaluated.

The variables selected for inclusion in the multilevel model, were those that were significant and had a strong association with the outcome. This is to some extent an arbitrary decision but the variables selected appeared to be biologically plausible risk factors. Forwards and backwards stepwise models were constructed examining all the potential options. The best models are those which explain most of the variability in the data but are not too complex. Very simple models tend to lack value due to loss of data and potentially omitting important factors and very complex models can be unstable or unreliable as a result of their complexity and tending to be less practically useful.

In both of the best models, control of temperature and larger tanks size were associated with better survival. While an observational epidemiology study such as this can never prove causality, these factors are certainly worthy of examination and were studied further in Chapter 3. The findings of the survey regarding temperature control appear to agree with several published studies. Kumlu *et al*. (2000) reported that the best water temperature for rearing of *Penaeus semisulcatus* for growth and survival was 30 °C and the salinity was 30 ppt. Ponce-Palafox *et al.* (1997) reported that the best survival and growth of *P. vannamei* postlarvae was found in the temperature range from 28 to 30 °C. Wyban *et al*. (1995) demonstrated a reduction in growth of *P. vannamei* when water temperature was less than 23 °C, whereas when the temperature was 30 °C or more shrimp growth improved.

There was a great deal of variability in the size of the tanks in all types of hatchery with most being small but a few being large. As survey reported above, in nursery sites, average size of tanks ranged from 3 to 20 m^3 and in broodstock sites from 0.5 - 32 m^3, this results is similar to those of Treece and Fox (1993) who reported that there were a variety of type of tank used in *P. monodon* and *P. vannamei* larval rearing section such as concrete, fiberglass, and size of tanks were from 2 – 20+ m^3. This would suggest that the size of tanks has remained consistent over a

protracted period of time, which in turn suggests that there are reasons for not changing. In Thai shrimp hatcheries, many factors may determine the size of tanks e.g. size of hatchery, budget, existing infrastructure and ease of management. While larger tanks are associated with improved survival, all of the complex factors affecting the size of tanks used would have to be better understood before recommendations could be made to farmers.

The association between better survival and larger average tank size is not surprising since larger tanks are inherently more stable and less susceptible to fluctuations in water quality and temperature. However, Wattanamahard (1993) demonstrated that in Thai hatcheries there are two types of tank; small or large tanks, and mentioned that advantages and disadvantages of both tank system depended on location of hatchery where located in a variety of environmental condition.

The data on survival from the survey was based comments from farmers. Some farmers were reluctant to provide accurate survival data. This might be due to the farmers have concerned about their production related to whether taxation or official government statistics.

2.5 Conclusion

An observational study of this nature requires further testing before recommendations can be made to farmers. However, there were promising indications from the study which may lead to improved guidance for farmers. While temperature control appears to result in better productivity, this is still not practiced by all farmers. It will be necessary to first prove this is a real association in experimental trials and then examine the cost benefits of implementing it on farms that do not currently use it. Similarly larger tanks size appeared to produce better results and again this would require a more detailed cost benefit analysis prior to making recommendations to farmers.

There remain some unexplained results, including the association between the presence of *V. harveyi* and EMS and improved survival. This result warrants further

study to determine if there is a biological process involved or if this is a statistical artifact.

This survey has produced the first systematic description of the Thai shrimp hatchery sector and has identified some associations that, with further work, may lead to practical recommendations for farmers.

2.6 References

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CHAPTER 3

Investigating impact of a probiotic strain *Bacillus licheniformis* on health status in whiteleg shrimp, *Penaeus vannamei* (Boone, 1931) larvae

3.1 Introduction

Marine shrimp belonging to Penaeidae are commercially valuable aquaculture species in Thailand. In 2018, over 90% of the farmed marine shrimp production in Thailand was from whiteleg shrimp (*Penaeus vannamei*) (Coastal Aquaculture Research and Development Division, Department of Fisheries; Thailand, 2019). Over the last 30 years the Thai warm water shrimp farming sector has continued to face a range of production level constraints. These include significant loses from infectious disease outbreaks due to Yellow Head Virus (YHV) in the early 1990's followed by White Spot Syndrome Virus (WSSV) in 2002 (Flegel, 2009). Farmed marine whiteleg shrimp continue to suffer from a range of microbial infectious diseases and since 2017 emerging diseases have contributed to major losses from parasite *Enterocytozoon hepatopanaei* (EHP) and viral outbreaks from Infectious Myonecrosis Virus (IMNV) and White Spot Syndrome Virus (WSSV) were reported (Songkhla Aquatic Animal Health Research Centre, Department of Fisheries; Thailand, 2019). Farmers apply a range of methods and intervention strategies to help control and/or prevent outbreaks on their farms. Whilst there are no known anti-viral treatments, and traditional vaccines are not applicable for invertebrate animals, these strategies are often a combination of medications and biosecurity practises.

These include the use of disinfectants and antibiotics as well as antiparasitic treatments.

In Thailand antibiotics and chemicals including malachite green and several antibiotics including chloramphenicol, nitrofurazone, nitrofurantoin, furazolidone and furaltadone are banned in the shrimp industry due to their adverse effects on human health and the environment (Fisheries Commodity Standard System and Traceability Division, Department of Fisheries; Thailand, 2017). Long term use of antibiotics can contribute towards the development of antibiotic resistance and alternative treatments are being sought. One alternative strategy to control bacterial disease in farmed warm water shrimp and/or boost health, is through the use of probiotics which are widely known and applied in shrimp aquaculture. Positive effects of using probiotics in aquaculture are reported to include increased growth or reduced disease outbreaks and these have been described by several authors (Hjelm *et al.*, 2004; Balcazar *et al*., 2006; Vendrell *et al.*, 2008; Li *et al*., 2007 ; Vine *et al*., 2006).

Probiotics are described as live but non-pathogenic, bacterial species which are often found in the environment or as commensals in the digestive tract of people and animals (Balcazar *et al*., 2006; Sahu *et al*., 2008). Intake of probiotics have been shown to be beneficial to improve health and wellbeing in people and they have been used more frequently in the aquaculture industry over the last 20 years (Tinh *et al*., 2008; Vine *et al*., 2006; Gildberg *et al*., 1995; Gatesoupe, 1994; Gatesoupe, 2002). In warm water shrimp farming, probiotics have been mostly applied to “boost” the immune response of the animals particularly during times of perceived stress e.g. temperature fluctuations, disease periods. One of the main reasons that there is such an interest in probiotics in warm water shrimp is these animals lack an adaptive or specific immune response, so a routine vaccine does not work. Therefore, probiotics along with other feed supplements have been considered as an alternative strategy to promote improved animal health. The exact mode-of-action of the probiotics for warm water shrimp is not well understood. However, experimental studies have shown improved immune response (Brunt and Austin, 2005; Nayak, 2010), better survival (Vendrell *et al*, 2008) and reduced disease losses (Vaseeharan and Ramasamy, 2003) when administered to shrimp. These

benefits have all been identified through experimental or field studies. In Thailand, although probiotics are widely used in the hatcheries, the type of product and the route of exposure is dependent on the hatchery owner and whether the hatchery wish to treat the water or the animals.

Many bacterial species and strains have been proposed as probiotics, however, the most prevalent species belong to *Lactobacillus, Bacillus, Enterococcus, Streptococcus*. There are several strains of non-pathogenic bacteria that have been used in the shrimp industry, particularly in the hatchery and these are often members of the *Bacillus* genus (Nayak, 2010). The *Bacillus* spp., comprise of a variety of species including Bacillus *subtilis*, *B. megaterium*, *B. licheniformis, B. coagulans*, *B. clausii*, and *B. cereus*, (Oggioni *et al*., 2003).

In Thailand, since some antibiotics have been banned in aquaculture industry, so there is a trend of using probiotic as well as biofloc as an alternative to antibiotics. A strain of *B. licheniformis* has been used by the Surat Thani Research centre and the Thai DOF to support the Thai shrimp farming sector, and the product is designed to be administered mixed with the shrimp feed. Strains of *B. licheniformis* are described as a rod shaped, Gram positive, spore-forming bacteria often found in the soil, as both spores and vegetative cells and has been used as a probiotic in a range of animals and people (Knap, 2019; Wikipedia, 2019; Webmd, 2017; Gomez-Gil *et al.*, 2000). Whilst the mode-of-action of this probiotic is not well elucidated, application of *B. licheniformis* provided to whiteleg shrimp*,* as a probiotic, specifically increased the number of the 3 different types of circulating haemocytes associated with the innate immune response (Li *et al*, 2007). In Thai shrimp hatchery, probiotics have widely been applied in an attempt to reduce antibiotic administration and these are often supplied as either a dietary additive or treatment for the water. Vaseeharan and Ramasamy (2003) reported a 90% reduction in cumulative mortality of juvenile *Penaeus monodon* exposed to *V. harveyi* at 10^3 – 10^4 cfu per ml. These shrimp had received a probiotic water treatment for 6 days prior to bacterial challenge using the probiotic strain *B. subtilis* BT23. Rengpipat *et al*. (1998) found that cultures of probiotic strain Bacillus S11 completely protected black tiger prawn (*P.monodon*) postlarvae against disease from *V. harveyi*. Garriques and Arevalo (1995) showed

that *V. alginolyticus* can improve survival rate of whiteleg shrimp after challenge with *V. parahaemolyticus.*

The overall success of any hatchery system is measured by the quality and quantity of the seed produced and this is no different for the Thai shrimp hatcheries. The grow out farms rely heavily on the availability of seed production and any threats to this supply must be controlled. Although the probiotics are used, evidence is lacking in understanding how well they perform. So, the aim of this study was to investigate the effect of a single probiotic strain of the bacterium *B. licheniformis* on the health and development of shrimp larvae in different growth stages and varied production systems within a hatchery.

In chapter 2 the size of the rearing unit and the control of temperature (maintaining optimal temperature) were identified as the parameters associated with survival. The data analysis in chapter 2, identified the use of probiotics as a significant factor contributing to improve survival in univariable analysis but this was no longer significant when adjusted for confounding and interactions in multivariable models. Despite this lack of significance in the survey data, it was considered appropriate to study probiotics further given the prevalence of their use and the interest in their activity form the shrimp production sector. This part of the study therefore aimed to test the efficacy of temperature control and probiotics, with tank size. The experiments were limited by the facilities available and therefore it was not possible to study all these variables equally.

3.2 Materials and Methods

3.2.1 Experimental Facilities and Systems

All experiments were conducted at the Coastal Aquaculture Research and Development Regional Centre 3 (Surat Thani in the south of Thailand), DOF, Thailand (from this point onwards called ST DOF) (Plate 3.1a). Two different systems (Figure 3.1) were used for the hatchery/nursing studies conducted in this chapter. These were 200L buckets or 7 tonne concentre tanks. The pilot studies were only performed in 200L plastic buckets with lids and aeration provided through air stone as seen in Plate 1b. These were held in a room with air conditioning which

lowered the room temperature and reduced the water temperature in each bucket (Plate 3.1b).

Large scale studies were performed using the 7 tonne concrete tank systems (Plate 3.1c) which were also located at the ST DOF facilities. These were performed at 30±1^{o}C with the temperature controlled using in-tank heaters. In these facilities, air supply was provided to each tank (1.5 x 5 x 1 m) using air stones. For all the studies (pilot and large scale) the water supply was provided from the local reservoir and was sterilised using calcium hypochlorite ($Ca(OCl)_2$) at 30 ppm for 3-5 days until chlorine level was neutralised. This was measured and confirmed using a chlorine test kit (IMPACT Test Kits, Thailand). Afterwards the treated water was filtered using 5µm mesh size bag filter prior to use.

The grow out studies were performed both in the large concrete tanks (Plate 3.1d) and in net cages held within earthen pond systems which belonged to the ST DOF facility (Plate 3.1e). The dimension of each net cage was 1.7 x 5 x 1.2 m and the water depth was 0.8 m for all cages. The water supply in the earthen ponds came from the mangrove area and was pumped directly into the pond with no disinfection for the inlet supply. Temperature control, as described for the studies using the 200L buckets and the 7 tonne concrete tanks was not possible in the earthen ponds. The temperature of the water in earthen pond ranged from 24 to 32^{o}C, this was measured by thermometer at 6 am, 12 noon and 6 pm daily.

a

b c

d e

Plate 3.1. (a) The shrimp hatchery of Coastal Aquaculture Research and Development Regional Centre 3 (Surat Thani), (b) 200L buckets tanks, (c) 7 tonne concrete tanks for large scale study (d) 7 tonne concrete tanks for grow-out study and (e) net cages in the earthen pond

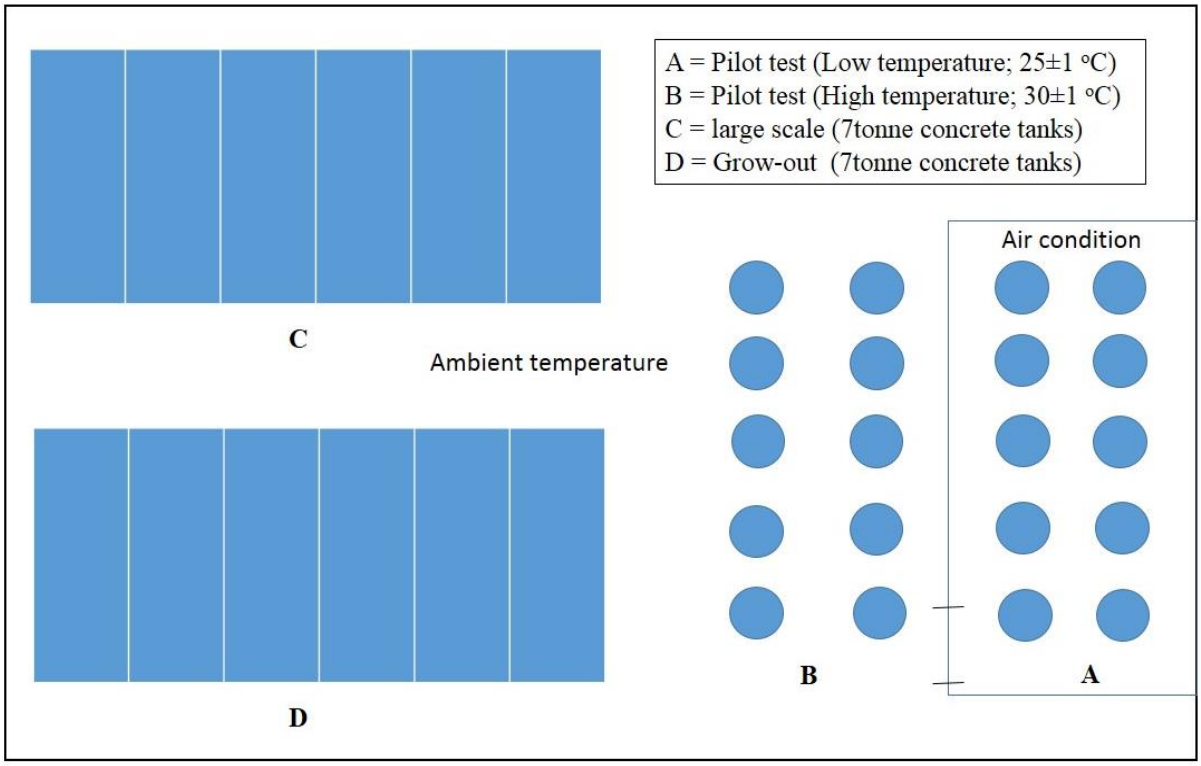

Figure 3.1. Diagrammatic representation of the experimental studies performed in ST DOF facilities.

3.2.2 Overview of Experimental Studies on Effect of Temperature and Probiotic Use in Shrimp

A total of 4 different experiments were conducted between April – June 2017. The two variables under investigation were temperature control, identified from the results of the questionnaire survey data provided in chapter 2, and probiotic use, because of the interest and prevalence of use in the shrimp industry in Thailand. Initially a 2 x 2 factorial design (Table 3.1) was produced for the pilot studies with 5 replicate tanks for each variable being tested. A total of 20 tanks of 200 L/tank were used, and each tank contained 150 L of treated sea water.

Table 3.1. Pilot study in small scale using 200L plastic buckets, 2 x 2 factorial design

Probiotic	**Temperature**	
	Low temp**.** control (25±1^{o}C) (**-**)	High temp**.** control (30±1^{o}C) (**+**)
Probiotic used (**+**)	(**+-**) T1	(**++**) T3
No probiotic (**-**)	(**--**) T2	(**-+**) T4

T **=** treatment group, **+ =** with, **- =** without**.**

Several issues occurred in the pilot phase of the experiments, which caused mortalities in these systems and these are listed in Table 3.2. It was considered prudent to stop the low temperature studies as this was causing significant health issues to the shrimp and instead to concentrate on the administration of probiotics at high water temperature (30±1^{o}C) in the large scale experiment.

Table 3.2. Overview and outcome of experiments performed in Surat Thani hatchery (April - June 2017)

Trial Number	Trial Date (start-finish)	Experiments			
		Low temperature (25±1 °C); (T1, T2)	**High temperature (30±1 °C); (T3, T4)**	**Stocking densities (larvae/L)**	**Number of initial stock (larvae/tank)**
1	19-27 April 2017	Started from nauplius -zoea2 (All treatments were dead within 6 days)	Started from nauplius - zoea3 (T3R1,T4R2 were dead within 6 days) Nauplius developed to mysis1(T3R2,T3R3,T3R4, T3R5,T4R1,T4R3,T4R4, T4R5 were dead within 9 days)	100	15,000
2	25-29 April 2017	Started from mysis1 -mysis3 (T1R1,T1R2, T2R1,T2R3 were dead within 5 days) Mysis1 - Post Larvae1 (T1R3,T2R2 were dead within 5 days)	Started from mysis1 - Post Larvae1 (All treatments were dead within 5 days)	20	3,000
3	16-18 May 2017	Started from nauplius -zoea1 (All treatments were dead within 3 day)	No trial	100	15,000
4	24 May-13 June 2017	Started from mysis1 (All treatments were dead within 6 day), No developing into Post larvae	Started from mysis1 - Post Larvae1. When larvae developed into pl ,pl of each replicate was combined into 1 tank of each treatment, and the total final amount of T3 and T4 were 666 and 489 ind. ,respectively.	30	4,500

T = treatment group, R = replicate e.g. T3R1 = Treatment group 3 replicate 1.

3.2.3 Experimental Design of Large Scale Study

In this experiment, there were 2 treatment groups with 3 replicates tanks each. Treatment group 1 (T1 BL-) the shrimp were not exposed to the probiotic (***B. licheniformis***) and Treatment group 2 (T2 BL+) the shrimp were exposed to the probiotic. The water temperature was 30 ± 1^{o}C for all tanks and was controlled using in-tank heaters. Animals were placed into each of the 6 concrete tanks containing 3 tonnes of treated sea water, as described previously in section 2.1 with initial stocking densities of nauplius at 40 larvae/L. This gave 40 larvae x 3000 L = 120,000 larvae/tank.

3.2.4 Grow-out Study

For the grow-out studies both cages and concrete tanks were used as described in Table 3.3. The pl 15 (post-larvae 15 days after metamorphosis) shrimp from the large-scale study were used in this experiment in order to trace their growth performance in grow**-**out phase after receiving the probiotic *B. licheniformis*. All of the pl 15 shrimp (BL+ and BL**-**) were separately acclimatized in 1 tonne fiberglass tanks for one week before being transferred to the concrete tanks and cages. After acclimatizing, the pl 15 developed to pl 22 in the first date of the grow-out studies.

Table 3.3. Experimental details for grow-out phase

Types of experimental system	Number of Replicate tanks	Stocking densities (pl/sq.m)	Number of initial stock (pl/tank or cage)	Size of cage or tank (W x L x H)	Cultured water depth (cm)
Cage	2	100	850	1.7 x 5 x 1.2	80
Concrete tank	3	100	750	1.5 x 5 x 1	45

pl=post-larvae

3.2.5 Data Collection and Statistical Analysis

The raw data were recorded into an excel spreadsheet and analysed using Microsoft Excel 2013^{TM} (Microsoft, USA). Statistically significant differences with 95% confidence limit interval in each treatment was compared using ANOVA and T-test for significant differences among treatment groups by looking more closely with

selected variable (probiotic) against the main outcome (larval developmental stage, health screening, survival rate, pathogen detection) were tested.

3.2.6 Animals

Nursery Study Phase

The animals used in this study were all *P. vannamei* which were supplied from a hatchery located in Surat Thani province, Thailand. Two shrimp populations were sourced from the hatchery but used as a single population for each experiment and were not mixed. The animals were purchased from the private hatchery as nauplius (Plate 3.2), transferred to the ST DOF facility by car in sealed plastic bags inserted into Styrofoam boxes with a bag of ice in the box to control temperature (23±1^{o}C). Aeration was provided prior to transportation by oxygen provided directly into the bag with the animals and seawater from the hatchery. The maximum transportation time was 1h and on arrival at the ST DOF facility the bags were removed from the Styrofoam boxes and floated in a container with tap water at ambient temperature for approximately 20 mins to ensure that the transportation and water temperature were similar and avoid temperature shock for the animals. After 20 mins, the bags were carefully opened and the contents poured into a 325 µm mesh size scoop net to collect the nauplius. A sub-sample of the population were removed immediately and sent for health screening at another DOF laboratory, with all procedures conducted according to the DOF health evaluation criteria. The remaining animal stock was cleaned by flushing chlorine treated sterilised seawater through the scoop net before stocking the animals into the appropriate tanks.

Plate 3.2. Image of a nauplius of *P. vannamei*, (4X magnification)

Grow-out Study Phase

Animals at pl22 stage were used and these shrimp had a weight range of 0.04-0.05g weight (Plate 3.3) and were used in the grow-out study and these animals came from the final pl stage of the large-scale nursery experiment.

Plate 3.3. The post larvae (pl) of *P. vannamei* which stage of pl22

3.2.7 Probiotic

The bacterium, *B. licheniformis* (BL) was provided by Coastal Aquaculture Research and Development Regional Centre 2 (Samut Sakhon), DOF,Thailand as a single strain of probiotic and was grown in the laboratory at ST DOF to provide a bacterial stock (BL stock culture). Growth of the BL stock culture was initiated in the microbiological laboratory of the ST DOF under aseptic conditions, who kindly provided 300 ml broth suspension (stock sample) at log phase growth after incubation for 12h to use in the experiment (Plate 3.4c) and from this point onwards the product will be called BL.

Bacterial purity and viable growth was checked where a single colony subculture was aseptically prepared onto Tryptone Soya Agar (TSA) plate (Oxoid Thailand) incubated at 32^{o}C for 24h in a static incubator before a single colony (Plate 3.4a) was aseptically removed and inoculated into 100 ml of sterile nutrient broth

(Difco™). This was incubated at approximately 28°C (room temperature) for a maximum of 48 h and moderate aeration was continuously provided from aquarium air pump model ACO-9905. Contamination checks were performed from the liquid bacterial suspension by aseptically sub-culturing onto the selective agar TCBS (Thiosulfate Citrate Bile Salt Sucrose Agar; Difco™) to check for any contamination from *Vibrio* species. If no growth was observed on the TCBS plates, then the BL suspension was considered suitable for use. The TCBS agar was used as a presumptive indicator for Vibrio species.

Higher volumes of the BL suspension were grown aseptically using 3L minimal media (MM) which composed of 30 g sucrose, 7.5 g Di-Potassium Hydrogen Orthophosphate (K_2HPO_4), 7.5 g Potassium di-Hydrogen Phosphate (KH_2PO_4), 3 g Di-Ammonium Hydrogen Phosphate [$(NH_4)_2HPO_4$], 0.6 g Magnesium Sulfate Hepta-Hydrate ($MgSO_4.H_2O$), 25.5 g Sodium Chloride (NaCl), 0.03 g, Iron (II) Sulfate hepta-Hydrate ($FeSO_4.7H_2O$), 0.021 g Maganese Sulfate Hepta-Hydrate ($MnSO_4.H_2O$), 3L distilled water at room temperature for 3-5 days to reach a target cell concentration of 1 x 10^{10} cfu per ml (Plate 3.4b). The chemicals preparing for minimal media were purchased from Ajax Finechem and this was made following manufacturers guidelines. Total viable colony counts were performed using TSA agar following the methods described by Miles *et al*. (1938). Contamination checks were performed on TCBS agar as previously described for each flask produced prior to enrichment of the artemia with the BL probiotic suspension.

a b c

Plate 3.4. (a) *B. licheniformis* on TSA plate (b) BL liquid form (c) DOF probiotic product

3.2.8 Feed Preparation & Feeding Regimes

Nursery Study

As the nauplius develop, the feeding regimes change depending on developmental stage of the larvae (Table 3.4). The shrimp larval feeding regimes used in this study follow normal practices of the DOF Surat Thani hatchery with the larvae being fed six times per day.

Table 3.4. Feeding regimes of warm water shrimp species *P. vannamei*

Feed	Stage							
	Nauplius	Zoea1	Zoea2	Zoea3	Mysis1	Mysis2	Mysis3	post larvae (pl1-pl15)
Phytoplankton		—	—	—	—			
Artemia					—	—	—	—
Micro encapsulated feed		—	—	—	—	—	—	—
Artificial diet (Flake)								—

pl = post larvae; Micro encapsulated feed used was from AQUALINE(CANADA).

