UNIVERSITY of STIRLING

USE OF NON-ABLATED SHRIMP (*Litopenaeus vannamei*) IN COMMERCIAL SCALE HATCHERIES

THESIS TO BE SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

By

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DECLARATION

This thesis has been composed in it’s entirely by the candidate. The work described in this thesis has been conducted independently and has not been submitted for any other degree.

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To my ever supportive father ZACARIAS SIMÃO SITHOLE and in memory of my beloved mother MARIA FRANCISCO and grandmother SARA PENICERA MACHAVA.

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Published Articles and Conferences

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- **Zacarias, S**., Fegan, D., Wagsoontorn, S., Yamuen, N., Limakom, T., Carboni, S., Davie, A., Metselaar, M., Little, D.C. Shinn, A.P., 2020. Increased robustness of post-larvae and juveniles from non-ablated Pacific whiteleg shrimp, *Penaeus vannamei*, broodstock post-challenged with pathogenic isolates of *Vibrio parahaemolyticus* (VpAHPND) and white spot disease (WSD)

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- **Zacarias, S**., Carboni, S., Davie, A., Little, D.C., 2018. Reproductive performance and offspring quality on non-ablated Pacific white shrimp (*L. vannamei*) under commercial scale conditions. 5th Institute of Aquaculture PhD Research Conference, University of Stirling, Stirling, UK, 17 April.
- **Zacarias, S**., Carboni, S., Davie, A., Little, D.C., 2017. Comparison of reproductive performance and offspring (larvae and juveniles) quality of eyestalk ablated and eyestalk intact Pacific white shrimp (*L. vannamei*) under commercial conditions. European Aquaculture Society Conference, Dubrovnik, Croatia, 17-20 October.

CONFERENCE ORAL PRESENTATIONS

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- **Zacarias, S**., Carboni, S., Davie, A., Little, D.C., 2019. Potential route to replace unilateral eyestalk ablation of shrimp (*L. vannamei*) in commercial hatcheries. 6th Institute of Aquaculture PhD Research Conference, University of Stirling, Stirling, UK, 24-25 October.

Abstract

The Pacific white shrimp (*Litopenaeus vannamei*), is currently the most cultured marine shrimp worldwide with 78% of global shrimp production in 2019 and it represents an important marine food source for consumers. Induction of rapid egg production in captivity of this shrimp is usually induced by unilateral eyestalk ablation which involves the removal or constriction of one eyestalk through cutting, cauterizing or tying to reduce the level of hormone that inhibit reproduction of shrimp in captivity. However, due to physiological imbalance and stress caused by this practice, it has attracted attention as an animal welfare issue. As a result the need of using eyestalk intact animals in hatcheries has become a priority for producers, retailers and seafood certifiers. The main goal of this thesis was to provide a body of evidence on potential of using non-ablated female in commercial shrimp hatcheries to support on-going dialogue around the continued use of unilateral eyestalk ablation in modern *L. vannamei* breeding practices.

First the feasibility of using shrimp non-ablated female (NAF) in modern egg production practices was evaluated and the quality of their offspring was assessed from early development stage to marketable size shrimp. NAF produced significantly more eggs and nauplii than ablated female (AF) ($p < 0.05$). However their level of productivity (eggs and nauplii per tank/day) was significantly lower than AF ($p < 0.05$). In addition, their offspring had similar growth and final survival to those from AF from early development stage to marketable size shrimp ($p > 0.05$). However, salinity stress tests indicated that postlarvae from NAF have significantly better resilience ($p < 0.05$). The second study assessed the effect of fresh-frozen natural feed (squid and polychaete) at pre-maturation stage on NAF productivity, their biochemical composition and offspring quality. Fresh-frozen natural feed did not improve NAF productivity as it was significantly lower to AF ($p < 0.05$). In addition, this practice did not affect their offspring quality (biochemical content, growth performance and survival) ($p > 0.05$). However, their fertilization rate increased and the hepatopancreas had significantly higher levels of total lipid, total saturated and monounsaturated fatty acids, linoleic acid (LA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA) when compared to other females ($p < 0.05$). After this, based on higher survival observed on postlarvae from NAF after salinity stress tests (SST), it was proposed to verify if the possible resilience of postlarvae produced from NAF broodstock would be expressed following a disease challenge, including at juvenile stage. There was a