The nauplii do not feed, from the Zoea 1 larval development stage onwards and in the Zoea 1 stage they were fed the phytoplankton diatom (*Chaetoceros* spp.) until they reached Mysis stage 1 when they can receive artemia (Table 3.4). The artemia were enriched following ST DOF standard protocols. Briefly, the artemia cysts were purchased from a commercial supplier (Red Leaf Co., Ltd) in a 425g can and hatched by incubating the cysts for 24h in 850 L of 30 ppt seawater in an artemia hatching tank to give 2g cysts/L. The hatched artemia (instar stage II) were recovered by passing the hatched animals through a 125 µm mesh net and mixing these with the BL probiotic for 6-hr at room temperature (Plate 3.5). The concentration of the BL probiotic was 1 x 10^6 cfu per ml. The probiotic enriched artemia were then fed to the shrimp larval from Mysis 1 to pl 15 (Table 3.4).

Plate 3.5. Preparation of artemia enrichment with BL

Grow-out Study

Shrimp from the large-scale nursery trial were transferred and separately grown in either the net cages for four months or in the concrete tanks for 5 months**.** The animals in both types of systems were fed 4 times daily using a commercial pellet feed which contained 35% protein (Thai Union Feedmill Company).

3.2.9 Health Assessment Assays

Several assays were performed to assess the health of the animals in each of the experimental systems. These criteria were all part of the routine DOF health screening protocols and are described below.

Larval Development

To determine the larval development process of the animals in each treatment group, a subpopulation of the shrimp larvae (n=10) were randomly sampled daily. This followed a time course where each day the sub sample of animals were observed using a compound microscope (Leica ICC50 HD, Switzerland) at 4X

magnification and the developmental stage recorded followed the stages shown in Plate 3.6. This goes from young (3.6a) to mature (3.6h).

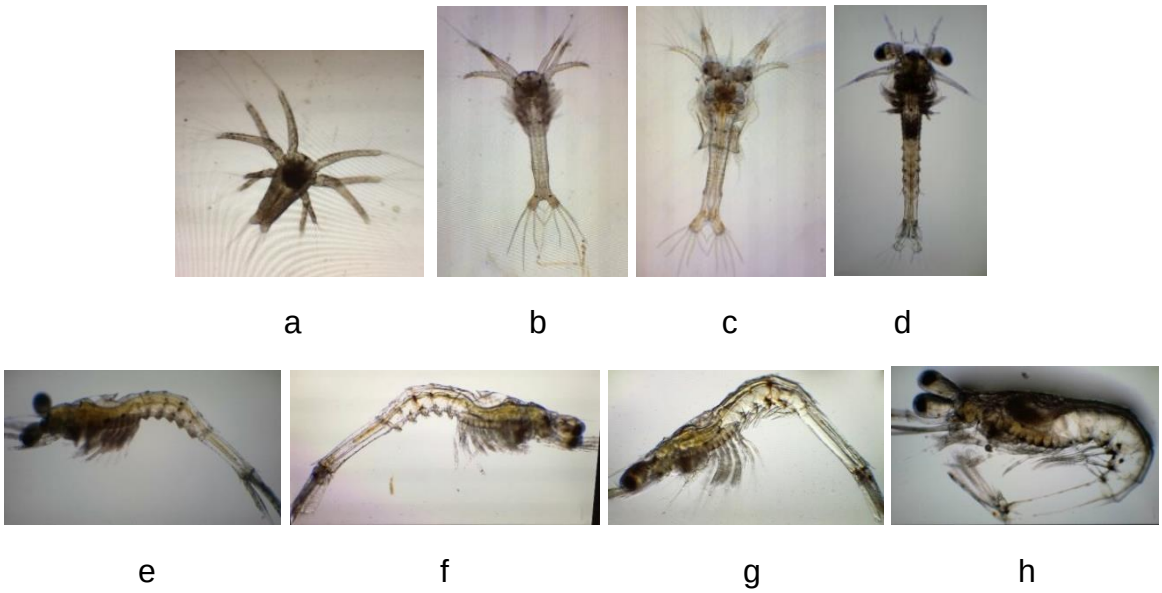

Plate 3.6. Shrimp larvae development stages; (a) Nauplius, (b) Zoea1, (c) Zoea2, (d) Zoea3, (e) Mysis1, (f) Mysis2, (g) Mysis3, (h) Post larvae1(pl1)

Developmental Screening and Health Check

Six tests were applied at the end of the large-scale nursery study (Table 3.5). Each test provides a single score and the final score is achieved by deducting the individual scores from each test from 100. There is an accepted range to allow the scores to be translated to pl quality. For tests 1-4, a total of 20 pl were sampled whereas for test 5 and 6 a total of 50 pl were used.

Table 3.5. Information on the pl health check criteria, DOF Thailand.

Test No.	Indicator	Description of test	Number of pl tested (ind.)	Total score	Scoring when pl do not meet the criteria				
					1 ind.	2 ind.	3 ind.	4 ind.	5 ind.
1.	Rostrum	Number of rostrum (≥3) visible under microscope	20	10	8	6	4	2	0
2.	Presence of ectoparasites	No ectoparasites attached body and appendages (5 score)	20	15	4	3	2	1	0
		No ectoparasites attached gills (5 score)	20		4	3	2	1	0
		Appendages must be complete and not corroded (5 score)	20		4	3	2	1	0
3.	Presences of hepatopancrease and lipid cells	Completed hepatopancrease, not pale & no atrophy (15 score)	20	35	12	9	6	3	0
		Full of amount of lipid cells (20 score)	20		16	12	8	4	0
4.	Muscle Gut Ration	≥4:1 (muscle of 6th appendages:intestine)	20	10	8	6	4	2	0
5.	Stress test	Using formalin 100 ppm for 30 mins	50 pl/ 5 litre	15	10	5	0	0	0
6.	Environmental Adaptation test	Using drinking water for 30 mins	50 pl/ 5 litre	15	10	5	0	0	0
Total score				100					
Interpretation of Final Score									
91-100				very good quality					
81-90				good quality					
71-80				Acceptable					
0-70				not acceptable					

Ind. = individual

Pathogen Detection Assays

The pooled sample of 2 g of either Nauplius or pl15 were collected to determine the presence of known viral, parasitic and bacterial pathogens a series of laboratory based tests were performed. Again, these are part of the routine DOF checks and were conducted following the laboratory protocols established in ST DOF facility (followed the manual method of Songkhla Aquatic Animal Health Research Centre, 2017). The molecular assays investigated the presence of 6 viruses including white spot syndrome virus (WSSV) using Nested PCR (improved from OIE method) (Lo *et al.*, 1996), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) using single PCR (improved from OIE method) (Tang *et al.*, 2007), Infectious Myonecrosis Virus (IMNV) using Nested RT-PCR (IQ2000), Yellow Head Virus

(YHV) using Nested RT-PCR (improved from OIE method) (Cowley *et al.*, 2004), Taura Syndrome Virus (TSV) using RT-PCR (improved from OIE method) (Navarro *et al.*, 2009), Covert Mortality Nodavirus (CMNV) using Nested PCR (Zhang *et al.*, 2014).

Extraction of all pathogen DNA/RNA was performed using a TACO™ 24 Plate & Comb Nucleic Acid Automatic Extraction System machine (GeneReach Biotechnology Corporation, Taiwan). Briefly, in the extraction process, reagent kits were used which composed of 200 µl ethanol added : 400 µl of sample (shrimp sample prepared by chemical lysis and centrifugation), Washing Buffer A 750 µl, Magnetic bead 50 µl, Washing Buffer B 750 µl, Eluting buffer 60 µl in each pathogen.

The parasite EHP (*Enterocytozoon hepatopenaei*) was detected using the nested PCR method described in Tangprasittipap *et al.* (2013). Briefly, the DNA was extracted from pooled tissue samples by adding 500 µl of Lysis Buffer kit and centrifuged at 12,000 rpm for 5 min at 4°C using a benchtop centrifuge (Brand Orto Alresa; Model Dig cen 20). A total of 400 µl aqueous solution was pipetted for DNA/RNA extraction. In the extraction process, the TACO™ DNA/RNA auto extraction kit was used following the manufacturer's instructions and any alterations followed those described by Tangprasittipap *et al*. (2013). All extracted samples were stored at -20C until required.

For bacterial pathogen detection, a PCR assay was performed to detect the presence of the shrimp specific bacterial pathogen *V. parahaemolyticus* using multiplex PCR methods (DOF inhouse-method modified from Tinwongger *et al*., 2014). To ensure enough bacterial DNA was recovered a 18h broth incubation step was performed prior to extraction of bacterial DNA. Briefly, 0.1 g of pooled tissues of either nauplius or pl15 was incubated in 0.9 ml TSB (+ 2% NaCl) at 35 ± 2 °C for 18h.This was the broth incubation step. After that, 700 µl of bacterial culture was transferred to the new tube and centrifuged at 12,000 rpm for 5 mins, 4 °C using benchtop centrifuge (Brand Orto Alresa; Model Dig cen 20). The supernatant was discarded, 100 µl of DEPC treated water is added to suspend the bacterial pellet. The bacterial suspension is boiled at 95-100 °C for 5 mins using a heat block. After

boiled, 400 µl of DEPC treated water was added. The suspension was centrifuged at 12,000 rpm for 5 mins, 4 ^{o}C and the solution containing the DNA (DNA template) was pipetted into a sterile 1.5 ml microcentrifuge tube and stored at -20 ^{o}C until required for PCR assays.

There were 3 different PCR assays performed using the bacterial DNA extracted. Each PCR used a different primer set to detect the presence of *V. parahaemolyticus* or AHPND *V. parahaemolyticus*. The first primer set is called Vp-flaE and this PCR assay (called Vp) amplifies a flagella gene that is located on the chromosome of *V. parahaemolyticus*, so it used to detect all *V. parahaemolyticus* DNA. The second PCR used a primer pair called TUMSAT-Vp1 and the PCR is designed to detect all *V. parahaemolyticus* which contain a plasmid. The final PCR is the most specific as this is designed to detect the shrimp specific *V. parahaemolyticus* DNA containing the plasmid harbouring toxin genes and was called TUMSAT-Vp3 (Tinwongger *et al*., 2014) or Vp3 for short. The last PCR assay provides a PCR product of 360bp and is specific for the detection of the plasmid toxic gene thorugh to cause AHPND pathology. This is an insect-like gene 'Photorhabdus insect-related' with proteins A and B called Pir-A and Pir-B (Sirikharin *et al.*, 2015). The primer sets used and the expected product size produced are provided in Table 3.6.

Table 3.6. Primers used for detection of bacteria *Vibrio parahaemolyticus*

Primers	Oligonucleotide Sequences (5´to 3´)	Product size (bps)	Target
Vp-flaE-79F	GCAGCTGATCAAAACGTTGAGT	897	*V. parahaemolyticus* (No plasmid); No AHPND
Vp-flaE-34R	ATTATCGATCGTGCCACTCAC		
TUMSAT-Vp1F	CGCAGATTTGCTTTTGTGAA	500	*V. parahaemolyticus* (Plasmid, non toxic gene); No AHPND
TUMSAT-Vp1R	AGAAGCTGGCCGAAGTGATA		
TUMSAT-Vp3F	GTGTTGCATAATTTTGTGCA	360	*V. parahaemolyticus* (Plasmid with toxic gene); AHPND
TUMSAT-Vp3R	TTGTACAGAAACCACGACTA		

Viable bacterial recovery was also performed to complement the molecular assays. This was performed on the animals at pl 15 development stage and the nauplius. Total viable colony counts were performed following the methods described in Miles *et al***.** (1938). Briefly, 0.1 g of shrimp tissue samples were macerated in sterile physiological saline (2% NaCl) and 10-fold serial dilutions performed and bacterial spread plates produced where each dilution inoculated ono the agar plates would enable colony counts between 30-300. Spread plates were then performed by aseptically removing 100µl from each serial dilution of 10^{-1} to 10^{-6} and plating onto TSA + 2% NaCl for total bacterial recovery, TCBS agar plate was also used for presumptive recovery of all *Vibrio* species and the green and yellow colonies on the TCBS agar were counted and finally samples were plated onto a Chromogenic agar (CHROME agar™) for specific detection of *V. parahaemolyticus* as these are purple coloured colonies on the chromogenic agar. All media was prepared following the manufacturers details. All plates were incubated at 35 ± 2°C for 18-24h and colony growth and numbers counted.

3.3 Results

3.3.1 Nursery Phase

3.3.1.1 Pilot Study in Small Scale Trials

In every pilot trail of small scale tanks, larval nursing was not successful with larvae that failed to develop and larvae with fouling on the appendages were observed in the low temperature treatments. The results are provided in Table 3.7 and Plate 3.7

Table 3.7. Results of the Pilot trials performed in small plastic containers

Trial No.	Low Temperature (25±1 ºC) (T1, T2)	High Temperature (30±1 ºC) (T3, T4)
1	Larvae in all replicate tanks in both treatment groups died within 6 days at the stage of zoea2	In some tank (T3R1, T4R2) nauplius died at zoea 3 Whilst, the rest of larvae in each treatment group developed into mysis1 all died within 9 days of the trial
2	Larvae reached development stage Mysis 3 for only 4 tanks (T1R, T1R2, T2R1, T2R) all animals died by day 5	All animals in both treatment groups metamorphosed into pl1 within 5 days but all died
3	All larvae in all replicate tanks in both treatment groups died within 3 day at zoea1	Not done
4	In both treatment groups, development stage reached was mysis 1 but all died within 6 days	All larvae developed to pl 15 and survived from both treatment groups pl15 of BL + group were 666 pl/all replicates, pl 15 of BL - group were 489 pl/all replicates

T1 = Treatment group 1, low temperature with probiotic used ; T2 = Treatment group 2, low temperature with no probiotic

T3 = Treatment group 3, high temperature with probiotic used ; T4 = Treatment group 4, high temperature with no probiotic

a b

Plate 3.7. The abnormal larvae in Pilot test at low temperature ;(a) arrow showing the carapace abnormal and (b) abnormal larvae with arrows showing fouling on the appendages

3.3.1.2 Large Scale Study

3.3.1.2.1 Health Assessment

Larval Development

Neither a biological nor a statistical difference was detected in the developmental stages reached between the animals tested in (BL-) or (BL+) (Figure 3.2).

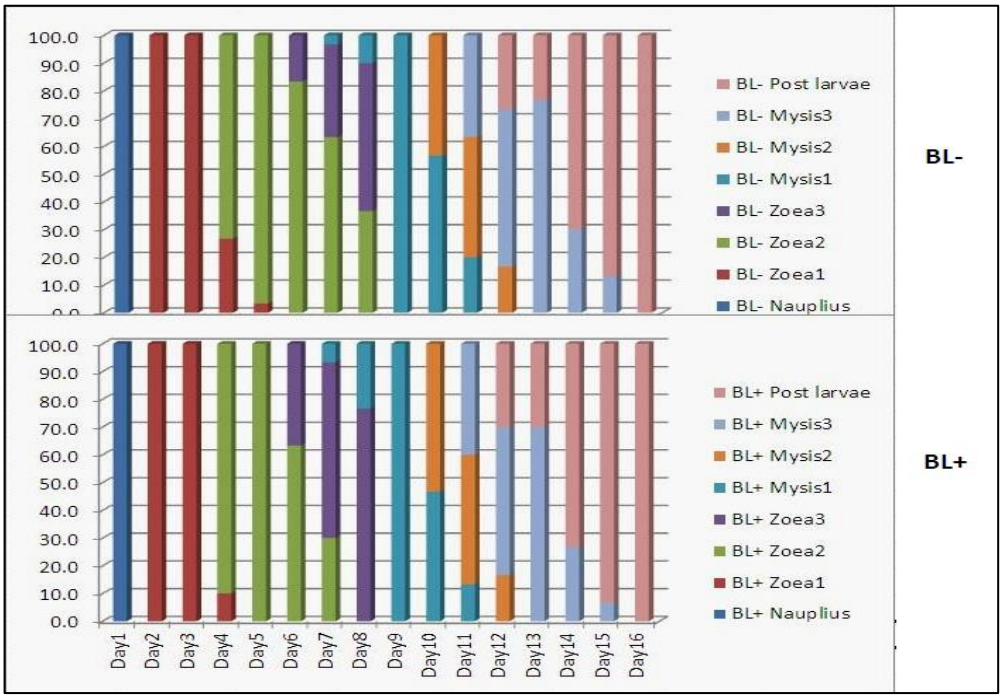

Figure 3.2. Developmental stages of shrimp larvae each day of Treatment group (BL-) and Treatment group (BL+)

Developmental Screening and Health Check

Shrimp that were administered the probiotic (BL+) achieved a health score of 83.67, whereas the animals in the (BL-) group received a health score of 80, but the difference was not statistically significant (Figure 3.3, P = 0.606). The group of shrimp eating artemia supplemented with the probiotic BL had a higher health score (good quality) than the BL- group (acceptable).

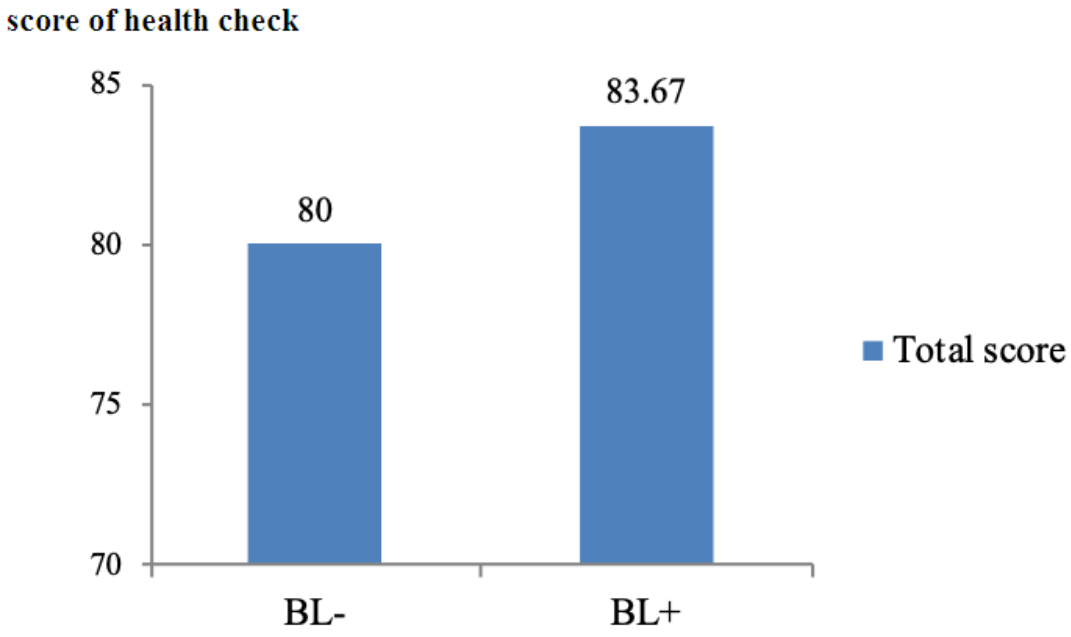

Figure 3.3. Total score of health of pl at the end of experiment of in group (BL-) and group (BL+)

Pathogen Detection

PCR

The PCR tests against the viruses (WSSV, IHHNV, YHV, TSV, IMNV, CMNV), parasites EHP and bacteria *V. parahaemolyticus* were negative for all samples taken including the animals from both BL+ and BL - treatment groups.

Bacteriology

Results of the viable bacterial counts (Table 3.8) show that there was no real difference in the total viable bacteria count (TVBC) and total vibrio count (TVC)

compared between the 2 treatment groups. In the samples plated onto the chromogenic agar, no *V. parahaemolyticus* (purple coloured colonies) were recovered from the (BL+) and purple coloured colonies were only observed in the shrimp samples from the (BL-) which was indicative of recovery of *V. parahaemolyticus.* There was no statically significant difference between TVBC (P=0.476) or the TVC (P=0.448) recovered between the animals tested with the probiotic (BL+) and non probiotic (BL-), whereas chromogenic agar counts of *V. parahaemolyticus* were significantly higher in the BL- group (P=0.025). A higher number of suspected *Vibrio* species were recovered from the BL+ group using TCBS agar selective for *Vibrio* spp. A higher number of yellow coloured colonies (associated with non-pathogenic *Vibrio* spp.) were recovered on the selective TCBS agar from the BL+ group compared with the BL- group but a lower number of green coloured colonies (associated with pathogenic *Vibrio* spp.) were recovered.

Table 3.8. Viable bacterial recovery and counts per Treatment group

		Total Bacteria Count (TSA)	Vibrio Count (TCBS)			*V. parahaemolyticus* (Chrome)
			Total Vibrio Count	Yellow colony	Green colony	
Nauplius	Mean	$5.2x10^6$	$2.45x10^4$	$2.35x10^4$	$1.00x10^3$	NT
	SD	$5.8x10^6$	$9.19x10^3$	$7.78x10^3$	$1.41x10^3$	NT
PL15						
BL-	Mean	$1.31x10^{7\,a}$	$7.70x10^{4a}$	$4.84x10^4$	$2.86x10^4$	$1.17x10^{2\,a}$
	SD	$5.99x10^6$	$1.19x10^5$	$6.99x10^4$	$4.89x10^4$	5.77x10
BL+	Mean	$1.70x10^{7\,a}$	$1.50x10^{5a}$	$1.45x10^5$	$5.00x10^3$	$0^{\,b}$
	SD	$6.36x10^6$	$9.23x10^4$	$8.81x10^4$	$7.81x10^3$	0

Note : The different letter shows a significant difference between the groups (P≤0.05) within column but the same letter shows no significant difference within column; NT = not tested

3.3.1.2.2 Survival Rate

The survival rate of animals of Treatment group 2 (BL+) was 77.63% whereas the Treatment group 1 were 67.71% (Figure 3.4), which was statistically significant difference (P=0.004).

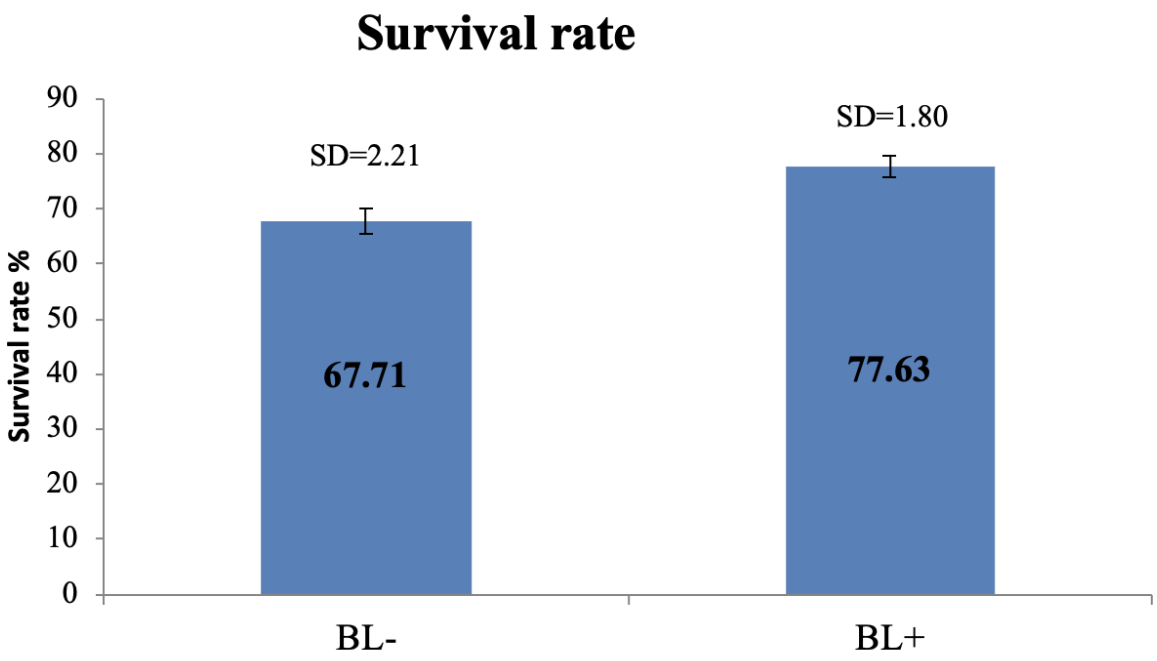

Figure 3.4. Graph shows the mean survival rate of the animals of Treatment BL- group and Treatment BL+ group in nursery study**.**

3.3.2 Grow-out Phase

3.3.2.1 Concrete Tank System

Growth Performance

Table 3.9 shows the weight gain and increased length of the pl shrimp when maintained in the concrete tanks over 5 months. Both weight gain and length of the animals were higher in the BL+ group but this was not statistically significant (P≥0.05).

Table 3.9. Growth of juvenile shrimp cultured in concrete tanks system

Month	weight (g)		length(cm)	
	BL-	BL+	BL-	BL+
0	0.05	0.04	1.96	1.75
1	0.40	0.52	4.14	4.59
2	1.81	2.20	6.71	6.82
3	3.82	4.01	8.44	8.48
4	6.08	6.15	9.62	9.72
5	10.23	10.26	11.54	11.6
M5-M0	10.18[a]	10.22[a]	9.58[a]	9.85[a]

Note : The same letter shows no significant difference within column; M = month

Survival Rate

The survival rate of animals in the concrete tanks that received the probiotic (BL+) was 25.2% whereas the BL- group was 19.8% (Figure 3.5). There were not a statistically survival rate was found in the animals receiving the probiotic (BL+) compared with the control or non-probiotic group (BL-) (P≥0.05), Figure 3.5, P = 0.517.

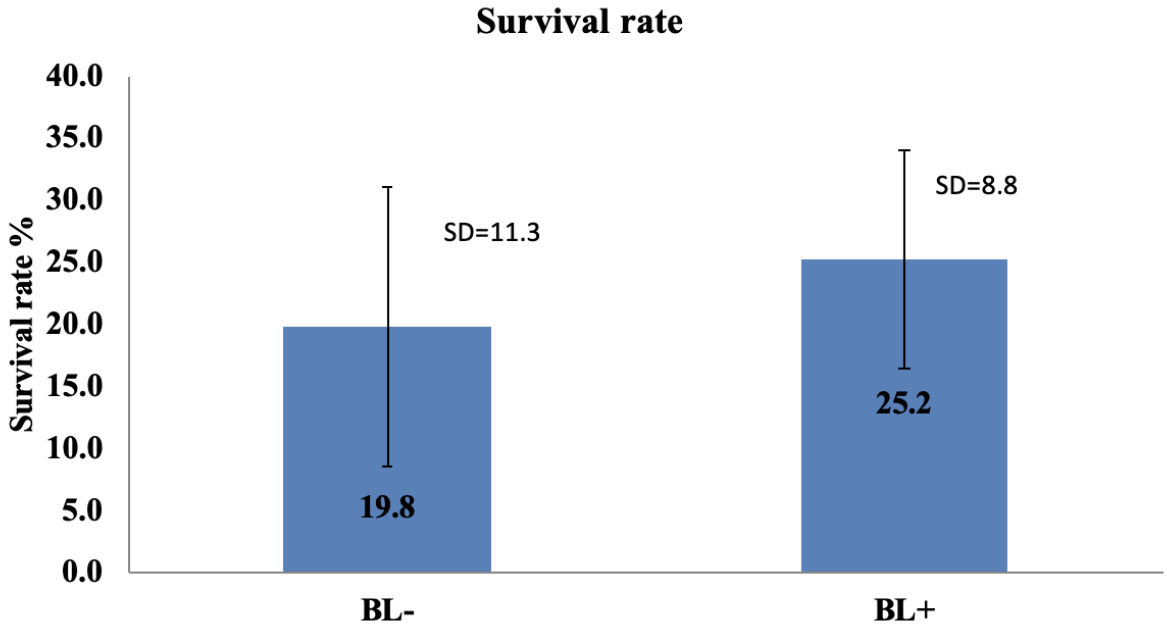

Figure 3.5. Survival rate of the animals of group of BL- and BL+ group maintained in concrete tanks system for 5 months.

3.3.2.2 Cage System

Growth Performance

The shrimp at pl 22 were maintained in net cages for 4 months and the results showed that the increased weight of juvenile shrimp of BL- group and BL+ group were 9.13 and 8.47g, respectively and the increased length was 9.26 and 9.14cm, respectively. Neither of these was statistically significant (P>0.05), (Table 3.10).

Table 3.10. Growth of juvenile shrimp cultured in cages both in weight and length

Month	weight (g)		length(cm)	
	BL-	BL+	BL-	BL+
0	0.05	0.04	1.96	1.75
1	1.16	1.02	5.99	5.6
2	2.61	2.21	7.82	7.37
3	4.71	4.21	9.16	8.74
4	9.18	8.51	11.22	10.89
M4-M0	9.13^{a}	8.47^{a}	9.26^{a}	9.14^{a}

Note : The same letter shows no significant difference within column; M = month

Survival Rate

The survival rate of grow-out animals cultured in the cages that received the probiotic (BL+) group was 4.5% whereas the BL- group was 3.5% (Figure 3.6). A statistically significant survival rate was not found in the animals receiving the probiotic (BL+) compared with the control or non-probiotic group (BL-) (P≥0.05), Figure 3.6, P = 0.376.

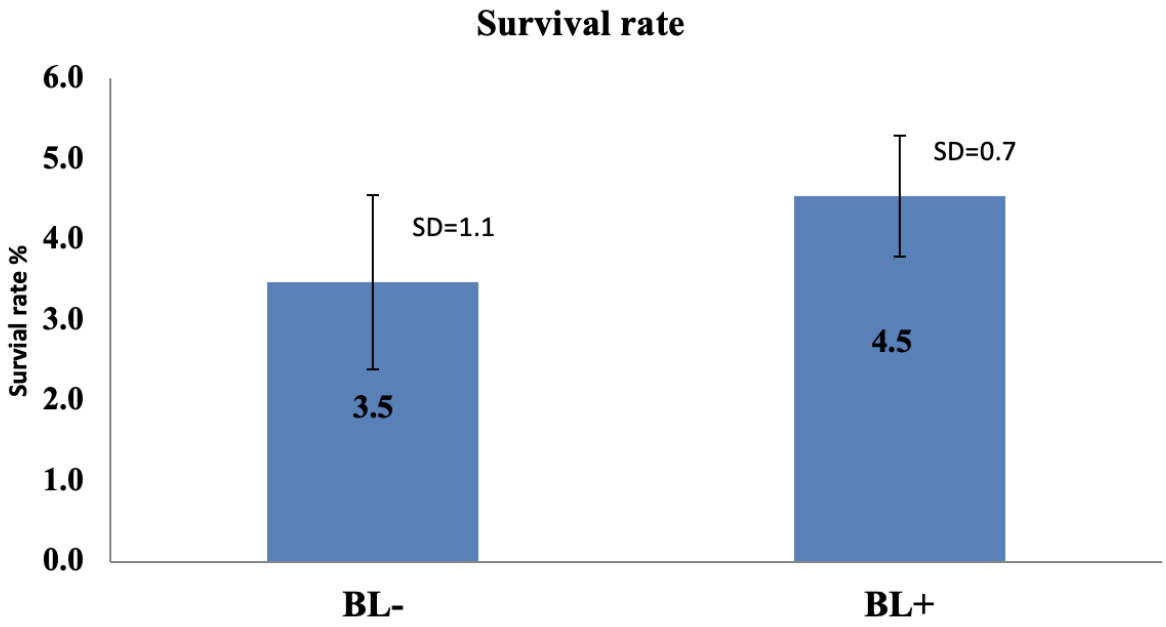

Figure 3.6. Graph showed the survival rate of the animals of group of BL+ and BL- cultured in cages for 4 month.

3.4. Discussion

This study aimed to investigate the effect of a probiotic strain of the bacterial species *B. licheniformis* on the survival rate and health assessment of whiteleg shrimp larvae. The animals were reared in the hatchery and their growth/health performance monitored as they were moved into 2 different grow out systems and monitored over 5 months. There were very few differences between the BL+ and BL- treatments with the exception of higher survival rate and lower pathogen load in the animals given the probiotic in the nursery phase. Significant differences were not detected in terms of reaching developmental growth stages of the pl or in achieving a higher health score between the 2 treatment groups.

From the data provided in chapter 2 of this study, controlling temperature within the optimal range was associated with better survival and was therefore included in the experimental study presented here. Temperature control is important for the development and survival of all ectothermic animals as they rely on the external temperature to drive many of their metabolic functions (Villarreal and Hernandez-Llamas, 2005). In Thailand the water temperature in the grow out shrimp farms can

fluctuate between 24 - 33^{o}C. If hatcheries use temperature control they avoid such extremes and this is thought to minimise thermal shock and support the development of the larvae to make them robust and ready for transfer. However, not all hatcheries practice temperature control and therefore it was considered worthwhile to further investigate the effects of temperature control under experimental conditions.

In the pilot study performed in the small-scale tanks, the effect of temperature on reaching development stage and survival of the shrimp larvae was examined. Animals were maintained in air conditioned room to lower the temperature. However, all of the 4 trials conducted as part of the low temperature condition were stopped. In these systems the shrimp larvae did not metamorphose into the pl stage whereas in the high temperature condition the larvae did develop into pl. The effect of low temperature was probably confounded by the use of small tanks since in chapter 2 there was increased survival in larger tanks. It was not considered ethical to continue using the low temperature in small tanks and therefore the study focussed on a high temperature (30 ± 1^{o}C) environment, only conducted in the larger tanks. In this system the nauplius reached pl 15. From these trials, it was concluded that the low temperature in small rearing containers were not be suitable for shrimp larval rearing and this result agreed with the survey chapter 2 data. The results from the temperature-controlled study performed would support the need for a consistent temperature suitable for the development of the animals to be applied at the hatchery and that the use of probiotic could not overcome the thermal shock.

A higher survival rate was observed in the shrimp larvae raised in the large-scale nursery system when fed the probiotic strain *B. licheniformis.* The probiotic in this study was administered to the shrimp at 10^6 cfu per ml via live artemia. The effect of the probiotic reduced the mortality of the animals in this study and was in agreement with the results of Jamali *et al.* (2015) who reported that survival rate of whiteleg shrimp larvae can be improved using rotifer and artemia supplemented with *B. licheniformis* and *B. subtilis* (1:1) at 10^6 cfu per ml feeding. The study described by Raida *et al.* (2003) found that probiotic strains of *B. subtilis* and *B. licheniformis* were administered as a feed additive to rainbow trout and improved the survival rate of the fish when they were exposed to a bacterial challenge from *Yersinia ruckeri*.

Similarly, studies of Sahandi *et al.* (2012) showed improved health and growth of *Cyprinus carpio* and *Ctenopharyngodon idella* larvae when adding probiotic strains of *B. circulans* and *B. licheniformis* at 1×10^6 cfu per ml directly into the rearing system. However, the evidence of Avella *et al.* (2010) showed there was no significant difference on survival when administered probiotic mixture (*B. licheniformis, B. subtilis, and B. pumilus*) mixed with live feed and given to gilthead sea bream larvae. Addition of the probiotics to the water did increase fish growth as measured by increased length and body weight (Avella *et al*., 2010). The data provided in this study is in general agreement with the published literature that the use of *Bacillus* probiotics have a positive effect on the overall health of the shrimp larvae, as judged by survival rate (Vendrell *et al.*, 2008 ; Nimrat, 2011 ; Nimrat, 2012 ; Rengpipat *et al.*, 1998). However, the grow-out trial in these studies the survival rate of animals was very low. The period of shrimp culture was long for 5 months. Also I had less experienced with growout. This might be one of causes leading to low survival rate.

The mode-of-action of probiotic strains is not clear for any species but a combination of improved host immune responses and pathogen inhibition in terms of adhesion and competitive exclusion have been suggested (Kongnum and Hongpattarakere, 2012 ; Chiu *et al.*, 2007). Whilst the cellular mode-of-action was not identified in this study, the results of the health assessment on pathogen detection found that probiotic *B. licheniformis* administration gave lower pathogen load in the tissues of pl whiteleg shrimp. Administration of the probiotics strain *B. licheniformis* in this study did not affect the total viable bacterial colony counts, but a lower number of *V. parahaemolyticus* colonies were recovered on the selective CHROME agar, as judged by the lack of purple coloured colonies. This was very encouraging given that strains of *V. parahaemolyticus* have been identified as aetiological agent of AHPND in shrimp (Tran *et al*., 2013).

In an attempt to understand the effect of the probiotic administered in this study on viable bacterial load in the animals, samples were grown on the selective TCBS agar, where strains of *V. parahaemolyticus* should be green coloured colonies (Thermo SCIENTIFIC, 2019). From the results presented in this study, a higher total number of colonies were recovered on the TCBS agar from the BL+ treatment

groups, but these gave a higher proportion of yellow coloured colonies. This result combined with the lack of detection of *V. parahaemolyticus* on the Chrome agar would suggest that the administration of the probiotic has “altered” the ability of the *V. parahaemolyticus* to attach and reproduce in the shrimp given the probiotic. However, a bacterial challenge was not performed and so this is a working hypothesis only, and further studies are required to elucidate these findings. However, this hypothesis may support a higher survival of the shrimp given probiotics by reducing the specific pathogen load. As Vaseeharan and Ramasamy (2003) reported probiotic administered to shrimp could reduce disease losses and studies performed by Zokaeifar *et al.* (2012) reported that administration of *B. subtilis* at a concentration of 10^8 cfu per ml orally to shrimp improved disease resistance. Similar results were reported by Li *et al* .(2007), where *B. licheniformis* at 10^5 cfu per ml was administered in water of *P. vannamei* culture inhibited Vibrio species and improved shrimp immunity.

In this study a single probiotic was administered whereas others have used combinations of probiotic strains which may give greater protection against colonisation from potential pathogens in the environment. In the study presented, a bacterial challenge was not administered so the bacteria recovered and identified would be a combination of organisms acquired from the environment or commensals within their microflora. Zhang *et al*. (2014) reported that administration of probiotic *B. subtilis* and prebiotic fructo-oligo-saccharide (FOS) had significantly higher on immune responses, growth performance and disease resistance of juvenile ovate pompano. No immune responses were measured during this study but should be included in future research to confirm if the *B. lichenoformis* can promote immune responses.

Feeding the probiotic to the larvae did not cause any negative side effects. An increase in survival rate was observed in larvae fed the probiotic which was found to be statistically significant. No other significant differences were observed in any of the criteria measured between the treatment groups. Although the larvae fed the probiotic was slightly faster in reaching the next development stage and the health check criteria score was higher, this was not significant between the group. It would appear from these data that feeding the larvae with the probiotic would be beneficial

in improving survival rates at the hatchery. However, how this is improving ie. mode-of-action was not investigated during this study, so it is unclear what is the positive effect. Nevertheless, when the larvae were transferred to the 2 grow out systems, they also showed a higher survival rate (although not statistically significant) in the animals from the probiotic group compared with the non-probiotic group. The results showed there is a difference between the 2 grow-out systems. In the animals that had received the probiotic and were then transferred to the concrete tanks there was a slight increase in the weight/size and the survival rates of the stocks from the BL+ probiotic group, but this was not statistically significant, so larger sample size may be needed. In the animals fed the probiotic and then transferred to the net cages the ones that had been fed the probiotic were smaller and lighter than those not receiving the probiotic but in the group that had received the probiotic they had no statistically significant survival rate, and even if it is not statistically significant it was biologically greater so this would all suggest that the production systems for the grow out can influence the benefit of the probiotic if there is one. In the concrete tanks they may retain the water heat better so less fluctuation and this may help the survival and growth of the animals.

However, one of the main reason to continue the study from the hatchery to the grow-out systems was to track the animal population and determine if the probiotic showed a negative impact or not. The grow**-**out study results showed that shrimp cultured in concrete tanks or net cages placed into the earthen pond had no significant difference on growth performance and survival rate irrespective of whether the shrimp seed had been fed the probiotic or not. So, in this study it would say that the BL probiotic did not get a negative impact on growth and survival of animal in farmed shrimp. The results produced from the study presented were in agreement with Farzanfar (2006) that proposed *Bacillus* spp**.** is not harmful when administrated to the aquatic animal. This is inability to cause disease is an important consideration when selecting a probiotic strain of bacteria.

In this study, the only single strain that was utilized. In future studies would investigate a combination of probiotics to explore and see how the combination of probiotics may beneficially act. The concentration of probiotic that was used in this study was similar with other reported studies (Sahandi *et al.* ,2012; Jamali *et al.*,

2015) but again it might be useful to explore the effect of concentration of the probiotic on animal health and survival. Future studies could look at a sequential sampling of the shrimp under varied probiotic administration regimes and to try to determine the mode-of-action samples for microbiome analysis could be included.

Luis-Villasenor (2013) stated that the intestinal microbiota of shrimp has limited bacteria diversity but after feeding a probiotic mix containing three Bacillus strains for 10 days, the gut microbiota of the shrimp were significantly changed. Our understanding of the role of the gut microflora and the microbiome research is only developing in aquaculture. However, Zhao *et al*. (2018) showed that both biotic and abiotic factors could influence the gut microbiome of freshwater shrimp species *Macrobrachium nipponense*. There is no doubt that the gut microbiota will play a role in supporting the health of the animals and having a more diverse range of bacterial communities within the gut are considered to be more robust. Therefore understanding the role that probiotics could play in supporting the gut microbiota may help clarify their role in promoting better health and survival in the shrimp. Starting probiotic administration early in the hatchery would be sensible to provide the pl with the best start before being stocked in the earthen ponds.

3.5 Conclusion

In conclusion, the results from this study supported the findings from chapter 2 that temperature control and larger tanks are associated with improved survival of the pl. It also showed that use of *B. licheniformis* enriched artemia administered as a probiotics and feed to shrimp larvae either improved the survival rate or reduced presence of *V. parahaemolyticus* in the animals. Attempts to understand the underlying mechanisms remain unclear as the results from this study did not show improved health criteria not did the probiotic fed animals reach developmental stages faster. Additionally, use of BL as a probiotic feed supplement in shrimp seed production had no demonstrable affect on growth performance or survival of the grow-out shrimp.

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CHAPTER 4

Effect of probiotic *Bacillus licheniformis* on bacterial infection in whiteleg shrimp, *Penaeus vannamei* (Boone, 1931)

4.1 Introduction

Bacterial infections occur in all aquaculture farming systems and in marine shrimp farms they are considered to cause approximately 20% of the total losses encountered from infectious diseases (Flegel, 2012). A wide range of bacterial species have been reported to cause disease outbreaks, leading to high levels of mortality (Tran *et al*., 2013 ; Flegel, 2012 ; Lightner *et al*., 2012 ; Lavilla-Pitogo and de la Pena, 1998) and/or morbidity (Thitamadee *et al*., 2016). A higher number of bacterial disease outbreaks in shrimp are reported from members of the *Vibrio* genus, contributing to the total infectious bacterial disease losses reported in Asia (Longyant *et al*., 2008). For many years, strains of *Vibrio harveyi* (Luminescent bacteria), *Vibrio vulnificus*, *Vibrio alginolyticus*, and *Vibrio penaeicida* (Longyant *et al*., 2008 ; Sung *et al*., 1999 ; Aguirre-Guzman *et al*., 2001) have been reported as the main aetiological agents causing high levels of shrimp losses or low production yields in aquaculture. Over the last 20 years, there have been more reports of disease losses in farmed shrimp due to viral aetiology (Flegel, 1997; Flegel, 2006; Flegel, 2012; Wang *et al.*, 1996; Sakaew *et al*., 2008; Pratoomthai *et al*., 2008 ; Sritunyalucksana *et al*., 2006) but in 2009 an emerging disease resulted in 100% losses of the shrimp larvae (Tran *et al*., 2013 ; Thitamadee, 2016). The disease is

described as Acute Hepatopancrease Necrosis Disease or AHPND (Lightner *et al*., 2012 ; Tran *et al*., 2013). The aetiological agent was identified as the Gram negative bacterium *Vibrio parahaemolyticus* which causes a toxin-mediated disease resulting in necrosis of the epithelial tubules in the hepatopancreas of infected shrimp (Tran *et al*., 2013). Outbreaks of AHPND have been widespread in shrimp aquaculture since 2009 and was first reported in Thailand in 2012 (Lightner *et al*., 2012 ; Flegel, 2012 ; Tran *et al*., 2013 ; NACA, 2014).

Acute Hepatopancreatic Necrosis Disease causes massive mortality in shrimp in the first 20 - 45 days after stocking into the farms. Both *P. vannamei* and *P. monodon* are affected by AHPND (Thitamadee, 2016 ; Reantaso, 2016 ; Lightner *et al*., 2012 ; Lai *et al*., 2015). Clinically, AHPND can be observed grossly by the naked eye as a complete loss of hepatopancreatic tissue (NACA, 2014) but recovery of the bacterium can be problematic, and so the actual diagnosis of AHPND requires the use of histopathology to show the cellular changes associated with the toxin released from the bacteria. The pathological changes associated with AHPND lesions include sloughing of hepatopancreatic tubule epithelial cells, degeneration of tubules lumen, and the hepatopancreas nuclei cell are enlarged with an associated reduction of R, B and F epithelial cells in the hepatopancreas tubules (Thitamadee *et al*., 2016 ; Manan *et al.*, 2015 ; Nunan, 2014 ; Lai *et al*., 2015). Soto-Rodriguez *et al*. (2015) reported that histologically, AHPND affected shrimp showed severe cellular necrosis in the hepatopancreas causing loss of tissue structure and described 3 stages of AHPND including initial, acute, and terminal stages.

The data from Songkhla Aquatic Animal Health Research Centre, Department of Fisheries; Thailand (2019) reported that in February 2017, AHPND was the biggest (48.4%) cause of sick or dead shrimp throughout the Thai shrimp farming regions. Animals between 1 - 30 days and 31 - 60 days old were most affected resulting in approximately 41% of sick shrimp.

To assist with a more rapid detection of AHPND in the farmed marine shrimp sector, several molecular assays were developed to detect the pathogen (Flegel and Lo, 2014 ; Tinwongger *et al*., 2014 ; Dangtip *et al*., 2015 ; Han *et al.*, 2015). The initial PCR methods were called AP1 and AP2 (Flegel and Lo, 2014) and have been

constantly updated. The assays were developed to detect DNA plasmid sequences present in the shrimp AHPND producing strains of *V. parahaemolyticus* (Sirikharin *et al*., 2015). The PCR assays target detection of the ToxA and ToxB genes located on the pVA plasmid, which is carried by the AHPND-causing bacteria (Dangtip *et al*., 2015). In the DOF Thailand, multiplex PCR assays (with 3 components) are routinely applied to first detect the presence of any *V. parahaemolyticus* from the bacterial recovered (PCR assay called Vp) and then to differentiate strains with and without the plasmid (PCR assay called C4). Finally, a third PCR (called Vp3) is performed to detect the presence of the toxin associated DNA and these all of the PCR methods were adapted from Tinwongger *et al*., (2014).

Many researchers have studied probiotics used to alter antibiotics in worldwide aquaculture system (Farzanfar, 2006 ; Ninawe and Selvin, 2009 ; Vinoj *et al*., 2013). A study by Kongnum and Hongpattarakere (2012) showed that diseases caused by the bacteria *V. harveyi*, *V. vulnificus*, *V. alginolyticus*, *V. anguillarum* were prevented by using probiotics in shrimp culture in intensive farming systems. The range of potential probiotic strains is continually increasing with a higher number of studies applying species belonging to the Gram positive *Bacillius* and *Lactobacillus* genera. Application of a probiotic strain of *B. subtilis* BT23 at a concentration of 10^6 - 10^8 cfu per ml given for 6 days to juvenile *P. monodon* produced a 90% reduction in cumulative mortality when the shrimp were exposed to pathogenic strain of *V. harveyi* at 10^3 - 10^4 cfu per ml (Vaseeharan and Ramasamy, 2003). Chiu *et al.* (2007) administrated a probiotic strain of *Lactobacillus plantarum* into the shrimp diet at 10^{10} cfu per kg and fed this to *P. vannamei* before exposing the animals to the pathogen *V. alginolyticus,* and found that the immune ability of shrimp was enhanced and the resistance of *V. alginolyticus* infection was increased. Further work by Ajitha *et al.* (2004) reported that administration of Lactic Acid Bacteria (*Lactobacillus acidophilus*, *Streptococcus cremoris*, *Lactobacillus bulgaricus*–56 and *Lactobacillus bulgaricus*–57) at 5 x 10^6 cfu per g mixed with moist feed and administered orally to *Penaeus indicus* produced better survival and resistance to disease when challenged with pathogenic *V. alginolyticus* at 3 x 10^9 cfu per ml.

The results from Chapter 2 identified the widespread use of probiotics within the Thai shrimp hatcheries. Furthermore, many Thai farmers were applying probiotics

during the grow out stage. Although probiotics were not significantly associated with improved survival in Chapter 2, they were thought worthy of further study given their widespread use in the industry. Therefore, the aim of this study was to investigate the effect of the probiotic *B. licheniformis* (BL) against a bacterial infection from pathogenic strains of AHPND *V. parahaemolyticus* in shrimp. The experimental design investigated the morbidity and mortality of the post larvae (pl) exposed to pathogenic *V. parahaemolyticus* after feeding with a single probiotic which was administered as described in Chapter 3. Two experiments were performed in this chapter. The animals used in Experiment 1 originated from the same stock described in Chapter 3, whereas the animals in Experiment 2 were from a new batch of shrimp treated as described in Chapter 3.