significant higher survival of postlarvae from NAF when challenged with *Vibrio parahaemolyticus* ($p < 0.05$). No significant difference was observed between the juveniles from NAF and AF when challenged with white spot syndrome virus (WSSV) ($p > 0.05$). At last, there was evaluated the impact of shifting from AF to NAF *L. vannamei* based maturation facilities system on production costs and profitability. This change increased nauplii production cost however this may not be reflected at larviculture level. The profitability modeling showed that if the farmers switch to source animals produced on NAF maturation facilities based system, they would reduce the level of financial losses caused by acute hepatopancreatic necrosis disease (AHPND) and WSSV. In summary, this thesis provides a new scientific knowledge and relevant evidence to support the phase out of unilateral eyestalk ablation in shrimp hatcheries and working toward improvement of NAF productivity.

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List of Abbreviations

% – Percentage

µg – Microgram

µm – Micrometer

5-HT – Serotonin

A – Ablation

AF – Ablated females

AG – Androgenic gland

AHPND – Acute hepatopancreatic necrosis disease

ANOVA – Analyses of variance

ARA – Arachidonic acid

BFT – biofloc technology

BIOMAR – Biocultivos Mariños

bn – Billion

$CaCO_3$ – Calcium Carbonate

CFU – Colony-forming units

CHH – Crustacen hyperglycemic hormone

CNS – Central nervous system

Crz – Corazonin

DA – Dopamine

DF – Dry feed

DF&NF – Dry feed and natural feed

DHA – Docosahexaenoic acid

Dmc – Meiosis related gene

DNA – Deoxyribonucleic acid

dpi – Days post-infection

dw – Dry weight

EHP – *Enterocytozoon hepatopenaei*

EMS – Early mortality syndrome

EPA – Eicosapentaenoic acid

F – Feed

FAME – Fatty Acid Methyl Esters

FAO – Food and Agriculture Organization

FAs – Essential fatty acids

FCR – Feed conversion rate

FVGAL – Fish Vet Group Asia Limited

g – Gram

GIH – Gonad inhibiting hormone

GIH-dsRNA – anti-GIH double stranded

GnRH – gonadotropin releasing hormone

GSI – Gonodassomatic index

h – Hour

Ha – Hectare

HSI – Hepatosomatic index

HUFA – Highlyunsaturated fatty acids

IHHNV – Haematopoietic necrosis virus

IMNV – Infectious myonecrosis virus

Kg – Kilogram

L – Liter

LA – Linoleic Acid

LARVIPAC – Larvicultura del Pacífico

lGnRH-III – lamprey gonadotropin releasing hormone

LI – Larviculture I

LII – Larviculture II

LIII – Larviculture III

LIV – Larviculture IV

LSI – Larval stage index

m – Meter

M.I – Maturation index

mAb – Monoclonal antibody

MF – Methyl farnesoate

mg – Milligram

MIH – Molt-inhibiting hormone

ml – Millilitre

mm – Milimeter

mRNA – Messenger RNA

n-3 – Omega-3 Fatty Acid (s)

n-6 – Omega-6 Fatty Acid (s)

NaCl – Sodium chloride

NAF – Non-ablated females

NAF^- – Offspring from ablated females without specific disease

NAF^+ – Offspring from non-ablated females with specific disease

ns – Not significant

OA – Octopamine

OctGnRH – Octopus gonadotropin releasing hormone

OD – Optical density

OIE – Office International des Epizooties

PCR – Polymerase Chain Reaction

PGs – Prostaglandins

pH – Potential of hydrogen

PL – Postlarvae

PMC – Pre-maturation conditioning

ppt – Parts per thousand

PUFA – Polyunsaturated fatty acids

qPCR – Quantitative PCR

RNA – Ribonucleic acid

RNAi – RNA interference

SE – Standard error

SGR – Specific growth rate

SPF – Specific pathogen free

spp. – Multiple species

SPR – Specific pathogen resistant

SST – Salinity stress test

T – Time

TSB – Tryptone soya broth

UK – United Kingdom

USA – United States of America

USD – United States Dollar(s)