4.2. Materials and Methods

4.2.1 Animal Stocks & Health Evaluation

The animals included in this study were from 2 batches of nauplii purchased and sourced as described in Chapter 3 (section 3.2.6). Animals used in pre-challenge 1 and Experiment 1 were from the pl15 stocks described in Chapter 3 and grown in 7 tonne concrete tank until they reached juvenile stage at 60 days post larvae. The shrimp used in pre-challenge 2 and Experiment 2 were purchased from a second batch of nauplii and administered the BL probiotic as described in Chapter 3, but they were only grown to pl22. Prior to any experimental work a subsample of each group of shrimp was randomly selected and sent to the DOF, Coastal Aquaculture Research and Development Regional Centre 3 (Surat Thani) laboratories and checked for prior exposure to the bacterium *V. parahaemolyticus* using the 3 different PCR methods as described in Chapter 3 (section 3.2.9, Table 3.6). Samples from the stock shrimp used in Experiment 2 were also taken for histopathology as described (section 4.2.10).

For Experiment 1, the juvenile animals were 60 days post-larvae (pl60), weight approximately 0.5 g (Plate 4.1a) and for Experiment 2, younger animals at pl22 weight approximately 0.05 g (Plate 4.1b) were used. Size of animals in both experiments were different. Size of animals were based on to be available at the

periods of experiment conducted, and these size of animal chosen are commonly found to be affected by AHPND in grow-out sites.

(a)

(b)

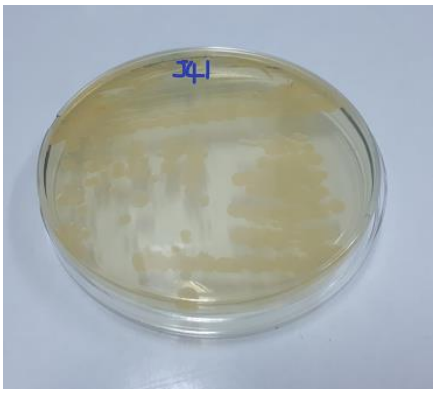
Plate 4.1. (a) The juvenile of *P. vannamei* weight approximately 0.5 g for the 1st challenge and (b) The post larvae (pl22) of *P. vannamei* for the 2nd challenge

4.2.2 Bacterial Isolates

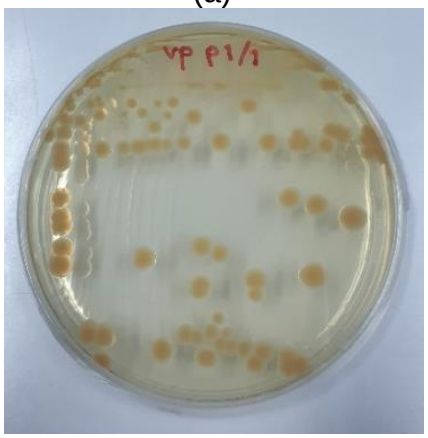
Two strains of AHPND-*V. parahaemolyticus* were used for the challenge studies. The isolate J41 was used for pre-challenge 1 and Experiment 1 (Plate 4.2a), and the isolate VPP1 was used for pre-challenge 2 and Experiment 2 (Plate 4.2b). These strains were provided by the Songkhla Coastal Aquatic Animal Health Research Centre, Department of Fisheries, Thailand as pure cultures, stored in Tryptone Soy Broth (TSB) with 2% Sodium Chloride (NaCl) + 25% glycerol as the cryoperservative. In experiment 2, the strain of AHPND-*V. parahaemolyticus* were changed from J41 to VPP1 due to J41 might not be virulent enough since the data showed it resulted in low mortalities.

The J41 strain had originated from clinical outbreaks of AHPND in farmed *P. vannamei* in Songkhla province and VPP1 was recovered from the hepatopancreas of moribund shrimp farmed in Pattani province in 2014. The isolates were identified using routine bacteriology methods following those described in Kaysner and DePaola (2004) with minor adaption as described in the DOF Thailand laboratories routine procedures. During the DOF identification tests both isolates were positive

for the presence of the AHPND plasmid mediate toxin detected by PCR (data not shown).



(a)



(b)

Plate 4.2. (a) and (b) Pure cultures of *V. parahaemolyticus* isolate J41 (a) and VPP1 (b) strains grown on TSA + 2% NaCl.

4.2.3 Bacterial Pre-challenge Study

To confirm pathogenicity of the 2 *V. parahaemolyticus* strains, a small pre-challenge study was performed to ensure that the 2 bacterial strains were able to cause disease in the shrimp. Animals that had not been exposed to the probiotic, were placed into 2 tanks with 30 shrimp per tank. For isolate J41, the shrimp were grown to juveniles at pl60 and for isolate VPP1, the animals were at pl22. The shrimp were exposed the bacterial strains via bath for 6 hours at 3 x 10^6 cfu per ml for J41 and 6 x 10^7 cfu per ml for VPP1. After this time, the moribund/dead shrimp and the surviving shrimp from each tank were aseptically removed and placed into separate sterile containers. These were then sent to the DOF laboratories for detection of *V. parahaemolyticus*.

At the laboratory, routine procedures were followed which briefly included, cleaning of the shrimp before macerating in sterile diluent (TSB) and the suspensions were then incubated for approximately 18h at 35 ± 2 ^{o}C and 2 aliquot per sample were aseptically removed. One aliquot was processed for bacterial detection by PCR and the other aliquot was processed for viable bacterial recovery using TCBS agar as described in Chapter 3 (section 3.2.9). Viable bacterial recovery was only performed on the shrimp exposed to strain J41 by plating samples onto the TCBS agar, incubating and then counting the number of viable colonies recovered. TCBS acted as a presumptive indicator of *Vibrio* species with green coloured colonies being indicative of *V. parahaemolyticus*. No further identification was performed on the colonies recovered and no histopathology samples were taken from any samples in the pre-challenge studies.

4.2.4 Experimental Design for Bacterial Challenge 1 and 2

Two bacterial challenge studies were performed: **Experiment 1** investigated the effect of the probiotic on bacterial infection when the animals were exposed to a single concentration of the pathogenic *V. parahaemolyticus* strain J41. **Experiment 2** investigated the effect of bacterial concentration on shrimp survival after receiving the probiotic. In Experiment 2 the shrimp were exposed to *V. parahaemolyticus* strain VPP1 at 10^5 or 10^7 cfu per ml. For each experiment there were 4 treatment groups (T1-T4), with 6 replicate tanks per treatment group. The experimental lay out for Experiment 1 and 2 are shown in Table 4.1**.** All bacterial pathogen exposure was via bath administration.

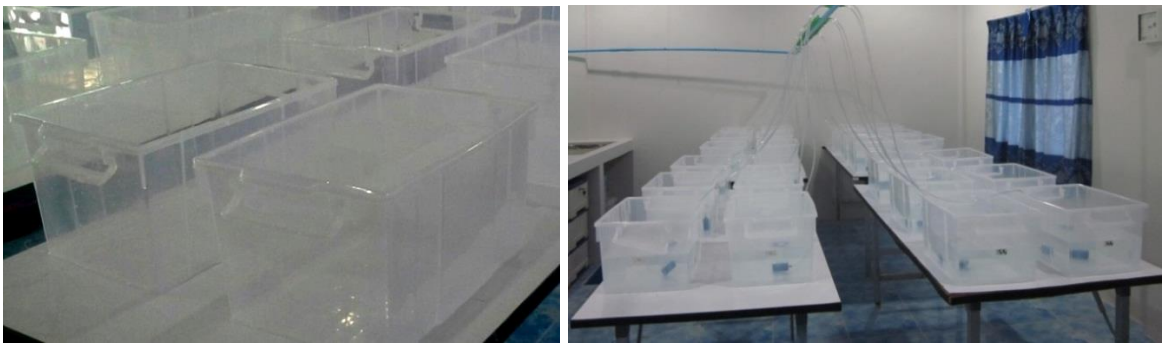
Table 4.1. Design description for Experiment 1 and Experiment 2.

Bacterial Challenge Experiment 1 (Isolate J41)			
Treatment Group 1	**Treatment Group 2**	**Treatment Group 3**	**Treatment Group 4**
BL+/VP- Probiotic only	BL-/VP- no probiotic, no bacteria (control)	BL+/VP+ probiotic and bacteria at concentration of 10^5 cfu/ml	BL-/VP+ no probiotic, bacteria only at concentration of 10^5 cfu/ml
Bacterial Challenge Experiment 2 (Isolate VPP1)			
Treatment Group 1	**Treatment Group 2**	**Treatment Group 3**	**Treatment Group 4**
BL+/VP+ probiotic and bacteria at concentration of 10^5 cfu/ml	BL+/VP+ probiotic and bacteria at concentration of 10^7 cfu/ml	BL-/VP+ no probiotic, bacteria at concentration of 10^5 cfu/ml (control)	BL-/VP+ no probiotic, bacteria at concentration of 10^7 cfu/ml (control)

BL+ : Probiotic; VP+ : *V. parahaemolyticus*; BL- : No probiotic; VP- : No *V. parahaemolyticus*

4.2.5 Experimental Facilities and Bacterial Challenge

Briefly, twenty-four transparent plastic containers (Plate 4.3a) were used for the bacteria challenge and these were on a static system applied throughout the duration of the study. The water salinity was 25 ppt which were measured using Salino-refractometer and daily temperature ranged from 26 to 28 ^oC using Thermometer measurement. Each 20 L tank had 10 L of treated seawater added with each container individually aerated using air stone (Plate 3b). The treated water was prepared for the experiment and sterilised using calcium hypochlorite [$Ca(ClO)_2$] for 3 - 5 days until chlorine was neutralised. This was confirmed using a chlorine test kit (IMPACT Test Kits, Thailand) and the treated water was filtered using 5µm mesh size bag filter prior to use. A stock tank of treated water was prepared in advance of the experiments.



a b

Plate 4.3. (a) Transparent plastic containers size of 27 x 37 x 20.5 cm (b) The static system

4.2.6 Preparation of the Bacteria for Challenge

Pure cultures of each bacterial strain were grown for 24h at 30 ± 2 ^{o}C on TSA (Difco™) + 2% NaCl and a single colony from each pure culture plate was aseptically removed and transferred into 9 ml of 2% sterile NaCl saline to produce a bacterial suspension. The optical density (OD) was measured using a spectrophotometer (model UV-1201, SHIMADZU) at wavelength 640 nm, and adjusted to give OD_{640nm} = 0.1 nm using 2% sterile saline as the diluent. This OD value was estimated to provide a bacterial concentration of 1 x 10^8 cfu per ml. For each bacterial suspension, viable colony counts were performed and plated onto TCBS (Difco™) agar, incubated 35 ± 2 ^{o}C for 24h and colonies counted following the methods described in Kaysner and DePaola (2004) adapted by the DOF Thailand laboratories routine procedures. This was performed for both *V. parahaemolyticus* strains used in the pre-challenge studies.

A higher volume of bacteria was required for the bacterial challenge studies performed in Experiment 1 and Experiment 2 and so 30 µl of the bacterial suspension at OD = 0.1nm was transferred into a sterile glass bottle containing 300 ml of TSB + 2% NaCl. This was then incubated for 16 - 18h at 30 - 32 ^{o}C on a shaking hot plate at 200 rpm (Brand IKA model C-MAG HS 7).

To harvest the bacteria, 2 different methods were used.

In Experiment 1, the bacterial suspension containing isolate J41 was centrifuged at 4,000 rpm for 10 min (Scanspeed model CPA 225D), washed once and resuspended 2% sterile saline. This gave washed whole bacterial cells only. Whereas in Experiment 2, no centrifugation or washing of the seed stock VPP1 was performed so this contained whole cells and media with any extracellular products or cell debris. The OD_{640nm} was measured again following Miles *et al*. (1938) to provide expected concentrations at 1 x 10^8 cfu per ml on TCBS agar.

4.2.7 Exposure of the Shrimp to the *V. parahaemolyticus* J41 and VPP1 strain

In Experiment 1, shrimp were exposed to J41 at 10^5 cfu per ml and the bacterial suspension was prepared and added into two, 60 L acrylic tanks (called bacterial challenge tanks) filled with 20 L treated seawater (Plate 4.4a). In Experiment 2 there

were four acrylic tanks in which 2 tanks received isolate VPP1 at 10^5 cfu per ml and 2 tanks received isolate VPP1 at 10^7 cfu per ml. After adding the bacteria to the tanks, the animals were carefully placed into the tanks and exposed to bacteria, as described above, for 6 hours without feeding. Subsamples of pooled shrimp (n=50) per treatment group in Experiment 2 were removed and sampled as described in Chapter 3 (section 3.2.9) for detection of the *V. parahaemolyticus* in an attempt to confirm uptake of the *V. parahaemolyticus*.

For both experiments after 6 hours, the shrimp were transferred and placed into pre-prepared 20L plastic containers (called holding tanks, Plate 4.4b) and fed with normal commercial pellet diet (without probiotic, Thai Union Feedmill Company) four times daily. The feeding times were spread throughout a 24h period at approximately 06.00, 12.00, 18.00, 24.00, and this was performed for 7 days**.** Feaces and uneaten food were quickly siphoned once every morning and water replaced.

a b

Plate 4.4. (a) 60 L acrylic tanks for bacterial challenge tanks (b) 20L plastic containers for holding tanks

4.2.8 Cumulative Mortalities and Biological Samples

Animals were checked up to 4 times daily and any moribund and dead animals were quickly removed out of holding tanks and numbers recorded. Samples of moribund/dead shrimp were removed and placed into a single sterile container on a daily basis. These pooled samples had a minimum of 1 animal to a maximum of 3 animals/per pooled sample. At the end of the study period, pooled samples of the

surviving shrimp (n=50) per treatment group were taken and placed into sterile containers for processing as previously described.

4.2.9 Detection of Bacteria

Pooled samples were processed as described for the pre-challenge study (section 4.2.3) where in Experiment 1, PCR assays only were performed on the moribund/dead and surviving samples. Whereas in Experiment 2, PCR was performed on the moribund/dead samples but both PCR and viable bacterial recovery on TCBS was performed. The methods for both were described in Chapter 3 (section 3.2.9).

4.2.10 Histopathology Samples

No histopathology samples were taken for any animal during pre-challenge 1 & 2. Histopathology samples were taken for Experiment 1 and Experiment 2 where individual whole shrimp bodies were fixed using Davidson's fixative (Distilled water 750 ml, 95% Alcohol 750 ml, 37% Formaldehyde 500 ml, Glacial acetic acid 250 ml) for 24 hours. After that shrimp were transferred into 70% ethyl alcohol for storage. All tissue samples were processed, wax embedded and tissue blocks cut to give 4 µm thick wax sections which were stained with Haemotoxylin and Eosin (H&E) following standard methods (Humason, 1979).

4.2.11 Data Collection/Data Analysis

The raw data were recorded and analysed using Microsoft Excel 2013^{TM} (Microsoft, USA). Data from treatment groups were tested for statistically significant differences at 95% confidence limit interval using ANOVA.

4.3 Results

4.3.1 Shrimp Stock and Animal Health

No *V. parahaemolyticus* was detected by any of the 3 PCR methods applied to the subsample of the shrimp taken for health checks and used in the pilot challenge studies or Experiment 1. In the shrimp population used for Experiment 2, a single positive PCR result was obtained for 1 sample. This was positive for the Vp PCR which detects all *V. parahaemolyticus* species and did not confirm AHPND strains

of *V. parahaemolyticus*. The other 2 PCR assays were all negative indicating that the shrimp stocks used in the pilot challenge study and in Experiment 1 or 2 did not have AHPND causing *V. parahaemolyticus* prior to the study, as detected by PCR.

4.3.2 Bacterial Pre-challenge Results

From the pre-challenge study performed with *V. parahaemolyticus* isolate J41 and VPP1, both strains were able to cause mortality in the shrimp exposed to the pathogens which were all PCR positive (Table 4.2). The PCR positive results were found in both the dead shrimp and moribund animals. The actual concentration of the J41 and VPP1 given to the shrimp during the pre-challenge studies were 3×10^6 cfu per ml and 6×10^7 cfu per ml, respectively. In the animals exposed to J41 a higher number of viable cfu were recovered from the dead shrimp compared with the surviving shrimp and this tested positive for all 3 PCR assays meaning that the *V. parahaemolyticus* challenge strains were able to cause disease and could be recovered from the sick/dead shrimp.

Table 4.2. Detection of bacteria from the moribund and surviving shrimp during pre-challenge studies

Bacteria Isolate	Total No. of Dead Shrimp	% of Dead Shrimp	PCR assay result from the dead/moribund shrimp			Number of Viable bacteria recovered (cfu/ml)	
			PCR 1 - Vp	PCR 2 - C4	PCR 3 - Vp3	Dead Shrimp	Surviving Shrimp
J41	15	50	+ve	+ve	+ve	3×10^{11}	1×10^8
VPP1	18	60	+ve	+ve	+ve	Not done	Not done

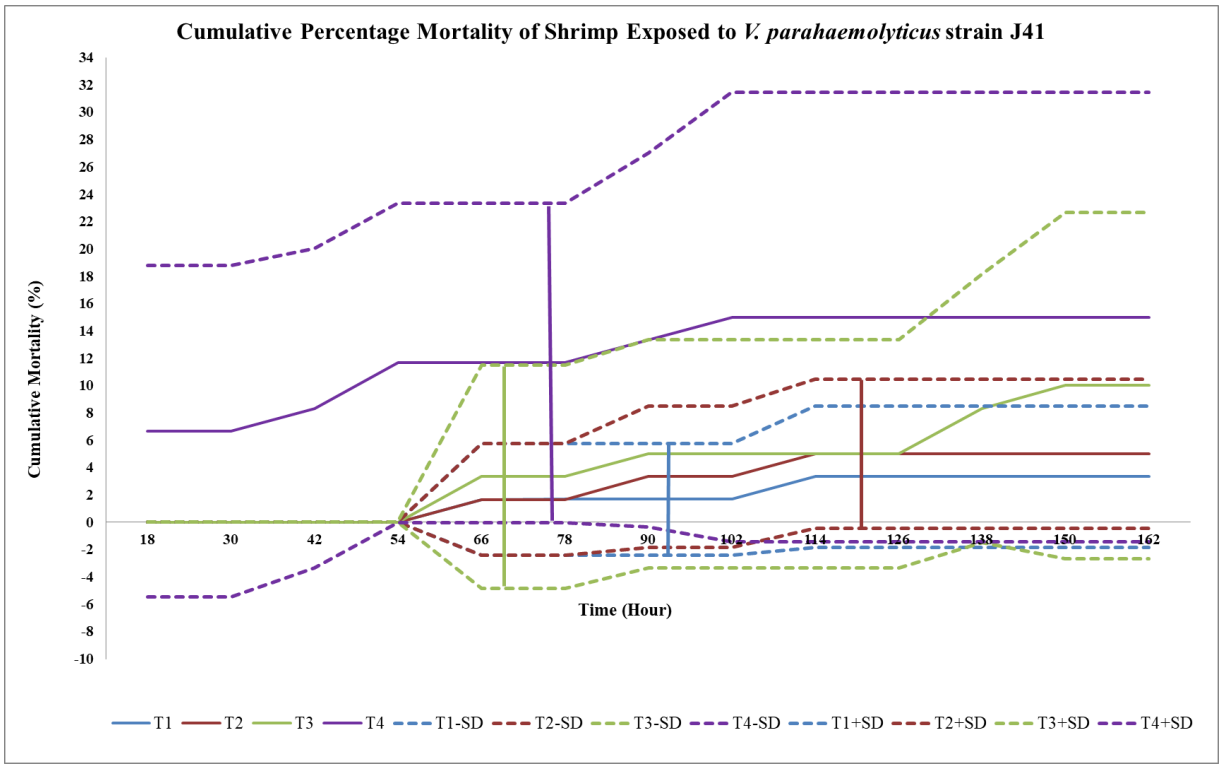
+ve = positive

4.3.3 Experiment 1 Results

4.3.3.1 Cumulative Mortalities

The lowest cumulative percentage mortality was found in the Treatment group 1 (BL+, Vp**-**) which was only slightly lower that the Treatment group 2 data (BL-, Vp-) (Figure 4.1). The highest percentage mortality was found in Treatment group 4

which were given the AHPND *V. parahaemolyticus* strain J41 but not fed probiotic (Figure 4.1).



T1 = Treatment group 1 (BL+/VP**-**) , T2 = Treatment group 2 (BL**-**/VP**-)** , T3 = Treatment group 3 (BL+/VP+) , T4 = Treatment group 4 (BL-/VP+) , SD = Standard Deviation

Figure 4.1. Cumulative % mortality of shrimp exposed to *V. parahaemolyticus* strain J41.

4.3.3.2 Recovery and Detection of the Bacteria

Vibrio parahaemolyticus was detected in dead/sick animals from each treatment group using the Vp PCR (Table 4.3). None of the moribund/dead shrimp samples were positive for the AHPND Vp3 PCR assay (Table 4.3) and positive PCR results for assay C4 was only detected in the Treatment groups 3 and 4, which were exposed to the J41 pathogen (Table 4.3).

Table 4.3. Detection of *Vibrio parahaemolytic*us from moribund/dead shrimp sampled during the Experiment 1.

PCR test	moribund/dead shrimp							
	T1:Vp$^-$BL+		T2:Vp$^-$BL-		T3:Vp+BL+		T4:Vp+BL-	
	T1R4	T1R6	T2R5	T2R6	T3R5	T3R3	T4R1	T4R2
Vp	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
C4	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
Vp3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Vp; *Vibrio parahaemolyticus* (No plasmid; No AHPND) , C4: *V. parahaemolyticus* (Plasmid with non toxic gene; No AHPND) , Vp3: *V. parahaemolyticus* (Plasmid with toxic gene ;AHPND)
+ve: positive ; -ve: negative

Vibrio parahaemolyticus DNA was detected in nearly all of the shrimp samples processed at the end of the 7 day study period. These were the surviving shrimp. However, AHPND causing *V. parahaemolyticus* was not detected in any of the surviving shrimp from any treatment group (Table 4.4).

Table 4.4. Detection of Vibrio parahaemolyticus from surviving shrimp, Experiment 1

PCR test	Surviving shrimp							
	T1:Vp$^-$BL+		T2:Vp$^-$BL-		T3:Vp+BL+		T4:Vp+BL-	
	sample1	sample2	sample1	sample2	sample1	sample2	Sample1	Sample2
Vp	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
C4	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
Vp3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Vp: *Vibrio parahaemolyticus* (No plasmid; No AHPND) , C4: *V. parahaemolyticus* (Plasmid with non toxic gene; No AHPND) , Vp3 : *V. parahaemolyticus* (Plasmid with toxic gene ;AHPND)
+ve: positive ; -ve: negative

4.3.3.3 Histopathology Results

A common finding in all of the histopathology samples screened included degeneration of the central hepatopancreatic tubules, which may have been caused by fixation artefact. Nevertheless, the histopathology results showed cellular changes indicative of AHPND in all of the samples taken from each treatment group, including the control (Treatment group 2).

Table 4.5 below, from the 8 histopathology samples of surviving shrimp taken in Experiment 1 in treatment group of BL- showed that all samples were infected with AHPND, whereas 6 of 8 of histopathological sampled of treatment group of BL+ were affected with AHPND. There were 2 samples of Treatment group 3 (Vp+BL+) showed there were no AHPND indicative (Plate 4.7). There is a tendency for BL to reduce risk of AHPND but this was not significant with these small samples.

Table 4.5. Histopathology samples of surviving shrimp taken in experiment1

Treatment	AHPND presence (no. of sample)	AHPND absence (no. of sample)
T1:Vp^{-}BL+	4	0
T2:Vp^{-}BL-	4	0
T3:Vp+BL+	2	2
T4:Vp+BL-	4	0

BL+ : Probiotic; VP+ : *V. parahaemolyticus*; BL- : No probiotic; VP- : No *V. parahaemolyticus*

The image below (Plate 4.5) showed degeneration of central hepatopancreatic tubules (DCHT) with some outlines of structures in the central lumen of hepatopancreas (Star). There were structures similar to the parasites gregorines but probably Aggregated Transformed Microvilli (ATM) (Spot) (Thitamadee *et al*., 2016). There were very few R-cells indicating poor nutritional reserves. There were some haemocyte aggregations round some of tubules (Arrow). This sample from surviving shrimp in the end of experiment 1 (162h) of Treatment group 1 that had not received bacterial challenge from *V. parahaemolyticus* J41.