UV – Ultraviolet

Vg – Vitellogenin

VIH – Vitellogenesis inhibiting hormone

Vp – *Vibrio parahaemolyticus*

WSD – White spot disease

WSSV – Whiste spot syndrome virus

ww – Wet weight

YHV – Yellow head virus

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Chapter 1: General Introduction

1.1 Overview

Over the past 4 decades commercial shrimp hatcheries have been using unilateral eyestalk ablation as a method to induce rapid maturation and spawning, hence it increase eggs production and meets the commercial demand. The increasing concern of consumers, retailers and standard setters on animal health and welfare has pushed the shrimp industry to work on viable alternatives. In this introduction chapter, a short overview of the current state of shrimp production, maturation and breeding practices in captivity of *Litopenaeus vannammei*, including challenges related to the use of unilateral eyestalk ablation and possible alternatives will firstly indicate the main context of this study. Endocrinology manipulation strategies to induce shrimp to maturation and spawning, their advantages and disadvantages will be reviewed. Then, recent practices reported to improve broodstock performance of AF, including supplementation of natural feeds and use of biofloc technology in pre-maturation will be discussed and demonstrate the potential these practices would have to improve eyestalk intact animals. Finally, from the foregoing discussion, knowledge gaps being identified, research hypotheses and objectives of the present study will be presented.

1.2 Shrimp production

Shrimp culture is the production of shrimp under controlled conditions in quantities for profit (Lawrence and Johns, 1983). This practice originated in Southeast Asia where for centuries farmers raised incidental crops of wild shrimp in tidal fish ponds (Whetstone et al., 2002). The shrimp were not considered of great value, but over time shrimp culture has grown into one of the largest and most important aquaculture crops worldwide (Whetstone et al., 2002). Shrimp culture consists of different stages, including maturation/reproduction, larviculture, nursery and grow-out. The first two stages usually occur in hatcheries (Wickins and Lee, 2002). Nowadays the shrimp sector is one of the most important of rapidly growing aquaculture globally. Recent shrimp production statistics indicate that more than half of shrimp production worldwide (i.e. nearly 4.5 million tons) is now farmed (Figure 1.1) (Sacton, 2018; Anderson et al., 2019). The Pacific whiteleg shrimp, *L. vannamei*, is currently the most cultured marine shrimp representing 78% of global shrimp aquaculture production in 2019 (Figure 1.2) (Anderson et al., 2019).

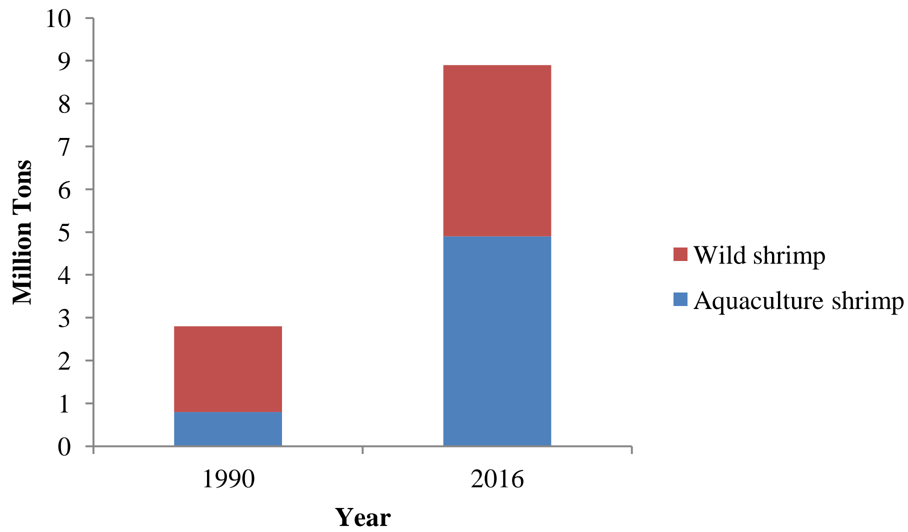

Figure 1. 1: overview of global shrimp production over 26 years. Source (adapted from Sacton, 2018)

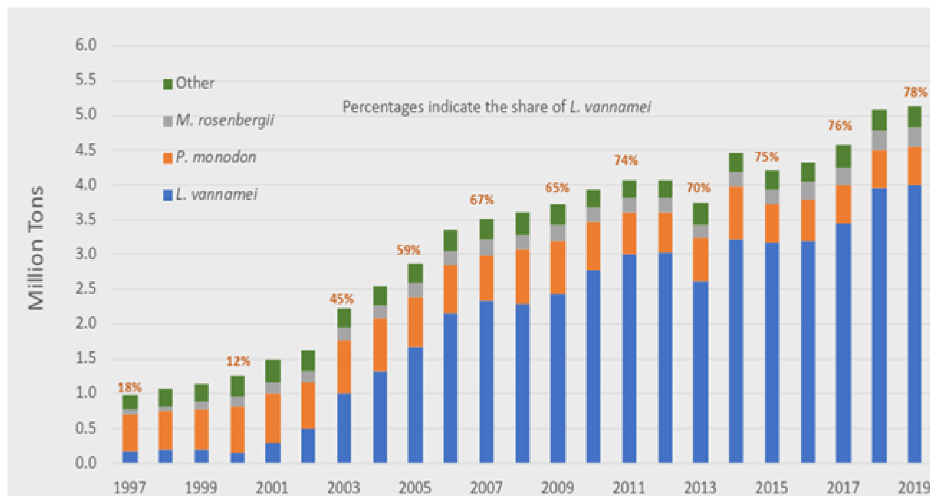

Figure 1. 2: Overview of global shrimp production by species. Source (Anderson et al., 2019)

1.3 The Pacific white shrimp (*Litopenaeus vannamei*)

The Pacific white shrimp grows fast, tolerates high stocking densities, has a relatively low dietary protein requirement, tolerates a wide range of salinities and is highly adaptable to culture conditions (Wickins and Lee, 2002), qualities that explain its global dominance. It has a similar life cycle to other Penaeid shrimp and is composed of four consecutive phases after egg hatching: larvae (nauplius, protozoea and mysis), postlarvae,

juvenile and adults. In the natural environment, *Litopenaeus vannamei* male and female adults mature and mate under typical oceanic conditions as they migrate offshore, where the female spawns at night in the water column and fertilization occurs. The eggs sink and after some hours (up to 24h) they hatch and the nauplius float to the surface. In general *Litopenaeus vannamei* spawns in zones with good currents which allows the transportation of larvae to areas with enough natural food and appropriate conditions for survival and growth, which are known as nursery areas such as estuaries, large bays and coastal lagoons. Developing shrimp remain in these areas for several months, and then begin maturing and moving offshore to complete their life cycle (Jory and Cabrera, 2003) (Figure 1.3).