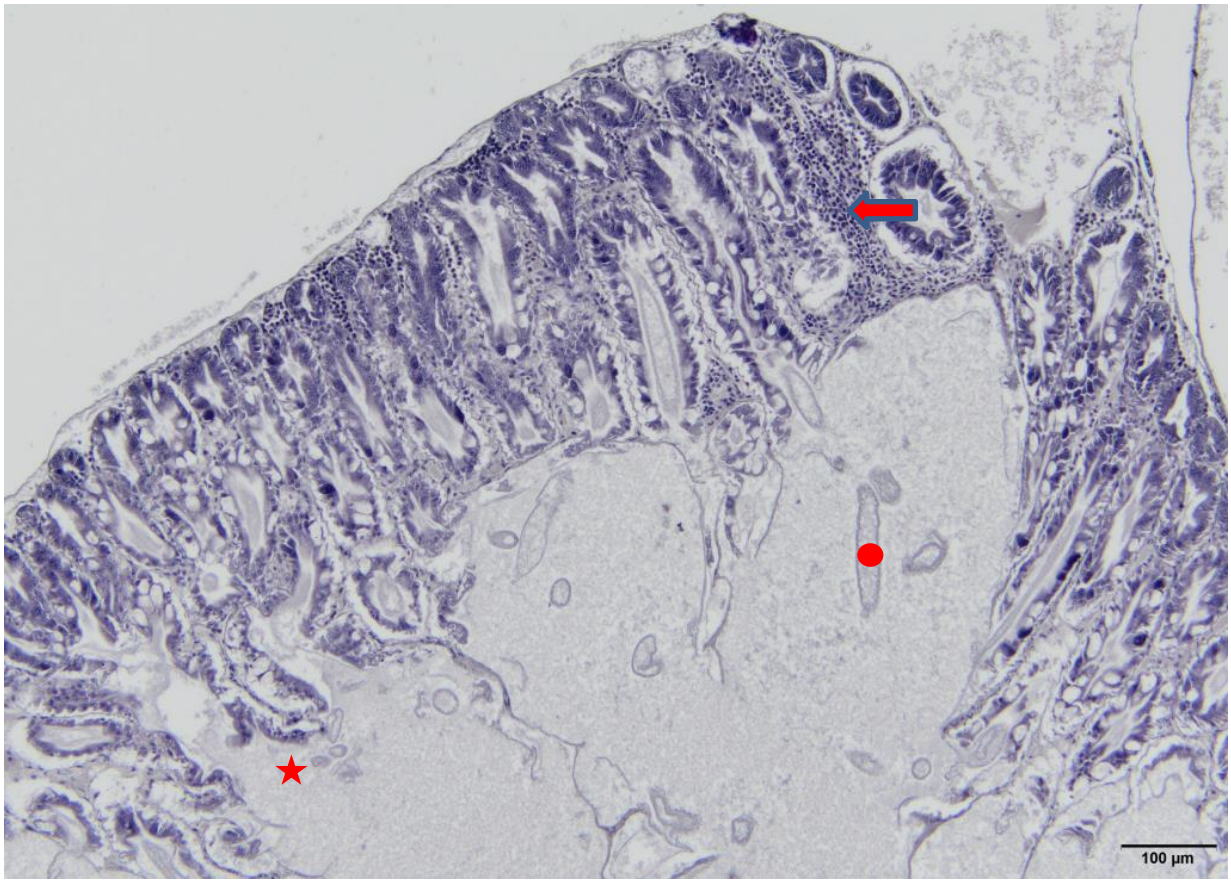


Plate 4.5. H&E sample of hepatopancrease from surviving shrimp of BL+ group in the end of experiment (162h) with no *V. parahaemolyticus* J41 exposure. Star = degeneration of central tubules. Spot = possible ATM. Arrow = haemocytic aggregations.

The image (Plate 4.6) is the same tissue of shrimp of Plate 4.5 but at higher magnification.

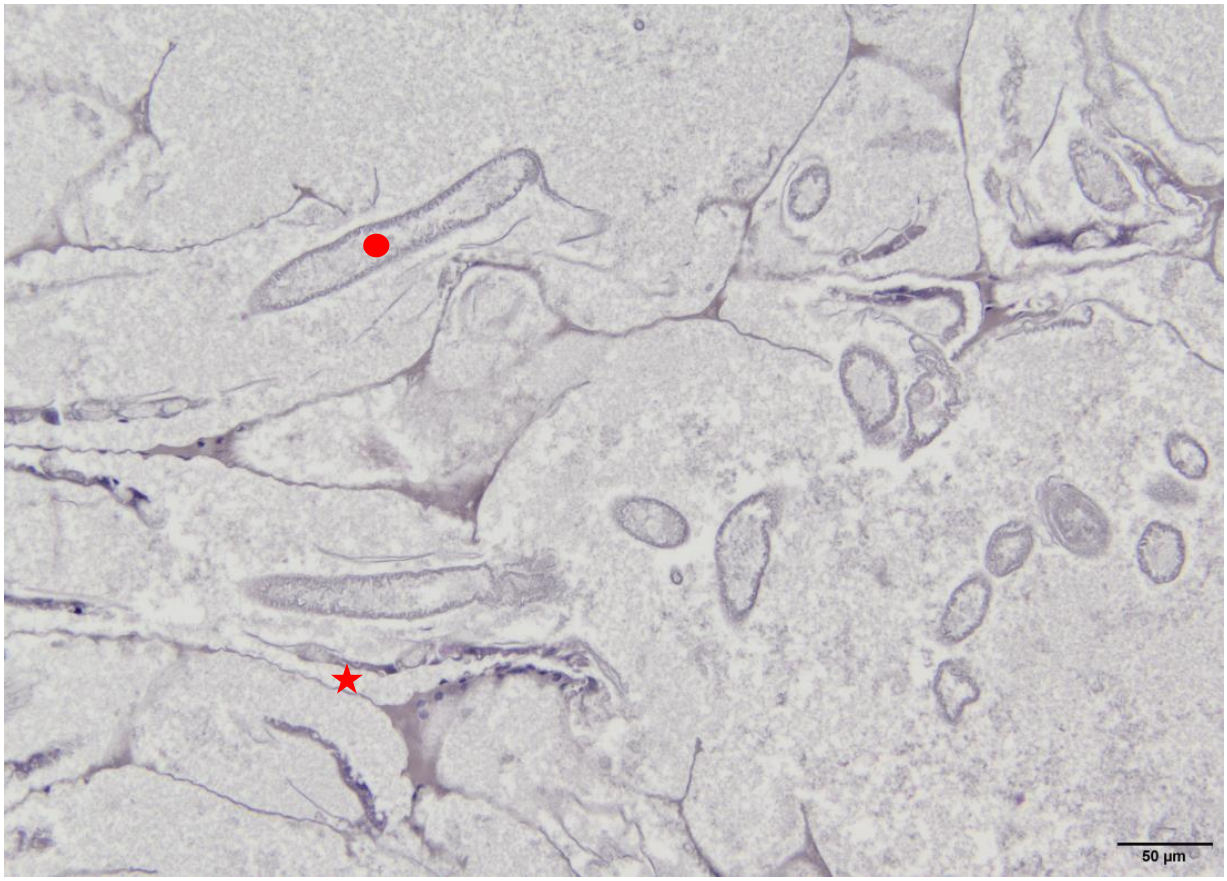


Plate 4.6. Hepatopancrease (H&E sample) from surviving shrimp of BL+ group with no *V. parahaemolyticus* J41 exposure. Star = degeneration of central tubules. Spot = possible ATM.

Plate 4.7 shows normal hepatopancreas from surviving shrimp (Treatment group 3, Vp+, BL+) sampled at the end of Experiment 1 study 162h post-exposure to the bacterial strain J41 administered at 10^5 cfu per ml. The central area of hepatopancrease was in good condition with some R-cells visible.

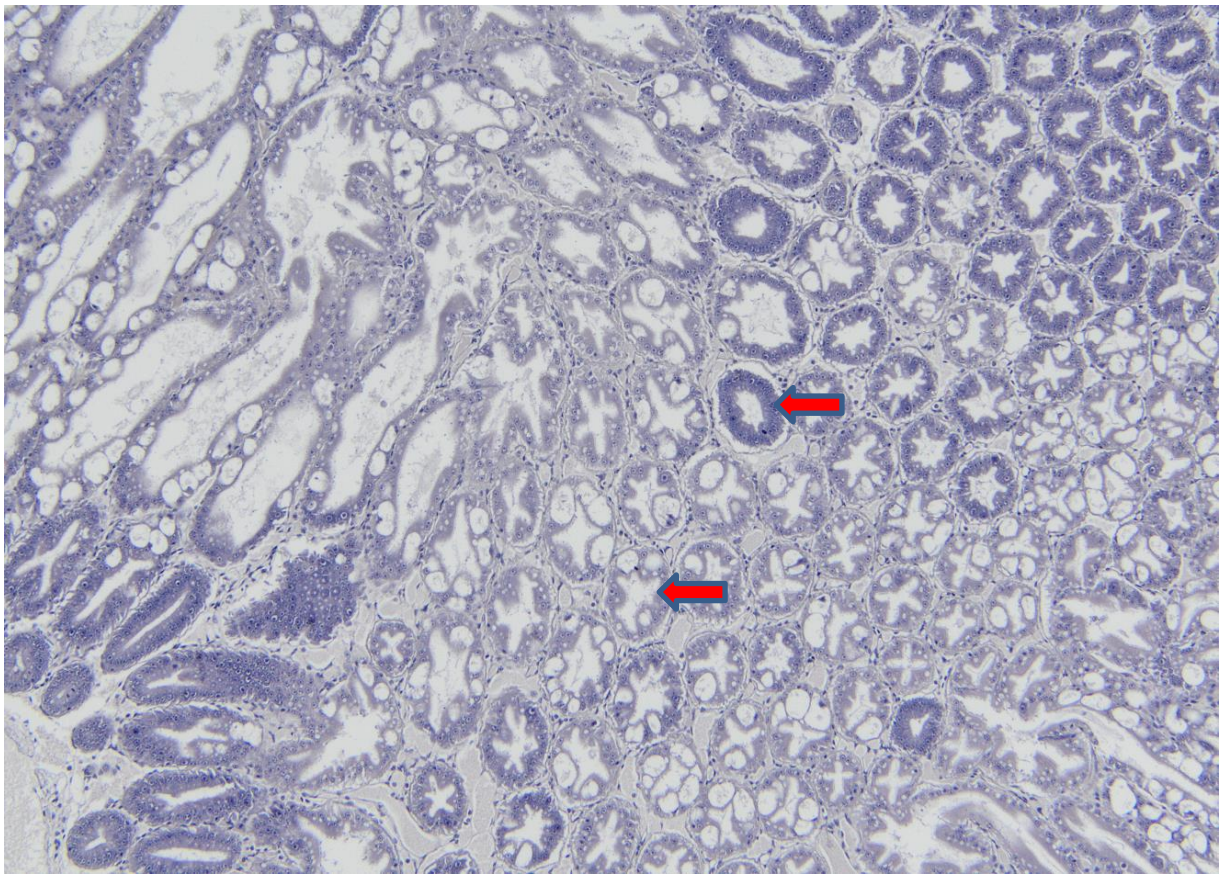


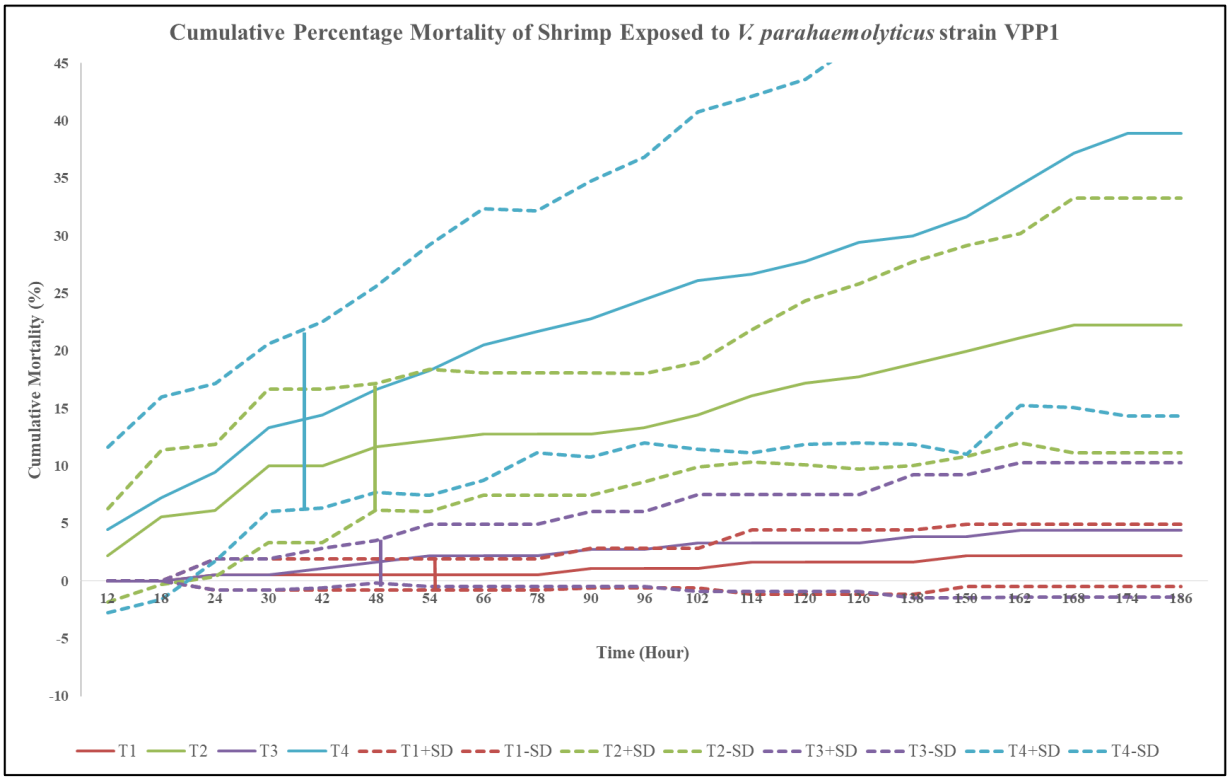
Plate 4.7. H&E stained section of apparently normal hepatopancrease from surviving shrimp sampled in Treatment group 3. Arrows show presence of normal tubule structure, upper arrow a distal tubule and lower arrow a proximal tubule with some R cells.

4.3.4 Experiment 2 Results

4.3.4.1 Cumulative Mortalities

The lowest cumulative percentage mortality was observed in treatment group 1(BL+/VP+) and 3 (BL-/VP+) which where both exposed to the bacteria at 10^5 cfu per ml. The cumulative percentage mortalities and mortality curves in these Treatment groups would suggest that administration of the probiotic did not cause any negative effects (Treatment group 3, Figure 4.2). The highest percentage

cumulative mortality was observed in Treatment group 4, which were exposed to the bacteria at 10^7 cfu per ml and not fed the probiotic (Figure 4.2). Mortalities were observed in Treatment group 2, which were also exposed to the higher bacterial concentration but were fed the probiotic.



T1 = BL+/VP+10^5 , T2 = BL+/VP+10^7, T3 = BL-/VP+10^5 , T4 = BL-/VP+10^7 , SD = Standard Deviation

Figure 4.2. Cumulative % mortality of shrimp exposed to *V. parahaemolyticus* strain VPP1

4.3.4.2 Recovery and Detection of Bacteria from Experiment 2

In the subsample of shrimp taken at 6h after exposure to the bacteria a positive PCR result was only found in the shrimp sampled in Treatment group 4 which had been given the *V. parahaemolyticus* at the highest concentration and not fed the probiotic (Table 4.6). No other positive PCR results were found.

Table 4.6. Detection of Vibrio parahaemolyticus from shrimp sampled after 6h exposure to VPP1

PCR test	shrimp sampled after 6h exposure to VPP1			
	T1:$Vp10^5$_BL+	T2:$Vp10^7$_BL+	T3:$Vp10^5$_BL-	T4:$Vp10^7$_BL-
Vp	-ve	-ve	-ve	+ve
C4	-ve	-ve	-ve	+ve
Vp3	-ve	-ve	-ve	+ve

Vp; *Vibrio parahaemolyticus* (No plasmid; No AHPND), C4: *V. parahaemolyticus* (Plasmid with non toxic gene; No AHPND), Vp3 : *V. parahaemolyticus* (Plasmid with toxic gene ;AHPND)

+ve : positive; -ve: negative

Data presented in Table 4.7 shows the PCR positive or negative results from the moribund shrimp samples taken in each treatment group over the 7-day period. The data show that a higher number of PCR positive samples were found in the treatment groups receiving the VPP1 *V. parahaemolyticus* at 10^7 cfu per ml.

Table 4.7. PCR results to detect the presence of V. parahaemolyticus and AHPND V. parahaemolyticus in the moribund/dead shrimp during the experiment

Treatment Group	Sampling time							
	12h	24h	30h	42h	48h	66h	168h	174h
1	NS	NS	NS	NS	NS	NS	NS	NS
2	NS	PCR-	PCR+	PCR+	NS	NS	NS	NS
3	NS	NS	PCR+	NS	NS	NS	NS	NS
4	PCR+	PCR+	PCR+	PCR+	PCR+	PCR+	PCR-	PCR-

NS = No sample, PCR+ = the sample was positive for all 3 PCR assays performed and PCR- = the sample was negative for all 3 PCR assays performed

Vibrio parahaemolyticus was not found by any of the PCR assays performed in the surviving shrimp sampled in Experiment 2 (Table 4.8). However, viable bacterial growth was only recorded on the surviving shrimp sampled onto TCBS agar from Treatment groups 2 and 4: these shrimp were exposed to the VPP1 strain at the highest concentration (Table 4.8). Recovery of green coloured colonies only occurred in the treatment group receiving the highest bacterial concentration without probiotic and was a proxy indicator of recovery of *V. parahaemolyticus*.

Table 4.8. The AHPND PCR analysis and bacteria results of surviving shrimp sampled in the end of the Experiment 2.

PCR test	Surviving shrimp samples in the end of the experiment 2			
	T1:$Vp10^5$BL+	T2:$Vp10^7$BL+	T3:$Vp10^5$BL-	T4:$Vp10^7$BL-
Vp	-ve	-ve	-ve	-ve
C4	-ve	-ve	-ve	-ve
Vp3	-ve	-ve	-ve	-ve
Total Vibrio Count (CFU/ml,g)	0	$2.60x10^6$	0	$2.60x10^6$
Vibrio yellow colony (CFU/ml,g)	0	$2.60x10^6$	0	0
Vibrio green colony (CFU/ml,g)	0	0	0	$2.60x10^6$

Vp; *Vibrio parahaemolyticus* (No plasmid; No AHPND), C4: *V. parahaemolyticus* (Plasmid with non toxic gene; No AHPND), Vp3 : *V. parahaemolyticus* (Plasmid with toxic gene ;AHPND),
-ve: negative

4.3.4.3 Histopathology Results

A common finding in all of the histopathology samples screened was degeneration of the central hepatopancreatic tubules, which may have been caused by fixation artefact.

Plate 4.8. is the shrimp stock used in Experiment 2, before any exposure to bacteria but these animals had been fed the probiotic. This was expected to be normal but the histopathology shows degeneration of central hepatopancreatic tubules (DCHT). There is no evidence of cellular inflammatory response but the structure is mostly absent with most of the tubular epithelium sloughed. This is indicative of AHPND.

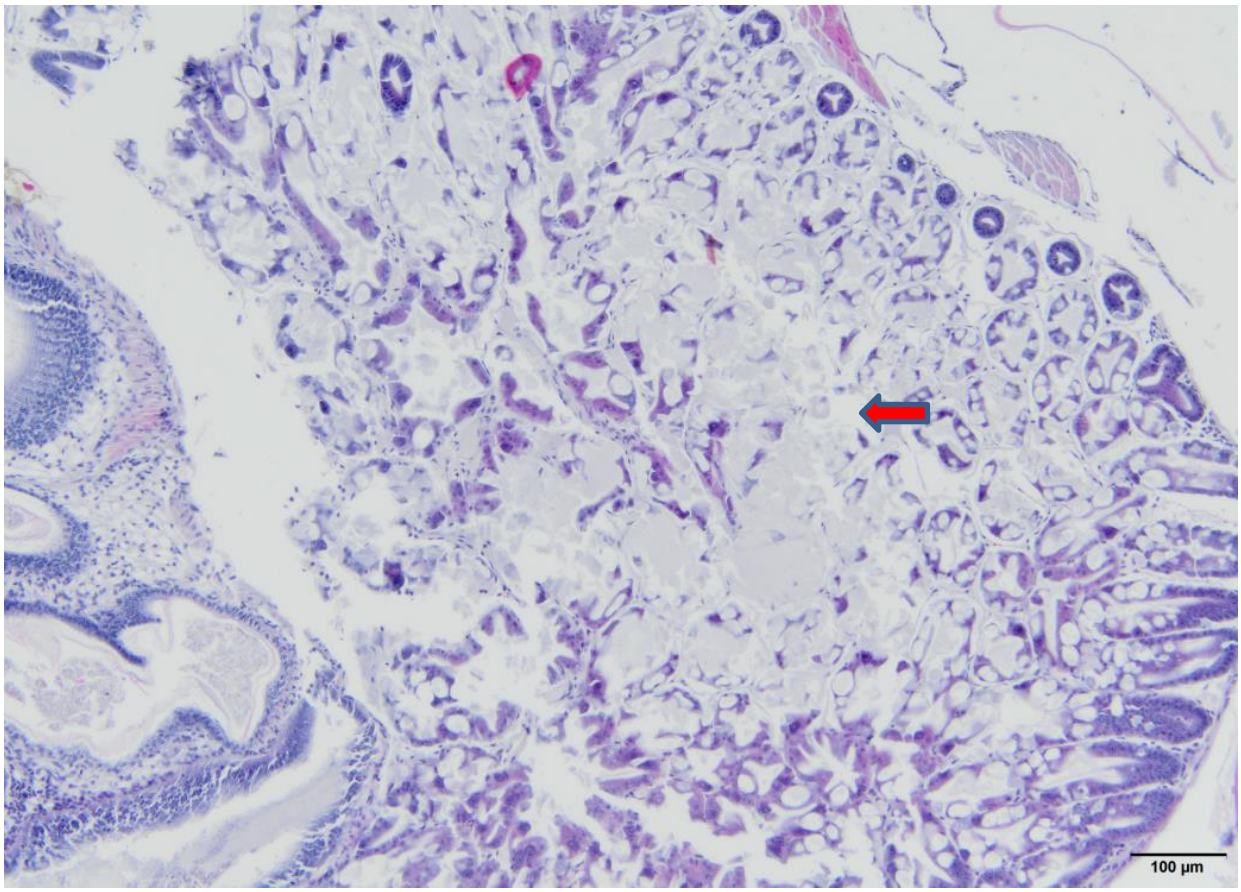


Plate 4.8. H & E sample of hepatopancrease from stock shrimp fed probiotic (BL+). The organ structure is mostly absent (arrow) and most tubular epithelium is sloughed.

Plate 4.9 shows apparently normal hepatopancrease structure but is lacking the presence of R-cells. This sample was obtained from the subsample of shrimp in Treatment group 1 (BL+/VP 10^5 cfu per ml) taken immediately 6h after bath administration of the *V. parahaemolyticus* strain VPP1. This is indicative of no AHPND.

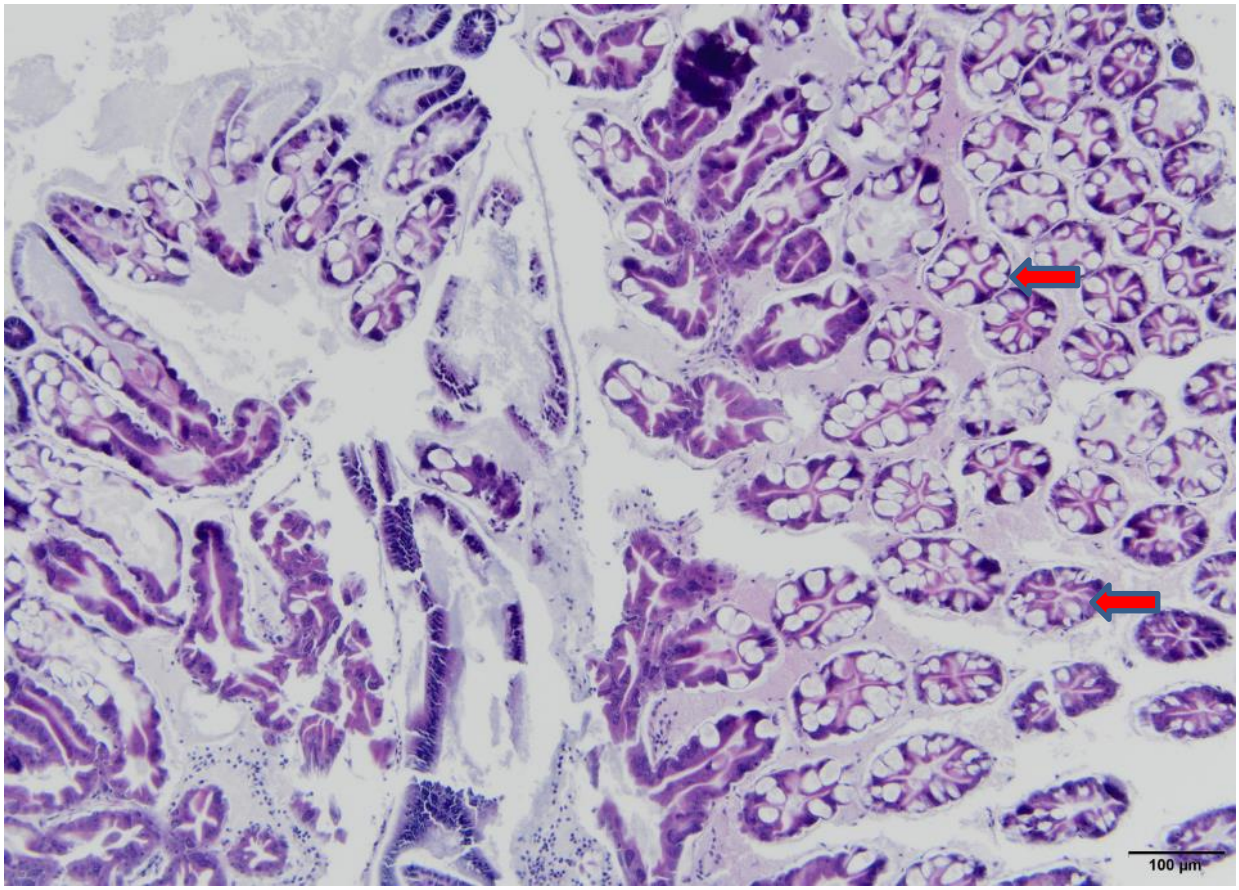


Plate 4.9. H&E stained section of apparently normal hepatopancrease from shrimp sampled in Treatment group 1 6h after exposure to *V. parahaemolyticus* Arrows show presence of normal tubule structure. Upper arrow in a tubule without R cells and the lower arrow a tubule with a small number of R cells.

The image (Plate 4.10) shows AHPND as observed by the chronic inflamed and shrunken hepatopancrease, with encapsulation and melanisation present. Bacteria can be observed in the section. This sample was taken from moribund shrimp in Treatment group 4, at 30h post exposure to the *V. parahaemolyticus* at 10^7 cfu per ml.

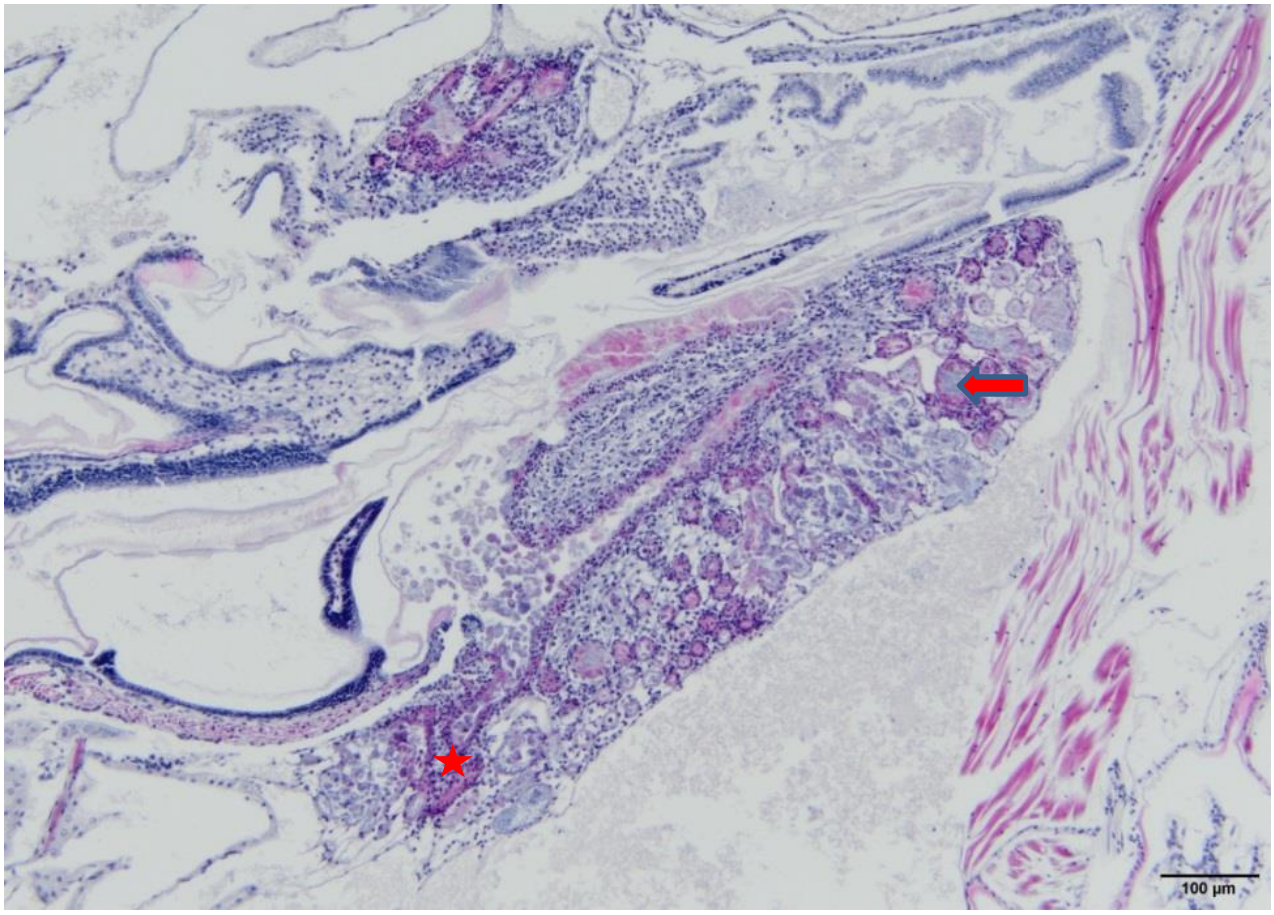


Plate 4.10. H&E stained tissue section showing AHPND-like lesion with arrow showing presence of bacteria and star showing areas of melanisation.

The image (Plate 4.11) shows more extreme chronically inflamed and shrunken hepatopancrease with encapsulation, melanisation and bacteria all visible. This sample was taken from moribund shrimp not fed the probiotic but exposed to the *V. parahaemolyticus* at 10^7 cfu and sampled 102h post-bacterial challenge.

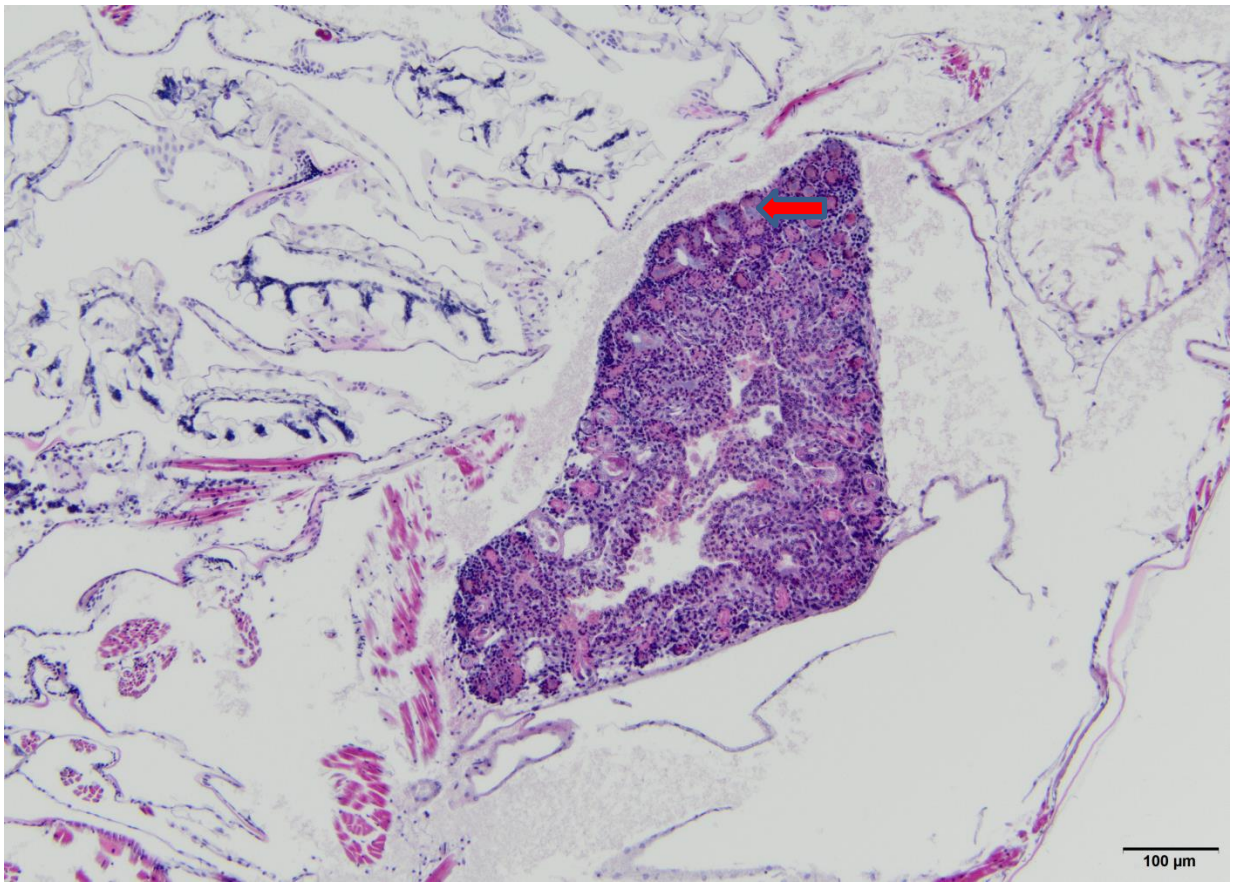


Plate 4.11 H&E stained tissue section shows more chronically inflamed and shrunken hepatopancrease with encapsulation, melanisation and bacteria with arrow showing presence of bacteria.

The image (Plate 4.12) is the same tissue of shrimp of Plate 4.11 but at higher magnification to show the presence of the bacteria (arrow) in the tissue.

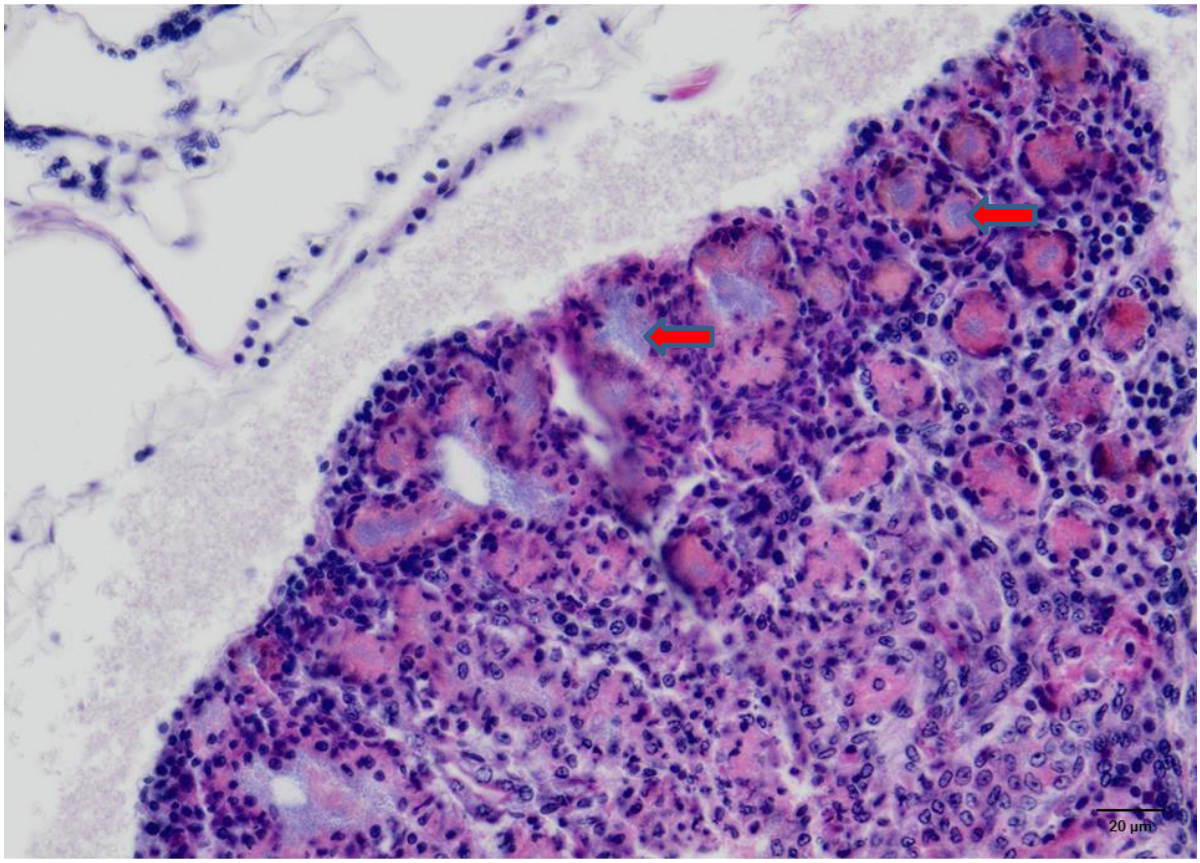
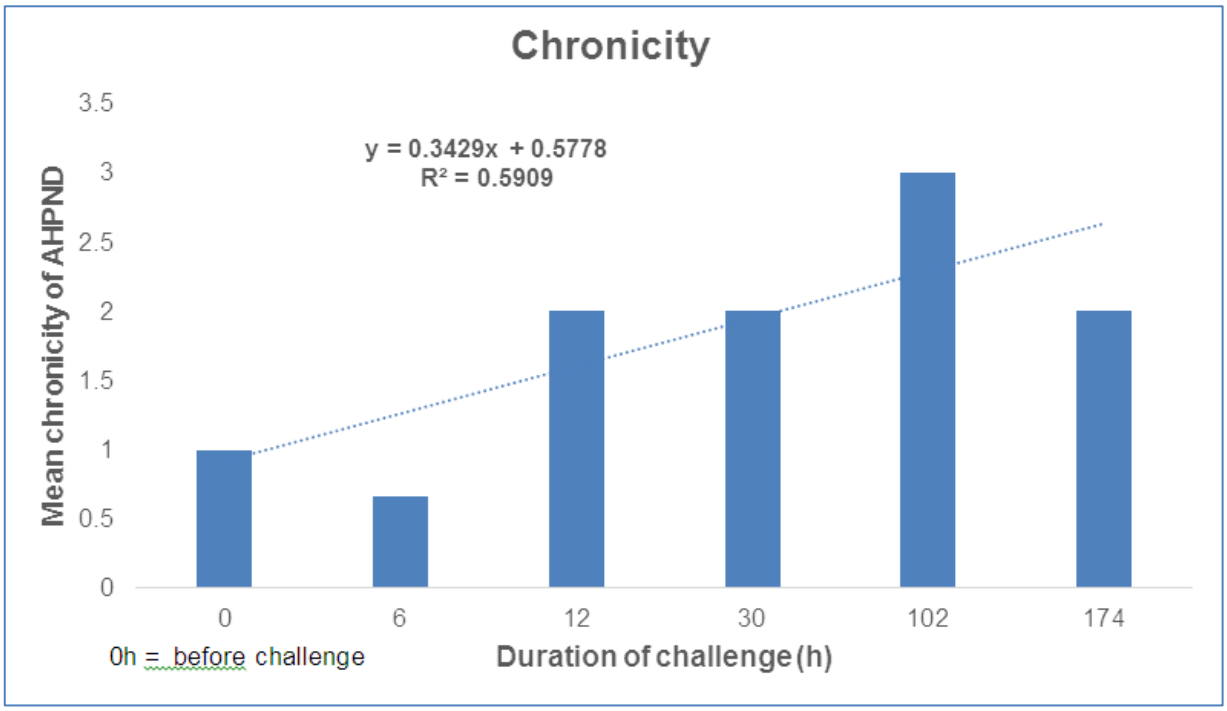


Plate 4.12. H&E stained tissue section shows more extreme chronically inflamed and shrunken hepatopancrease with encapsulation, melanisation and bacteria. Arrow showing presence of bacteria.

The impact of presence of bacteria in histopathology of moribund shrimp which was not fed the probiotic but exposed to the *V. parahaemolyticus* at 10^7 cfu/ml after 102h post-bacterial challenge maybe due to toxins.

Although a sequential pathology study was not performed in this experiment, data in Figure 4.3 shows the chronicity (how advanced the lesions were) of the AHPND-like pathology from the histopathology samples observed in Experiment 2. The chronicity of AHPND was related to the progression of the pathology from (acute – initial changes to long standing chronic changes). The cellular changes became more advanced as the study continued, suggesting a more chronic development of the disease over time which it can be seen the chronic AHPND was shown at 12h of post challenge, and the duration of 102h post-bacterial challenge a severe advanced chronic AHPND was found.



mean chronicity of AHPND; 1=AHPND ,2=chronic AHPND, 3=severe advanced chronic AHPND.

Figure 4.3. Chronicity of AHPND-like lesions observed from moribund shrimp (Experiment 2)

4.4. Discussion

The aim of this study was to investigate the effect of a bacterial challenge on shrimp after feeding them with the probiotic substance containing the Gram positive strain *B. licheniformis.* The 2 strains of *V. parahaemolyticus* that were used for the

bacterial challenge were both recovered from natural infections in shrimp and were able to cause disease as shown in the pre-challenge studies.

Establishing an infectious challenge model is problematic and in aquaculture there are few reproducible and reliable infection challenge models. This is important given the fact that suitable alternatives, e.g. mathematical models or computer simulations do not currently exist and so there is a continued reliance on the need for reproducible infection models performed *in vivo*. Currently several authors have performed infection challenge studies with AHPND-*V. parahaemolyticus* strains (Tran *et al.*, 2013 ; Vinoj *et al*., 2013 ; Li *et al*., 2008 ; Sajali *et al*., 2019) but the concentration of the bacteria and the exposure route often varies. For the initial AHPND-challenge models performed by Tran *et al*. (2013), gavage was used as the transmission route but in this study the animals were exposed to the bacteria by static bath. From the data presented in this study in the pre-challenge experiment, the route/duration of exposure and the bacterial concentration appeared to cause mortalities and AHPND-causing bacteria were detected by PCR. However, although mortalities were detected in the main challenge studies (1 & 2) described, the AHPND-like bacteria was not identified. This shows the complexity of performing infectious bacterial challenge studies and the need to have robust challenge models.

AHPND infectious studies are very difficult to do and to reproduce. In the studies described, replicate tanks per treatment group were always included. By including replicate tanks in the study design was an attempt to reduce in-tank variation in the final cumulative mortality. However, the rate of bacterial uptake will vary per shrimp and so further studies are required to identify the optimal concentration and duration of bacterial exposure using the bath challenge model.

Whilst the pre-challenge study was promising, no histopathology samples were taken in the shrimp during the pre-challenge nor in the apparently healthy stocks and this was an unfortunate oversight. Whereas, the stocks of shrimp in the experiment 2 were examined by histopathology before using in the experiment and these samples indicated AHPND in shrimp stock. The histopathology results were in contrast to the PCR results for these samples, as the PCR were all negative, suggesting that there was no AHPND present in the stocks examined.

In the BL+ shrimp stocks sampled immediately after 6h bath administration of *V. parahaemolyticus* strain VPP1, no AHPND was observed in the histopathology sample. In this case, we thought that different shrimp sample may got different histopathology results. This would support the hypothesis above meaning that not all shrimp stocks were infected with *V. parahaemolyticus* AHPND at the same rate. In this particular sample both the histopathology and the PCR were negative. In the studies, the result of histopathology was provided after the challenge experiment started, so in this study the results of Vp3 PCR had only confirmed the healthy shrimp stocks before bacterial pathogen exposed.

In addition, the histopathology results provided from the bacterial challenge study performed in Experiment 1 showed AHPND-like lesions observed in the hepatopancreas of the surviving shrimp in the end of experiment, and these groups had not been exposed to the bacterial pathogen. Again, these results were found to be PCR negative for the AHPND-PCR. It is recognised that sampling errors could have occurred during the study which may have confused the biological sample results. Given the small number of animals used and the fact that the size of the animals were so small, the shrimp samples for PCR and histopathology were not the same shrimp, even if they came from the same treatment group. It is quite acceptable that when dealing with small sized animals that the samples are taken whole and pooled. Of course the pooling of the samples may reduce the sensitivity of the results but it improves the chance of detecting a single positive response.

It could be that although animals in the same treatment group were affected, the level of infection or the stage of the disease varied between individuals and this impaired the detection of AHPND. It may also be that the level of AHPND bacterial pathogen in that sample was low so there was not enough bacterial DNA present to be detect by the AHPND-PCR.

Although technical errors in the study have not been identified, consideration must be given to the possibility that samples were wrongly labelled or identified and that perhaps during the experiment when the shrimp were in the holding tanks, the treatment groups were exposed to the bacteria from fine water from air stone supply

spreading out to neighbour tanks. There is a higher chance that unwanted bacterial exposure could have occurred in the Experiment 1 system which there were no lids covering the holding tanks. However, in experiment 2 the likelihood of this problem was reduced by covering the lids though out the holding tanks.

It is recognised that recovery of the viable AHPND-bacterium is not always possible or can be problematic and that the diagnosis of AHPND requires histopathology. However, in the stocks used in this study only PCR methods were used to confirm that the shrimp had not been previously exposed to the AHPND-*V. parahaemolyticus* and this appeared to be true. Furthermore, the source of the nauplii and the pl used in the studies were considered “apparently” healthy with no records of AHPND occurring and yet these stocks still presented with AHPND-like lesions. Therefore in future studies histopathology and PCR assays should be run together on the same stocks if possible, to ensure that there is no low level AHPND within the animals stocks. In future studies, no experimental bacterial challenge should be performed until the histopathology samples are taken to confirm the disease status of the population. Whilst this is a sensible and simple change to make, in the context of the study present it was not possible because the samples were sent to another laboratory and screened later.

Application of the PCR assays alone were performed correctly and there was no question of these results produced in the study. However, if the animal stocks have a very low level of AHPND then it may be too low for the detection level of the PCR assays used. Tinwongger *et al.*, 2014 determined that the Vp3 PCR assays was 100% accurate in the detection of AHPND *V. parahaemolyticus*, but did not provide details on the sensitivity of PCR detection. If the PCR assays are to be used as a screening tool, then further work is required to determine the sensitivity (lowest detection level of bacterial DNA) as well as the sensitivity of the PCR using a range of samples. These should include whole viable bacterial colonies as well as tissue samples from shrimp exposed to the bacteria over a range of times and concentrations. These data would significantly improve the application of PCR-only screening tools and ideally, they could be used on a larger samples size with lower numbers of samples taken for histopathology to confirm absence of AHPND.

It may also be that as the animals appeared to have low levels of AHPND-like lesions, these may not have come from *V. parahaemolyticus*. Other bacteria have been identified as causing AHPND-like lesions (Restrepo *et al*., 2018 ; Ahn *et al*., 2017 ; Liu *et al*., 2015 ; Dong *et al*., 2017) and as the bacterial toxin is located on a mobile plasmid, this can be transferred to other *Vibrio* species in the aquatic environment. Future work should include identification of the bacterial species which could be through viable bacterial recovery but also 16S r RNA PCR analysis could be performed.

Whilst further work is required to clarify the use of the PCR methods, overall, the experimental challenge studies performed in this study clearly showed that the shrimp who had been fed the probiotic and then received the pathogen had a lower mortality compared with the shrimp groups not receiving the probiotic. This is in agreement with several other authors (Rengpipat *et al*., 2000 ; Kongnum and Hongpattarakere, 2012 ; Vaseeharan and Ramasamy, 2003).

In the study presented, infectivity with different concentrations of *V. parahaemolyticus* strain VPP1 were tested and showed that the highest cumulative mortality was found in the shrimp group receiving the highest bacterial concentration. Furthermore, the shrimp that had received the probiotic had a lower number of mortalities when exposed to the *V. parahaemolyticus*. Thus, suggesting that the probiotic may have a protective effect against the infection under these conditions. The studies of Li *et al*., 2008 found *Vibrio* counts in intestine of *P. vannamei* fed with Arthrobacter XE-7 probiotic bacterium against *V. parahaemolyticus* was significantly lower than shrimp control and immune parameters e.g. percentage phagocytosis and total hemocyte counts, increased in the shrimp exposed to the probiotic. Rengpipat *et al*. (2000) who fed a probiotic bacterium (Bacillus S11) to *P. monodon* and exposed them to *V. harveyi*, reported that there was a significantly higher survival rate and enhanced immune response in the animals receiving the probiotic.

Similar results have been identified for fish species using the same probiotic species as the one applied in this study. Gobi *et al*., (2016) fed Asian catfish species *Pangasianodon hypophthalmus* the probiotic *B. licheniformis* Dahb1 and these fish

had enhanced immune and antioxidant responses as well as the growth compared with the animals not fed the probiotic. Furthermore, the probiotic fed fish showed higher resistance to disease from *V. parahaemolyticus* Dahv2 infection.

Different detection methods were applied in this study to confirm the recovery of the challenge strain (*V. parahaemolyticus*) using PCR assays as well as viable bacterial recovery. The 3 PCR assays all perform different but complimentary functions in the detection of *V. parahaemolyticus* strains. It is only PCR assay Vp3 that can detect the plasmid with the toxin which is required to cause the AHPND outbreaks in shrimp.