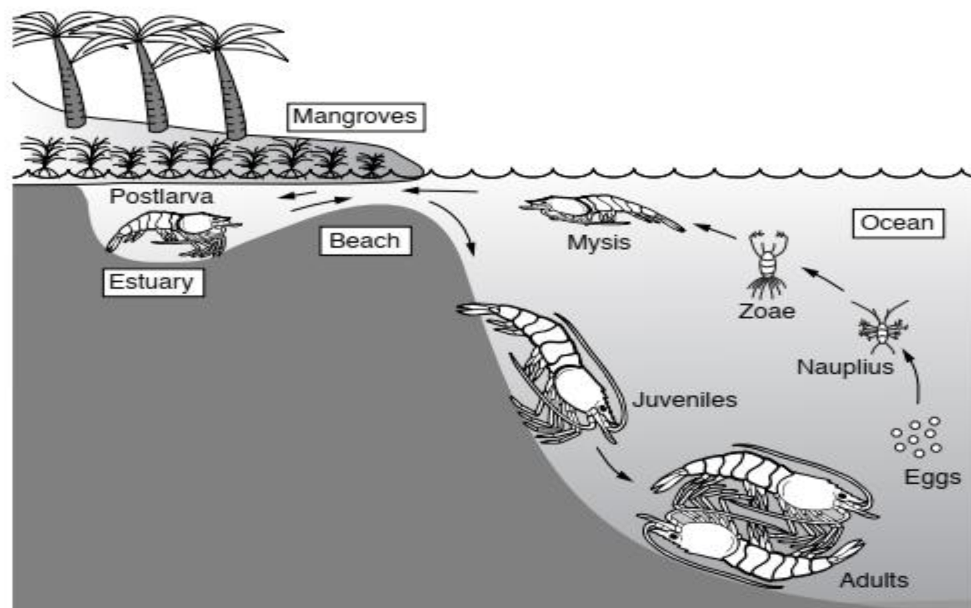

Figure 1. 3: A common lifecyle of marine shrimp in natural environment. Source: Jory and Cabrera, 2003.

The production cycle of *L. vannamei* in captivity starts with stocking domesticated broodstock in maturation tanks which are either Specific Pathogen Free (SPF) or Specific Pathogen Resistant (SPR). The common weight of individual broodstock are at least 30 g and >40 g for male and female, respectively (FAO, 2003). After the female has undergone unilateral eyestalk ablation, the broodstock remain in the maturation tanks between 3 to 5 months producing eggs (FAO, 2003). The fertilized eggs are transferred from spawning to hatching tanks and 12 to 18 h later nauplii are harvested. After that nauplii are stocked in

larviculture tanks to produce postlarvae after which they are then transferred to nursery first and then, subsequently, grow-out tanks. The nauplius stage is composed of 5 sub-stages: nauplius I, II, III, IV and V. Depending on the environmental conditions (i.e. water quality and feed), a nauplius might take 2 to 3 days to advance to the protozoea stage, which has 3 sub-stages (protozoea I, II and III) and take between 3 to 5 days to get to the mysis stage. Postlarvae begin to appear after 3 to 4 days of mysis sub-stages (mysis I, II and III) and they might take up to 35 days to become juveniles, a further 90-180 days to become sub-adults or 300 days to become mature adults capable of reproduction and continue the production cycle in aquaculture (Figure 1.4) (FAO, 2009).

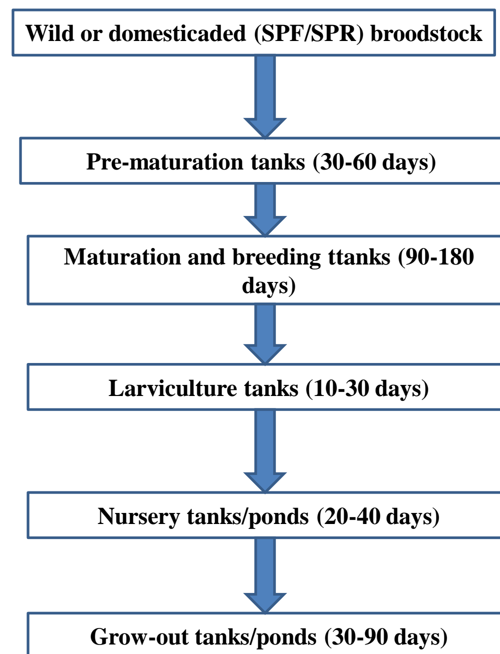

Figure 1.4: A typical lifecycle of *L. vannamei* in captivity. Source: adapted from FAO, 2009.