In the Experiment 1 study described, in terms of PCR detection of AHPND, the sample of animals in all treatment groups were negative for AHPND as detected with Vp3 PCR. However, the group of animals challenged with the AHPND-Vp bacterial strain, showed a PCR positive results detected by C4 PCR assay which detects the presence of the plasmid but not the AHPND-causing toxins. These results were unexpected and one consideration is in the pathogenicity of the *V. parahaemolyticus* challenge strain may have been altered during preparation, as the pathogenic bacteria secrete a toxin. In the experiment 1 study, only whole bacterial cells with no media were used for the challenge and if the toxin was in the media this could reduce the pathogenicity of the bacterium. However, the pre-challenge and Experiment 1 challenge results are conflicting as the bacterial challenge was prepared in the same way for both studies and yet in the pre-challenge studies a positive PCR was detected, especially from the Vp3 assay which detects the toxin. To help clarify this further, we need to look at the actual concentration of bacteria given to the shrimp in the pre-challenge and the experiment 1 study which was different at $3x10^6$ and 10^5 cfu per ml, respectively.

It may well be that the lack of the toxins in the media impaired the ability of the AHPND-bacterium to be detected in the Experiment 1 moribund shrimp but it is more likely that the higher concentration of the bacteria used in the pre-challenge study influenced the PCR detection. This needs further exploration but given that in Experiment 2 study higher mortalities and better PCR detection of the AHPND-

causing bacteria were found only in the treatment groups exposed to the higher bacterial concentration.

In Experiment 1, histopathology samples of treatment group of BL- showed all samples were infected with AHPND, whereas 2 samples of histopathology of treatment group of BL+ were no AHPND indicative. In this study it could be concluded that there is a tendency for BL to reduce risk of AHPND but not significant with these small numbers.

Experiment 2 was similar to Experiment 1, but was developed to investigate how bacterial concentration may influence any protective effect of the probiotic during experimental exposure of the pathogen. We recorded the early detection of *V. parahaemolyticus* AHPND VPP1 strain by Vp3 PCR in Treatment group 4 at the highest concentration and shrimp not fed the probiotic after 6h exposure to VPP1, whereas the other treatment had negative result all VpPCR tested. These data suggests that higher concentration of bacterial pathogens could cause more susceptible to disease, and probiotic could help to resist the *V. parahaemolyticus* AHPND.

Correlation between AHPND histopathology and PCR detection of the AHPND-*V. parahaemolyticus* strains was not simple. Several errors occurred which would have influenced this including cannibalism as in some tanks only tails were left. In Experiment 2, the moribund/dead shrimp sampled towards the end of the study period and the surviving animals showed no AHPND using PCR detection. One hypothesis may be that the bath exposure route of the AHPND-causing bacteria was not sufficient to cause disease to be established and the bacteria may have attached to the outside of the shrimp and were not ingested internally. If the bacteria were attached externally to the shrimp and these animals placed into the holding tanks then over time the uptake of the *V. parahaemolytoicus* would have increased, but perhaps not enough to cause chronic AHPND lesions. Future studies should evaluate the exposure route and uptake of the bacterium.

Attempts were made in Experiment 2 to recover the viable bacteria which was not possible in the samples taken from the shrimp exposed to the lower concentration

of *V. parahaemolyticus*. Viable bacteria was only possible from those shrimp exposed to the higher concentration and yellow coloured colony only was recovered from the shrimp fed the probiotic. Whereas recovery of green coloured colonies only occurred in the treatment group receiving the highest bacterial concentration without probiotic and this was a proxy indicator of recovery of *V. parahaemolyticus*. No further identification tests were performed and this should be included in future work. A simple step here would be to test the viable green coloured colonies for the detection of the AHPND toxin using the Vp3 PCR. However, this was not possible during this study but would help to confirm if the bacteria recovered were able to cause the disease.

Several issues occurred during the bacterial challenge studies, however, in Experiment 2 there was a clear correlation between the shrimps that were exposed to *V. parahaemolyticus* strain VPP1 at concentration of 10^7 cfu per ml and chronic AHPND at 12h post challenge. The pathology and bacterial pathogen detection varied a correlation was shown in increased chronicity of AHPND in the animals exposed to the higher bacterial concentration. Although the sample sizes in this study presented are small, a further study needs to be performed to investigate bacterial exposure times against infection stage of AHPND. In this study in some treatment the sample is not enough to inspect both in PCR assays and histopathology. A sequential pathology study would certainly help clarify the situation as we can see there is a clear trend for the chronicity (how long the pathology has been developing) to increase with the duration of the study.

Further work should also investigate isolation of bacterial species from the infected shrimp. Furthermore, sensitivity of the PCR assays should be performed under the DOF conditions and various time of exposure against shrimp infected tissues using histopathology sections should be continuously performed.

4.5. Conclusion

From this study, short-term bath challenge experiments for 6 hours with pathogenic bacteria, the results found that shrimp treated with the probiotic BL had a survival rate higher than shrimp in the control group. There is a trend for BL to be associated

with less AHPND but no significant relationship in the results of histopathological sampled. The results from this study showed that there is a tendency for BL to reduce risk of AHPND but not significant with these small numbers. In conclusion, probiotics (BL) could be beneficial to be used to reduce pathogenic bacteria (*V. parahaemolyticus*) in the shrimp hatchery as well as improving of survival rate.

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CHAPTER 5

General discussion

5.1 Principal Aim of the Study

Marine shrimp industry in Thailand has grown rapidly since 1972 (FAO Fisheries and Aquaculture Department, 2014) the main seafood export being marine shrimp (Fisheries Statistics Analysis and Research Group, 2018). However, Thai marine shrimp production declined in 2000 (Flegel, 2009) and 2012 (Flegel, 2012; Lightner *et al*., 2012). The main causes of lost production was disease outbreaks (Flegel, 2006 ; Flegel *et al*., 2008 ; Flegel, 2009 ; Flegel, 2012 ; Joshi *et al*., 2014 ; Bondad-Reantaso, 2016 ; Lightner *et al*., 2012 ; Thitamadee *et al*., 2016 ; Longyant *et al*., 2008 ; Tran *et al*., 2013).

Grow-out farmers rely on seed or post larvae (pl) supplied from the hatchery sector but the pls may be a source of disease. The good quality pl which are free from specific pathogens would be a major contribution to improved productivity in grow-out farms. Therefore, the main aim of this PhD study was to focus on the health management in hatchery sites. The aim was to combine theoretical with applied knowledge to provide realistic strategies to improve the current health management approaches within Thai hatchery systems. The information from this scientific study will be rapidly disseminated to the relevant beneficiaries by journal publication.

Health management plays an important role in all aquatic animals, however, there was a lack of reliable information about the practices in the Thai hatchery sector and the health management practices in particular. Therefore, in this study a systematic survey of the hatchery sector was performed to explore current practices and look for associations between differences in practices and productivity or health of the shrimp pl.

The survey covered most aspects of the production systems in Thai shrimp hatcheries but the focus of interest in this PhD study was the health management practices and their strengths and weaknesses.

5.2 The Thai Hatchery Survey

From a list of all the Thai shrimp hatcheries a representative sample of 78 marine shrimp hatcheries from 9 provinces within 3 regions areas (Central & East, Gulf of Thailand and Andaman Sea). The main findings were an association between both maintenance of optimal temperature control (30 ± 2 ^{o}C) and larger size of tanks with improved survival of the pl. These findings were further supported by the experimental results in chapter 3.

The finding relating to temperature control was not surprising since the Thai shrimp hatchery farmers were aware that optimal temperature should be controlled. This information we got from the interviews, however some hatcheries could not control the temperature for a variety of reasons e.g. budget, location etc. The survey showed there was apparently lower survival in hatcheries who could not control the temperature. This agrees with the findings of other studies (Hennig and Andreatta, 1998 ; Wyban *et al*., 1995 ; Kumlu *et al*., 2000 ; Staples and Heales, 1991 ; Villarreal and Hernandez-Llamas, 2005). While both size of tank and temperature were significantly associated with improved survival it may also be that it is easier to maintain a constant temperature in larger tanks. This information could help the farmers who intend to set up the new hatcheries and/or the existing hatcheries who would like to improve their survival of pl. The findings of the study will be discussed with the DOF and farmers to investigate further the cost benefit of potential changes

in tank size and temperature control to develop practical strategies and advice for farmers.

In addition, from the survey results approximately 70% of Thai shrimp hatcheries used probiotics despite the lack of objective evidence for the efficacy of the probiotics. Therefore, further investigation of the efficacy of probiotics was also included in the study.

The data from survey were explored further in chapter 3 and the results supported the conclusions from survey chapter (2). Low temperature in small rearing tanks were found to be unsuitable for shrimp larval rearing. Furthermore, given both the global concern and impact of climate change, the need for temperature control may increase as the climate becomes more unpredictable. Thailand has seen increasing temperatures and changes in rainfall pattern over the last 30 year (United Nation Development Programme, 2019) with an increasingly unpredictable climate the need for controlling optimal temperature could become more important.

In chapter 2, both of the best multivariable models identified that control of temperature and larger tanks size were associated with better survival. While, probiotic use was only found to be significantly associated with improved survival in the univariable analysis. However, in chapter 3 there was better survival of the pl when given probiotic compared with pl not fed probiotic in the large scale study performed in the 7 tonne concrete tanks with a consistent optimal controlled temperature (30±1 ^{o}C). Therefore, the results from chapter 2 and 3 combined would suggest that the probiotic tested may have a beneficial effect but only when tank size and temperature are also appropriate. The larvae did not develop into pl with or without probiotic at low temperature (25 ^{o}C) in the small container condition. However, in large scale experiment using the 7 tonne tanks, with controlled temperature there was a positive effect.

The survey found that in broodstock hatchery, using detergent to clean the broodstock tanks had a significant association with better hatching rates compared with cleaning the tanks with just water. It may be that those using detergent were more careful with their stocks or paid more attention to their stock. Therefore further

work should be conducted to explore how the cleaning material is associated with the better hatching rate. This may lead to useful advice for Broodstock site management.

The survey result also concluded that there was an association of poorer survival or more mortality events when several treatments and prophylactics were used. While this finding might warrant further investigation, the most likely explanation is that treatments or prophylactics were used on sites that had disease problems and therefore the use of the chemical was an effect of disease outbreaks rather than a cause. There are alternative explanations such as the inappropriate use of chemical directly harming the shrimp. This might, for example, be use of an incorrect dose or repeated treatments leading to cumulative problems. It is also possible that there might be some previously unknown side effects or interactions between chemicals.

There was also an association between the presence of *Zoothamnium* spp. or unidentified bacteria and increased number of mortality events but not poorer survival. The result was unclear. Therefore, future work might include some form of monitoring the background levels of bacteria in these systems and correlating these data with mortality events and see what the *Zoothamnium* spp. and unidentified bacteria mortalities could cause or effect the hatchery system.

While the findings of the survey need further investigation, as is the case with all observational studies, they have the potential to lead to practical recommendations for Thai shrimp hatchery farmers.

5.3 The Probiotic Associated with Improving Survival of the pl Shrimp

In the Department of Fisheries (DOF), Thailand a probiotic product is available for use in the hatchery and on the grow out farms. This DOF product is supplied both in powder or liquid form which can contain a maximum of 3 Bacillus species:
B. subtilis, *B. megaterium* and *B. licheniformis.* This product is not sold but provided free of charge under the name Pormor1 (P.M.1), the allocation is limited by the area of the farm.

The relationship between the use of probiotics in the survey and in the experimental studies in mentioned above. The probiotic tested may only have a beneficial effect when tank size and temperature are also appropriate. It was not possible to obtain comprehensive data on the use of probiotics but it appeared that the probiotics which they used included commercial probiotics not just the DOF product. Many of interviewees said that they would recommend the use of probiotics in the hatchery but there was a lack of information regarding how they work. The experiment in chapter 3 tried to combine the information from the survey and theoretical knowledge to identify realistic strategies to improve the current health management approaches within Thai hatchery systems. The result obtained from chapter 3 showed use of *B. licheniformis* probiotic at concentration of 10^6 cfu per ml supplemented to live feed and fed to the shrimp larvae could improve the survival rate and reduced the presence of *V. parahaemolyticus* in the pl. The concentration used was similar to that of other published studies (Sahandi *et al.* ,2012; Jamali *et al.*, 2015) and the result was in general agreement of many studies (Jamali *et al*., 2015 ; Raida *et al*., 2003 ; Vendrell *et a.l*, 2008; Nimrat, 2011; Nimrat, 2012 ; Rengpipat *et al.*, 1998). In addition, in the chapter 4 it was demonstrated that probiotic could also reduce mortality of shrimp when they were exposed to *V. parahaemolyticus* pathogenic strains J41 and VPP1. Both of these strains were recovered from shrimp naturally infected with AHPND and so were considered pathogenic. However, histopathology results were not conclusive but suggested an association between use of probiotics with less *V. parahaemolyticus* AHPND. The problems with AHPND pathology in the stock population and lack of agreement between PCR and histopathology make it difficult to draw any clear conclusions.

There was no significant effect of the probiotic on the rate of development of the larvae, however these are not easy data to analyze statically and there was a trend for the probiotic treated larvae to achieve developmental stages earlier. In addition, the health check of the probiotic treated pl were also higher but not significantly. These trends may suggest some benefit from the probiotic during the post larval stages. However, these trends were not significant and would not justify the promotion of the probiotic to farmers without further evidence and quantification of the costs and benefits of use.

In the grow out studies in earthen ponds there was no apparent benefit from the probiotics in either the shrimp grown in net cages or in concrete tanks net cages or the. Neither was there any apparent negative impact.

5.4 Probiotic Against the Bacterial Pathogen *V. parahaemolyticus* and AHPND

The bacterial pathogens used in this part of the study was considered to be significant problems in Thailand, both being associated with clinical outbreaks of AHPND. The data from Songkhla Aquatic Animal Health Research Center, Department of Fisheries; Thailand (2019) reported that in February 2017, AHPND was the biggest cause of sick or dead shrimp throughout the Thai shrimp farming regions.

There were some methodological issues in chapter 4. Different methods were used to prepare the bacteria in the 2 experiments. Preparation with washing and centrifugation would have removed any extracellular product and without centrifugation would have left these in the challenge inoculum. Therefore, the capacity to compare between the two experiments is limited.

The shrimp were exposed to the bacterium by bath, which is the only possible route with such small animals but is perhaps not representative of the natural route of infection via ingestion. Also the bath method may have led to external contamination and confusion between infected and contaminated shrimp.

The concentration of the challenge dose was also increased in the second experiment due to the lack of mortalities in the first. The second bacterial concentration was more effective in producing mortalities and would form a better base line for future studies.

Due to lack of resources, the presence of green colonies on TCBS agar was used as a proxy indicator for the presence of *V. parahaemolyticus.* In future it would be preferable to confirm this with PCR, to ensure the challenge bacteria were recovered from the shrimp. Given the ubiquitous nature of *V. parahaemolyticus* in the marine shrimp systems, it is important that any future studies look at methods to mark the

challenge bacterial strain so that mortalities or morbidities occurring during the challenge period can be confirmed to come from the actual challenge strain and not from other sources, e.g. shrimp microflora or environment.

Given that AHPND diagnosis relies on histopathology interpretation, more rapid methods of screening the shrimp populations are required. This has led to the development of PCR assays to detect the presence of the toxin-mediated plasmids which is only present on the *V. parahaemolyticus* strains causing AHPND. The method used was adapted from Tinwongger *et al*. (2014) and is widely used in the DOF health screening procedures. This was also found to be effective in the pre-challenge shrimp (chapter 4) but was not replicated in the moribund/dead samples during the larger experimental trials (chapter 4). When producing this assay, Tinwongger *et al*. (2014) provided evidence of high detection specificity but not sensitivity. Therefore, it may be prudent for any future work that the sensitivity of the Vp3 PCR assay is confirmed. Dangtip *et al*. (2015) tested an updated nested PCR method called AP4 to detect presence of AHPND *V. parahaemolyticus* strains and found their new method was 100 times more sensitive than the one step AP3 PCR method. They suggested that the AP4 method may be more useful in detecting AHPND causing *V. parahaemolyticus* strains in samples with limited material. It was not possible in this study to test these hypothesis but the results from the studies performed would support the need for a robust and sensitive tool to detect the AHPND causing bacteria.

In the chapter 4 studies, a sequential pathology study of *V. parahaemolyticus* AHPND should be examined as this would help identify how the infection stage of AHPND observed in the histopathology sections have been developed with duration of study.

5.5 The Main Conclusions of the Study and Recommendation

1. Temperature control within larger tanks had higher survival in larval rearing section.
2. Probiotic supplementation in live feed can significantly improve survival and lower pathogen load.

3. The administration of probiotic, BL strain into shrimp larvae could improve the survival of animal and there is a trend of probiotic had relationship to lower *V. parahaemolyticus* AHPND.

4. The benefits of probiotic (BL) used could be recommend to reduce *V. parahaemolyticus* pathogenic bacteria in the shrimp hatchery. To some extent, the probiotic is an alternative source in supporting animal health in the shrimp hatchery and reduce the application of antibiotics that have been banned and more restrict used in shrimp aquaculture.

5.6 Future Perspective Research Work

In this study (chapter 3) a single probiotic (BL) was administered whereas others have used combinations of probiotic strains (Zhang *et al*., 2014). Future research should be carried on investigation of microbiome in shrimp gut to see how community of probiotic and other bacteria are. Tzuc *et al*. (2014) pointed that *Pseudoalteromonas* and *Vibrio* genera were found in the digestive tract microbial community of shrimp. Luis-Villasenor *et al*. (2013) also reported that shrimp after receiving the mix of three Bacillus strains probiotics, their gut microbiota was significantly changed. Cornejo-Granados *et al*, (2017) pointed that shrimp do not have specific immune response. Its digestive track is an open system which the bacterial colonization occurs from the surrounding water and the microbiome function reflect the microbial sources found from their surrounding environmental and can be influenced by physiological responses of the shrimp, as well as feed intake including probiotics, antibiotics, developmental stage etc. It would appear from these data that feeding the larvae with the probiotic would be beneficial in improving survival rates at the hatchery. However, the mode-of-action was not investigated during this study, so it is unclear what mechanisms is causing the positive effect. Therefore the further work also would look at the mechanisms of interaction could be and how the probiotics might be influencing the immune response or microbiomes of the shrimp and how this relates to a more healthy or robust animal**.**

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APPENDICES

Appendix I Current shrimp health management strategies within Thai hatchery

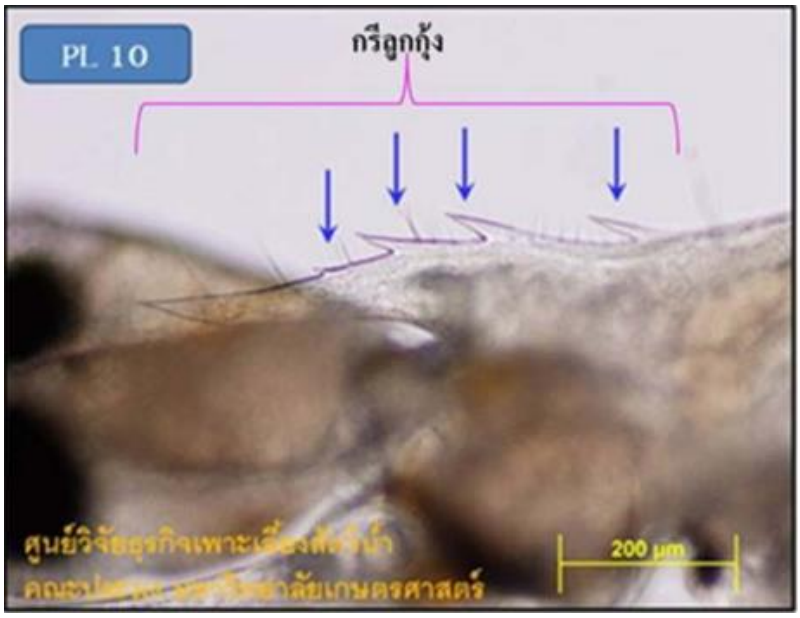
Item	Type of Test	Purpose or Description of Test	Reason for Assay	References
Broodstock	Specific Pathogen Free (SPF) breeding programme	- Broodstock are sampled and screened for the detection of potential pathogen through PCR assays (e.g. WSSV, TSV, YHV, and IHHNV) applied to broodstock reared under any biosecurity.	To check that the broodstock are specific pathogen free	http://www.shrimpaqua.com/index.php/component/content/article/2-demo1/158-manual-control-and-reduce-the-risk-of-disease-ems-in-shrimp
Post larvae (pl) for whiteleg shrimp	≥ pl 10	Observe a rostrum with at least 3 spine development	If animals are ≥ pl 10, gill is completely developed to ensure balanced osmoregulation	Marine Shrimp Culture Research and Development Institute, Coastal Aquaculture Research and Development Bureau, Department of Fisheries Thailand (2014)

	check for physical deformity in appendages and body	Observe gill and body - Clean and no ectoparasite - Complete appendages		
	Colour and condition of hepatopancrease	Visually observe (naked eye) and observe under microscope - Hepatopancrease, colour, size as well as physical characteristic should be completely developed. - Lipid cell in hepatopancrease should be fulled of nutrition. - Amount of lipid cell in hepatopancrease should be high.	Hepatopancrease is one of the index that can be used to identify the quality of pl	
	Muscle gut ratio; (MGR)	Observe ratio between size of muscle and intestine in the 6th	If the width of muscle is > 4, it means that pl get high feedrate and its strong.	

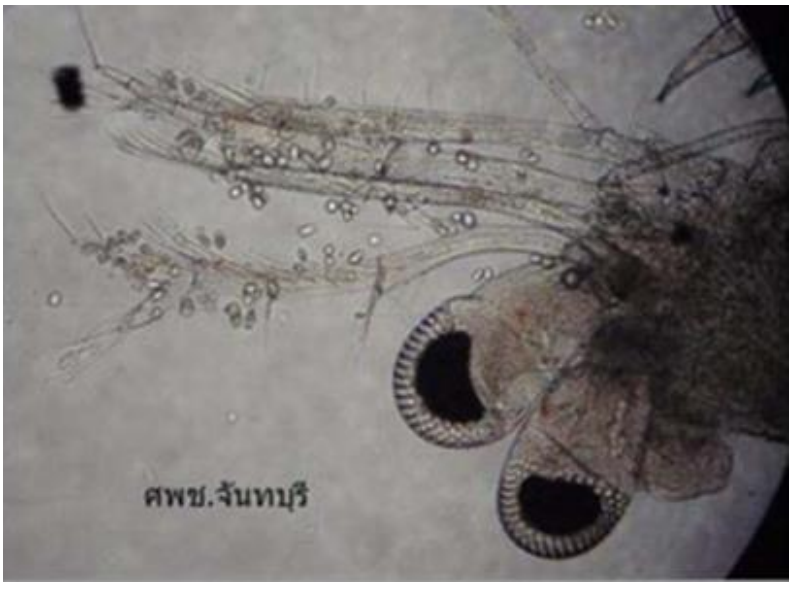
		body appendages. The ratio should be more than 4:1		
	Stress test	**2 methods:** 1) Salinity stress test using freshwater: randomly select sample of 50 individual animals and place into 5 L of freshwater (0 ppt) and leave them for 30 mins with aeration. Check for mortalities. 2) Chemical stress test using formalin As above place 50 individual animals into 100 ppm formalin and leave them for 30 mins with aeration. Check for mortalities.	To confirm the robustness of the pl which is a proxy indicator for quality of pl	

	Pathogen Detection (White Spot Syndrome Disease:WSSD, Taura Syndrome Virus:TSV, Yellow Head Virus :YHV, Infectious Hydrodermal and Haematopoietic Necrosis Virus :IHHNV, Infectious Myonecrosis Virus:IMNV)	Molecular diagnostic methods using PCR to screen viral diseases	To prevent disease outbreak and transmission of viral diseases	Coastal Fisheries Research and Development regional Centre 3 (SuratThani), Department of Fisheries Thailand (Naparat, Pers. Comm., 2014)
	Total bacteria count and total vibrio including including specific PCR test for detection of *Vibrio parahaemolyticus,* C4, Vp3	Subsamples of the animals are removed from the stock and processed for viable colony counts (total) and selective (Vibrio). Further work is performed using a specific PCR assay to detect the presence of the toxin genes from *V. parahaemolyticus*	To prevent disease outbreak and transmission of bacterial diseases	

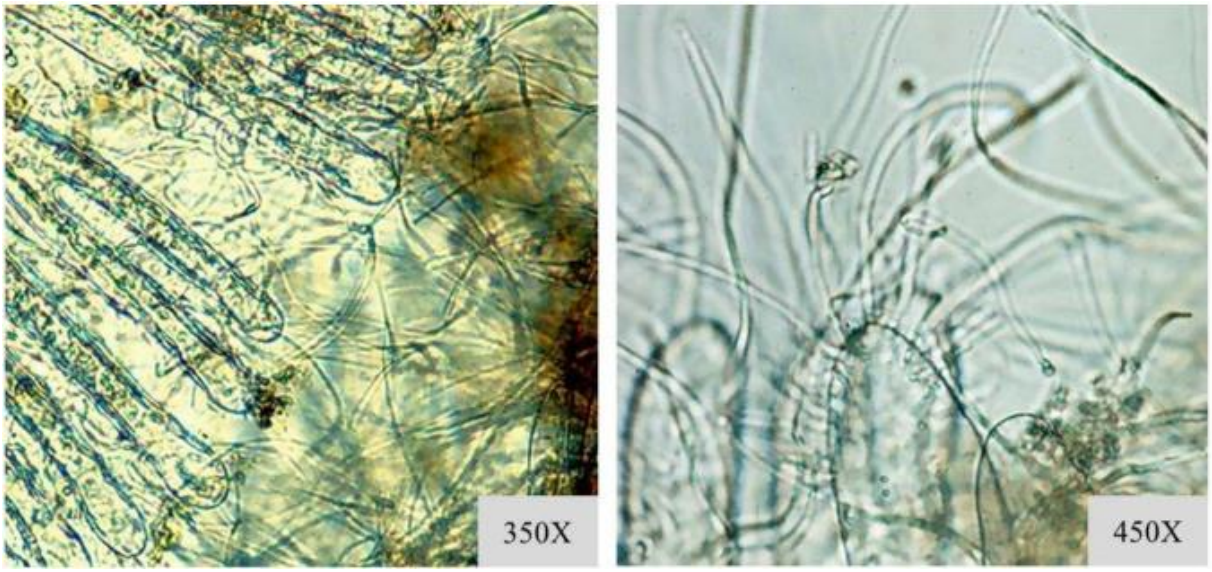
Appendix II The pl health check criteria of DOF, Thailand



Rostrum with 3 spines of pl10 [Source : Adapted from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from Faculty of Fisheries, Kasetsart University)]



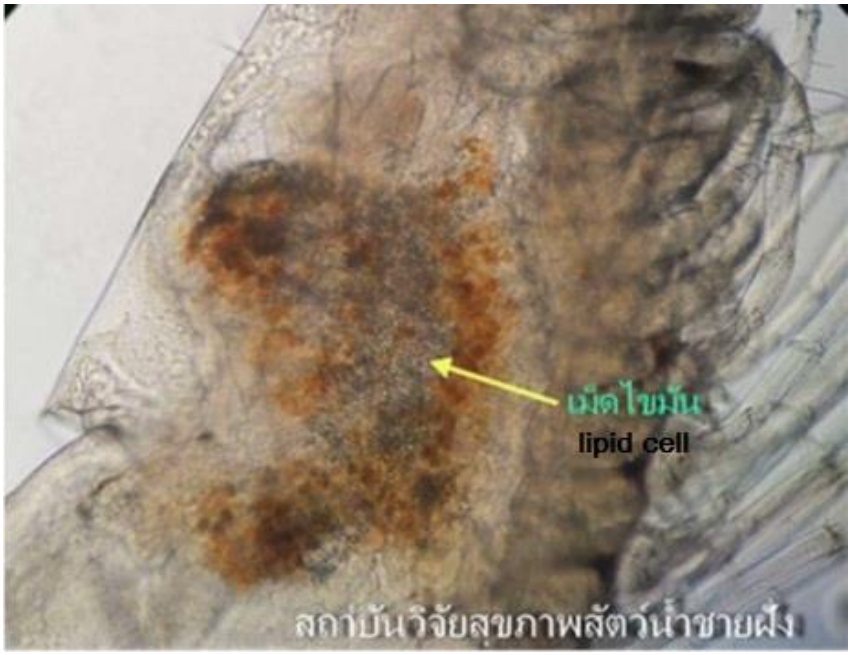
Ectoparasites attached body and appendages of shrimp larvae [Source : Adapted from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from Chantaburi Coastal Aquaculture Research and Development Centre, Department of Fisheries; Thailand)]



Gill observation with parasites attached [Source : Recreated from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from A handbook of pathology and diagnotic procedures for disease of Penaeid shrimp)]

Abnormal swimming leg appendages observation [Source : Adapted from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from Chantaburi Coastal Aquaculture Research and Development Centre, Department of Fisheries; Thailand)]

Healthy hepatopancrease observation [Source : adapted from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from Songkhla Coastal Aquatic Animal Health Research Institute, Department of Fisheries; Thailand)



Healthy lipid cell observation [Source : reproduced from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from Songkhla Coastal Aquatic Animal Health Research Institute, Department of Fisheries; Thailand)

Ratio between muscle of the 6th appendages and gut (Muscle gut ratio, MGR) observation [Source : Adapted from Marine Shrimp Culture Research and Development Institute, 2014 (Original source from Rayong Coastal Aquaculture Research and Development Centre, Department of Fisheries; Thailand)]

Appendix III Standards and certification schemes for Thai shrimp hatcheries

No.	Standards/Certification schemes	Description of the test	Reason	References
1	Standard of aquatic animal quarantine detention facilities for the importation of live aquatic animals for aquaculture	- DOF officer visit and inspect the detention place - Focus on hygiene and biosecurity	- The main purpose of this area is to quarantine live aquatic animal from importation in order to observe clinical sign and disease analysis to control disease outbreak	http://www.shrimpaqua.com/index.php/component/content/article/5-demo5/139-standard-marine-detention-or-accommodation-aquatic-animals
2	Certificate of white leg shrimp (*Penaeus vannamei*) hatchery	- DOF officer audit the hatchery - Judgement criteria	The aim of white leg shrimp hatchery	http://www.shrimpaqua.com (Coastal Fisheries Research and Development Bureau, 2014)

		5 standard requirements must be all complied. 1. Water filtration system, water treatment and reservoir must be provided. 2. Waste water treatment and filtation system before discharging to natural must be done. 3. Hatchery facilities, walk way, sewers as well as the equipment must be cleaned. 4. Hygiene area for preventing pathogen like it might come with the workers.e.g. foot bath with disinfectant to clean	inspection is to certify the standard of white leg shrimp (*Penaeus vannamei*) hatchery certificate.	

		the shoe before entering and out must be required. 5. Record keeping such as health, patients died including food, drugs and chemicals		
3	Good Aquaculture Practice (GAP), Department of Fisheries for shrimp hatchery	- DOF officer audit the hatchery. - Judgement criteria for 7 items 1. Hatchery site and registration 2. General management 3. Input factor such as use of drugs, chemicals, probiotics as well as feed 4. Health management 5. Farm sanitation	- To guide as the first step to fulfil the hatchery operations in order to produce good quality of Post larvae - To certify hatchery system in order to get GAP Department of Fisheries standard	- Booklet of Aquaculture of Development and Certification Centre, Department of Fisheries, Thailand - Department of Fisheries. B.E. 2548 (2005). Good Aquaculture Practices (GAP) for Marine Shrimp Hatchery.

		6. Harvesting and transportation 7. Record keeping e.g. water preparation, growth, survival rate, feed and feeding rate, water quality, health, drug and chemical used ect.		
4	Good Aquaculture Practice (GAP), Thai Agriculture StandardTAS 7422-2010 for marine shrimp hatchery and nursery	- DOF officer audit the hatchery. - Judgement criteria of 11 items must be all complied. 1. Hatchery site and registration 2. Broodstock management 3. General management 4. Use of veterinary drugs, chemicals, hazardous substances and probiotics	- To be as a guide to fulfil the operations along the supply chain to be recognized by both domestic and international consumers in order to produce good quality of Post larvae by	http://www.acfs.go.th/standard/system_standards.php?pageid=8 National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives Published in the Royal Gazette Vol.127 Section 147D Special, Dated 21 December B.E. 2553 (2010)

		5. Effluent management 6. Energy and fuel 7. Farm sanitation 8. Harvest, collecting and post-harvest handlings prior to distribution 9. Labour and welfare 10. Social and environmental responsibilities 11. Record keeping e.g. broodstock, pond preparation, preventive measures to control disease outbreak, veterinary drugs and chemicals, hazardous substances, employment and wage.	concerning food safety, environmental and social responsibility. - To certify hatchery system in order to get GAP TAS 7422-2010 standard	
5.	Code of Conduct (CoC) Department of Fisheries	- DOF officer audit the hatchery.	- To guide as fulfilment of the	- Department of Fisheries. B.E. 2546 (2003). Code of Conduct (CoC) for Responsible Marine Shrimp Hatchery.

	for shrimp hatchery and nursery	- Judgement criteria of 10 items must be all complied. 1. Hatchery site and reg istration 2. General management 3. Broodstock management 4. Feed and feeding 5. Health management 6. Use of drugs and chemicals 7. Effluent management and rubbish 8. Social responsibilities 9. Group and training 10. Record keeping system e.g. site selection, broodstock, culture management, feed and feeding, drugs and chemicals, health, market ect.	hatchery operations in order to produce good quality of Post larvae - To certify hatchery system in order to get CoC Department of Fisheries standard	

Appendix IV List of marine shrimp hatchery survey in 9 provinces

Province	Total (unit)	Broodstock (unit)	Broodstock+nursery (unit)	nursery (unit)	species	locations
Chachoengsao	239	4	33	202	black tiger 14, white leg 221, macrobrachium 4	Central
Chonburi	134	7	37	90	black tiger 53, white leg 80, macrobrachium 1	East
Nakhon Pathom	36	3	20	13	white leg 36	Central
Nakhon Si Thammarat	54	8	34	12	black tiger 14, white leg 39, green tiger 1	the Gulf of Thailand
Songkhla	108	14	57	37	black tiger 15, white leg 93	the Gulf of Thailand
Prachuap Khiri Khun	21	3	4	14	black tiger 2, white leg 19	the Gulf of Thailand
Phang Nga	32	3	23	6	black tiger 11, white leg 21	the Andaman sea
Phuket	94	10	45	39	black tiger 21, white leg 73	the Andaman sea
Satun	17	0	11	6	black tiger 5, white leg 12	the Andaman sea
total	**735**	**52**	**264**	**419**		

Note : Information from DOF Thailand in 2014 (pers.comm., 2014)

Appendix V Questionnaire

(insert code)

Broodstock	B+N	Nursery

Questionnaire for broodstock hatchery

Investigation of shrimp larvae quality in Thai hatchery

Please tick (/) all that apply in the right hand side boxes or fill details

1. Name of hatchery	1 ……	
2. Name of owner	2 ……	
3. Location of the hatchery	3 ……	
(address/province+GPS if possible)	……	
	……	
	……	
	……	
	……	
4. Date of interview	4 ……..	
5. Species of shrimp	5	
	1. *Penaeus monodon* (Black tiger prawn)	
	2. *Penaeus vannamei* (White leg shrimp)	
	3. *Penaeus merguiensis* (Banana shrimp)	
	4. Others (specify scientific name if possible)	
	……..	
A. Background of person interviewed		
1A. Responsibility	1A	
	1. Owner	
	2. Manager	
	3. Worker	
	4. Family member	
2A. Person interviewed name	2A ……..	
3A. Age	3A ……years old	
4A. Sex	4A	
	1. Male	
	2. Female	
5A. Address	5A……	
	……	
	Tel. ……	
6A. Have you attended any training or	6A	

seminar course recently (time 1-2 years)?	1. Yes 2. No	
If yes, please give details including who provided the training DOF, company ect.	- …… - …… - …… - ……	
7A. Education	7A 1. Illiterate 2. Primary School 3. Secondary School 4. High School 5. Undergraduate 6. Post graduate 7. Others	
B. Hatchery profile		
1B. Has your hatchery got the official certification?	1B 1. Yes 2. No	
If yes, which certificate?	1. Good Aquaculture Practice (GAP), DOF 2. Good Aquaculture Practice (GAP), TAS 7422-2010 3. Code of Conduct (CoC), DOF 4. Others(specify) ……	
2B. Is the certificate still viable?	2B 1. Yes 2. No	
If yes, what is expire date?	……	
3B. Total area of your hatchery	3B……Rai	
4B Total of broodstock tanks	4B……..tanks	
How many m^3 of each tank /number	……..... m^3.....tank ……..... m^3.....tank ……..... m^3.....tank ……..... m^3.....tank ……..... m^3.....tank	
5B How many broodstock do you have?	5B.....inds.	
6B Where do you get the broodstock from?	6B 1. Wild 2. Which country ……..	
7B What about domestic /Thai sources?	7B……. ……..	

8B Others	8B…… ……..	
9B How often do you obtain the broodstock?	9B 1. Once 2. Monthly 3. Others ……	
10B How many times do broodstock spawn?	10B .....time	
11B Do your broodstock get a SPF certification?	11B 1. Yes 2. No	
12B Methods of broodstock transportation to your hatchery	12B 1. Transfer by yourself 2. Transfer by seller	
13B Packaging of broodstock	13B 1. Plastic bag with aeration 2.Tank with aeration 3. Others……..	
14B How long do your broodstock take in transferring period?	14B From shortest …….to longest…….hrs.	
15B Stocking density in broodstock tank	15B…….ind. /m^2	
16B Do you grow the Post larvae to be brooder in your hatchery?	16B 1. Yes 2. No	
17B For female broodstock, Do spawners get ready eggs to spawn before capture or do they have to mate with males after arrival to hatchery?	17B 1. Get ready from wild capture 2. Have to mate them	
18B. Do you select brood stock to improve quality?	18B 1. Yes 2. No	
If so what are you trying to improve?	1. Selective breeding programme 2. Domestication 3. Other…….	
19B. How many years since you started your hatchery?	19B.....years	
20B. What is the source of water supply for your hatchery?	20B 1. Sea 2. Transfer sea water from other province 3. Others……..	

21B. How many time do you produce annually?	21B ……times	
22B. How long does it take per time?	22B ……days/time	
23B. What is egg hatching rate of broodstock?	23B	
March - June	1……%	
July - October	2……%	
November - February	3……%	
C. Husbandry, feed and water management		
1C. Do you use probiotic in your hatchery?	1C	
	1. Yes	
	2. No	
2C. Do you use chemical in your hatchery?	2C	
	1. Yes	
	2. No	
3C. What probiotic is used for prevention?	3C	
4C. What chemical is used for prevention?	4C	
5C. When is it used for preventing?	5C	
	1. Initial	
	2. During broodstock fattening period	
	3. Others……..	
6C. What treatment is used for each disease?	6C	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
7C. How do you know when to start using treatment?	7C	
	1. Whenever broodstock suffer morbidity	
	2. When observing the broodstock die	
	(specify, how many % of dead shrimp).….%,	
	Over how many days?.....days	
	3. Others…….	
8C. Do you clean or treat the tanks with chemical before and after using them?	8C	
	1. Yes	
	2. No	
If yes, what kind of chemical is used?	.....	
9C. What type of feed is used for broodstock?	9C	
	1. Fresh feed (specify) ……	
	2. Artificial diets	

	3. Both fresh and diets
	4. Others…….
10C. How often do you feed the broodstock?	10C
	1. Once daily
	2. Twice daily
	3. Others……..
11C. What feeding rate do you provide?	11C .....% of BW
12C. How often do you check the water quality?	12C
	1. Daily
	2. Twice a week
	3. Weekly
	4. Once a crop
	5. Others…….
13C. Do you use probiotic or chemical after changing the water?	13C
	1. Yes
	2. No
If yes, what probiotic or chemical do you use	……
14C. Have you recorded your work details?	14C
	1. Yes
	2. No
D. Disease problems and Health management	
1D. Can you describe your health management regime?	1D ……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
2D. Do you know if broodstock get disease?	2D
	1. Yes
	2. No
3D. How can you recognise the disease?	3D .....
	.....
	.....

4D. Does your hatchery ever suffer any high mortality problem?	4D 1. Yes 2. No	
If yes, how often have you had the problem?	.....	
5D. Who do you contact to confirm mortality problem?	5D 1. Solve problems by yourself 2. University 3. DOF officer 4. Others……	
6D. What disease or abnormality is found?	6D	
7D. Do your inspect your Nauplii before selling?	7D 1. Yes 2. No	
If yes, what methods do you use to inspect them?	1. Visual observe 2. Observe under microscope 3. PCR test 4. Microbiology lab 5. Others…….	
8D. How often is your Nauplii sample detect the problem?	8D 1. Every spawning time 2. once/2-3 spawning time 3. once/4-5 spawning time 4. once/6 or more spawning time 5. Never	
9D. Do you get some Nauplii problem comments from your customers?	9D 1. Yes 2. No	
If yes, what is the comment	…… …… …… …… ……	
10D. How does your hatchery deal with it for the customer?	10D 1. Refund money 2. Give the Nauplii instead next selling 3. Others…….	
E. Hygiene and Biosecurity		
1E. What methods are applied for hygiene protection before entering your hatchery?	1E …….. ……	

	……	
	……	
	……	
2E. Do the government officers audit sanitation	2E	
in your hatchery?	1. Yes	
	2. No	
If yes, how often do they visit your hatchery?	1. Weekly	
	2. Twice a month	
	3. Monthly	
	4. Others……..	
3E. How often do you clean your whole hatchery?	3E …….....	
4E. How do you clean?	4E.....	
	.....	
	.....	
F. Market		
1F. How much the price of Nauplii do you sell?	1F .....Baht/million Nauplii	
2F. Product purpose	2F	
	1. Sell to nursery section	
	2. Nauplii still continue nursing in your own hatchery.	
	3. Restocking	
	4. Others	

Questionnaire for nursery hatchery

(insert code)

Broodstock	B+N	Nursery

Questionnaire for nursery hatchery

Investigation of shrimp larvae quality in Thai hatchery

Please tick (/) all that apply in the right hand side boxes or fill details

1. Name of hatchery	1 ……	
2. Name of owner	2 ……	
3. Location of the hatchery	3 ……	
(address/province+GPS if possible)	……	
	……	
	……	

	……
	……
4. Date of interview	4 …….
5. Species of shrimp	5
	1. *Penaeus monodon* (Black tiger prawn)
	2. *Penaeus vannamei* (White leg shrimp)
	3. *Penaeus merguiensis* (Banana shrimp)
	4. Others (specify scientific name if possible)
	……..
A. Background of person interviewed	
1A. Responsibility	1A
	1. Owner
	2. Manager
	3. Worker
	4. Family member
2A. Person interviewed name	2A …….
3A. Age	3A ……years old
4A. Sex	4A
	1. Male
	2. Female
5A. Address	5A……
	……
	Tel. ……
6A. Have you attended any training or seminar course recently (time 1-2 years)?	6A
	1. Yes
	2. No
If yes, please give details including who provided the training DOF, company ect.	- ……
	- ……
	- ……
	- ……
7A. Education	7A
	8. Illiterate
	9. Primary School
	10. Secondary School
	11. High School
	12. Undergraduate
	13. Post graduate
	14. Others
B. Hatchery profile	
1B. Has your hatchery got the official certification?	1B
	1. Yes
	2. No

If yes, which certificate?	1. Good Aquaculture Practice (GAP), DOF	
	2. Good Aquaculture Practice (GAP), TAS 7422-2010	
	3. Code of Conduct (CoC), DOF	
	4. Others(specify) ……	
2B. Is the certificate still viable?	2B	
	1. Yes	
	2. No	
If yes, what is expire date?	……	
3B. Total area of your hatchery	3B…….Rai	
4B Total of nursery tanks	4B……..tanks	
How many m^3 of each tank /number	……..... m^3.....tank	
	……..... m^3.....tank	
	……..... m^3.....tank	
	……..... m^3.....tank	
	……..... m^3.....tank	
5B Where do you get the Nauplii from?	5B	
	1. Your own hatchery	
	2. Other hatchery(specify) ……	
6B Do your inspect your Nauplii before nursing?	6B	
	1. Yes	
	2. No	
If yes, what methods do you use to inspect them?	1. Visual observe	
	2. Observe under microscope	
	3. PCR test	
	4. Microbiology lab	
	5. Others…….	
7B Stocking density in nursery tank	7B…….ind. /m^3	
8B. How many years since you started your hatchery?	8B.....years	
9B. What is the source of water supply for your hatchery?	9B	
	1. Sea	
	2. Transfer sea water from other province	
	3. Others……..	
10B. How many crops do you produce annually?	10B ……crops	
11B. How long does it take per crop?	11B ……days/crop	
12B. What is survival rate from Nauplii to PL?	12B	

March - June	1……%	
July - October	2……%	
November - February	3……%	
C. Husbandry, feed and water management		
1C. Do you use probiotic in your hatchery?	1C	
	1. Yes	
	2. No	
2C. Do you use chemical in your hatchery?	2C	
	1. Yes	
	2. No	
3C. What probiotic is used for prevention?	3C	
4C. What chemical is used for prevention?	4C	
5C. When is it used for preventing?	5C	
	1. Initial	
	2. During nursing period	
	3. Others……..	
6C. What treatment is used for each disease?	6C	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
	- disease……..	
	treatment used……	
7C. How do you know when to start using treatment?	7C	
	1. Whenever larvae suffer morbidity	
	2. When observing the larvae die	
	(specify, how many % of dead shrimp)…..%,	
	Over how many days?.....days	
	3. Others…….	
8C. Do you clean or treat the tanks with chemical before and after using them?	8C	
	1. Yes	
	2. No	
If yes, what kind of chemical is used?	.....	
9C. What type of feed is used during nursery period? (can tick (/) more than 1)	9C	
	1. Live feed (specify)	
	1.1 Phytoplankton (specify species)	

	…….	
	1.2 *Artemia nauplii*	
	2. Egg custard	
	3. Artificial diets	
	specify brand, company…….....	
	protein level of the diets……%	
	4. Others feed ……..	
10C. How many time do you feed the larvae daily?	10C	
	1. Once	
	2.Twice	
	3. Four	
	4. Six	
	5. Others……	
11C. What is feeding rate of the larvae?	11C .....% of……..	
12C. What factors affect the growth rate?	12C	
13C. Do you monitor your water quality during nursing time?	13C	
	1. Yes	
	2. No	
If yes, which parameters do you measure?	1. Salinity	
	2. pH	
	3. Alkalinity	
	4. Temperature	
	5. Dissolved oxygen	
	6. Ammonia	
	7. Nitrite	
	8. Nitrate	
	9. Others ……..……	
14C. How often do you check the water quality?	14C	
	1. Daily	
	2. Twice a week	
	3. Weekly	
	4. Once a crop	
	5. Others…….	
15C. Do you exchange water during nursing period?	15C	
	1. Yes	
	2. No	
If yes, what is the exchange rate/how often?	……%……..time	
16C. Do you use probiotic or chemical after changing the water?	16C	
	1. Yes	

	2. No
If yes, what probiotic or chemical do you use	……
17C. Have you recorded your work details?	17C
	1. Yes
	2. No
D. Disease problems and Health management	
1D. Can you describe your health management regime?	1D ……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
	……
2D. Do you know if your larvae get disease?	2D
	1. Yes
	2. No
3D. How can you recognise the disease?	3D .....
	.....
	.....
4D. Does your hatchery ever suffer any high mortality problem?	4D
	1. Yes
	2. No
If yes, how often have you had the problem?	.....
5D. Who do you contact to confirm mortality problem?	5D
	1. Solve problems by yourself
	2. University
	3. DOF officer
	4. Others……
6D. What disease or abnormality is found?	6D .....
	.....
	.....
	.....
7D. Do you test the health status of your	

PL sample before selling?	7D	
	1. Yes	
	2. No	
8D. What tests are used?	8D	
	1. PCR test	
	2. Bacteria analysis	
	3. Others……	
9D. If you test, how often is your sample tested?	9D	
	1. Every crop	
	2. 1 time/2-3 crops	
	3. 1 time/4-5 crops	
	4. 1 time/6 or more crop	
10D. How often is your sample detect the problem?	10D	
	1. Every crop	
	2. 1 time/2-3 crops	
	3. 1 time/4-5 crops	
	4. 1 time/6 or more crop	
	5. Never	
11D. Do you do stress test your PL before selling?	11D	
	1. Yes	
	2. No	
If yes, how quality of PL?	1. Passed	
	2. Fail	
12D. Do you get some EMS issue comments From your customers?	12D	
	1. Yes	
	2. No	
If yes, what is the comment	……	
	……	
	……	
	……	
	……	
	……	
13D. How does your hatchery deal with it for the customer?	13D	
	1. Refund money	
	2. Give the PL instead next selling	
	3. Others…….	
E. Hygiene and Biosecurity		
1E. What methods are applied for hygiene protection before entering your hatchery?	1E ……..	

	……	
	……	
	……	
	……	
2E. Do the government officers audit sanitation in your hatchery?	2E	
	1. Yes	
	2. No	
If yes, how often do they visit your hatchery?	1. Weekly	
	2. Once per crop	
	3. Monthly	
	4. Others……..	
3E. How often do you clean your whole hatchery?	3E …….....	
4E. How do you clean?	4E.....	
	.....	
	.....	
F. Market		
1F. How much the price of each PL do you sell?	1F .....Baht/ind.	
2F. Market purpose	2F	
	1. Sell to on-growing farm	
	2. Restocking	
	3. Others……..	
3F. Which PL do you sell to farmer?	3F	
	1. Less than PL10	
	2. PL10 up	
	3. Others	