1.4 Endocrine regulation of reproduction

Endocrine system is a chemical messenger system comprised of glands that produce hormones which regulate biological process including growth and reproduction (Kumar and Kumari, 2018). The X-organ sinus gland complex is the principal neuroendocrine gland in crustaceans where hormones are synthesized, stored, and secreted to the hemolymph to regulate several metabolic processes (Sainz-Hernández et al., 2008) such as homeostasis, growth, development and reproduction (Primavera 1985; Fingerman,

1987). Thus, to date, significant efforts have been made just to understand the functionality of endocrine systems of these animals, mainly those with high commercial preference such as Pacific white shrimp (*L. vannamei*) (Huberman, 2000).

The major neuroendocrine control in shrimp is the X-organ-Sinus Gland Complex situated in the optic ganglia of the eyestalk (Primavera, 1985; Fingerman, 1987; Raviv et al. 2006; Nagaraju, 2011; Webster et al., 2012). Therefore reproductive processes, assimilation efficiency and oxygen consumption, blood glucose levels and molt frequency of these animals are deeply affected by hormones secreted by the sinus gland (Raviv et al. 2006; Marsden, 2008) and regulations of energy metabolism in response to stress (Shinji et al., 2012). The first general rule for endocrine control of reproduction in fish starts with environmental stimuli such as a change in temperature, salinity, photoperiod and/or diet, also known as external factors; this is applied for shrimp as well. These stimuli have an influence on the neurosecretory centres of the X-organ-Sinus Gland Complex to secret hormones (Mazurova et al., 2008; Swetha et al., 2011). In addition, the regulation of reproductive hormones is also controlled by internal factors (neuroendocrine and non-neuroendocrine secretions) (Huberman, 2000). Therefore, both factors work together to regulate reproduction in Pacific white shrimp.

A group of hormones which control part of reproductive process in Pacific white shrimp are known as CHH (Crustacean hyperglycemic hormone) family neuropeptides and are produced and secreted by the X-organ-Sinus Gland Complex (Fingerman, 1987; Raviv et al. 2006; Marsden, 2008; Shinji, et al., 2012; Treerattrakool et al., 2014; Li et al., 2015). They are composed of shrimp hyperglycemic hormone (CHH), the molt-inhibiting hormone (MIH) and the gonad inhibiting hormone (GIH), also referred as vitellogenesis inhibiting hormone (VIH) (Primavera, 1984; Nagaraju, 2011; Swetha et al., 2011; Li et al., 2015). VIH seems to have a primarily physiological role in the inhibition of ovarian development (Primavera, 1984; Fingerman, 1987; Li et al., 2015).

1.4.1 Endocrine manipulation strategies applied to Pacific white shrimp (*L. vannamei*) maturation

In natural environments, shrimp spawn with specific environmental cues stimulating ovary development and spawning by neurosecretory centres. Hence,

controlling environmental conditions is an essential maturation facility protocol. Parameters such as water temperature, salinity and dissolved oxygen; nitrogen compounds, photoperiod, light intensity, maturation room and tank characteristics, and diet may be manipulated and adjusted (Browdy, 1998; Whetstone et al., 2002; Jory and Cabrera et al., 2003; FAO, 2003). Each species has optimum ranges at which maturation will be facilitated and the value of these parameters and their rate of variation over time are critical to stimulate the reproductive process. Chamberlain and Lawrence (1981) found an effect of light intensity on maturation of Pacific white shrimp where broodstock matured and spawned more frequently under bright sun-light conditions than with moderate light intensity. Perez-Velazquez et al. (2001) observed that an adequate sperm count and percentage of abnormal sperm of *L. vannamei* broodstock are optimised at a mean water temperature of 26ºC, but not at 29ºC or 32ºC.

In general *L. vannamei* should be held for maturation under the specific conditions (Treece and Fox, 1993; FAO, 2003) that include low light (10-30% of natural or artificial light), ideally with a system to control photoperiod (maintained at about 10-12 dark and 12-14 hours light). Noise (particularly loud or intermittent noise), movement and other disturbances should be kept to a minimum level. Maturation tanks should be round, dark-colored, smooth sided, and of approximately 3-5 m diameter (FAO, 2003). The broodstock should be held with flow-through (new and/or recycled) water exchange of a total of 250–300% per day and a continuous, but not too vigorous air supply (FAO, 2003). In recirculation systems, water compensation levels of 5-10% per day are normally required. Water depth is generally around 0.5–0.7 m and the tanks are gently sloped towards a central drain to facilitate removal and siphoning of uneaten food and other undesirable debris (FAO, 2003). Shrimp stocking densities of around 6–15 shrimp/m^2, with a male to female ratio of 1:1, 1:1.5 or 1:2 are optimal. In relation to water quality parameters, temperatures are usually maintained in the range of 28–29 °C, salinity of 30–35 ppt and pH of 8.0–8.2. It is also important to make sure that the broodstock have good nutritional status (details below), appropriate size and age, and good source of the broodstock (see Racotta et al., 2003 for further details). Ceballos -Vázquez et al. (2003) found that 12-month-old males are mature and have high sperm quality that results in improved offspring production. The external factors mentioned above are considered to improve success in inducing sufficient spawning to meet commercial production schedules, but insufficient to

ensure consistent and predictable production scheduling, leading the sector to continue reliance on manipulating the endocrine system of shrimp using other methods to improve reproductive performance.

1.4.1.1 The common endocrinology manipulation technique: Eyestalk ablation

In the previous section it has been shown how important environmental conditions are to stimulate ovary development and spawning of *L. vannamei* females. Generally it is possible to meet production demand by inducing broodstock via external factors, however the female takes a long period to mature and begin reproducing, and the low level of the outcome is considered acceptable for hatcheries dedicated only to selective breeding programs and supply of broodstock for other hatcheries On the other side, where the hatcheries need to supply postlarvae for grow-out, the consistency of success obtained by environmental manipulation alone is not enough to meet commercial production demand, thus eyestalk ablation has been used to help to control internal factors regulating reproduction.

Ablation of eyestalk is broadly used in commercial hatcheries as a crude method of hormonal manipulation to induce maturation and spawning in many crustaceans (Chamberlain and Lawrence, 1981; Palacio et al., 1999a; Zhang et al., 1997; Sainz-Hernández et al., 2008; Das et al., 2015), including *L. vannamei* (Lawrence, 1983; Palacio et al., 1999a Racotta et al., 2003; Li et al., 2015). It involves the removal or constriction (through cutting, cauterizing or tying) of one (unilateral) or two (bilateral) eyestalk to reduce the level of gonad inhibiting hormone (GIH/MO-IH) which is produced by the X-organ and sinus gland complex situated in the optic ganglia of the eyestalk (Alava and Primavera, 1979; Reviva et al., 2006; Pillai et al., 2010; Bae et al., 2013; Treerattrakool et al., 2014; Das et al., 2015). Eyestalk ablation is important as ovarian maturation of Pacific white shrimp is controlled by GIH hormone and is presumed to inhibit Vitellogenesis (Treerattrakool et al., 2014; Urtgam et al., 2015), thus the eyestalk ablation can accelerate ovarian maturation in female resulting in spawning (Emmerson, 1983; Palacio et al., 1999a; Li et al., 2015) (Table 1.1) and in the male can cause an increase in sperm duct weight and hypertrophy of the androgenic gland (AG) (Li et al., 2015). As a consequence of the negative effects of bilateral ablation on shrimp females, such as high rates of mortality (Chu and Chow, 1992) only unilateral eyestalk ablation ablation is used.

1.4.1.2 Other effects of Eyestalk ablation

Ablation is still the main method used for commercial hatcheries in order to increase egg production which is directly related to improve spawning frequency (Chamberlain and Lawrence, 1981; Palacio et al., 1999a; Bae et al., 2013). Both unilateral and bilateral ablations also have other effects on females (Table 1.1), impacting on practically all aspects of shrimp physiology that are regulated by the X-organ Sinus Gland Complex. Over time, a physiological imbalance develops and female reproductive performance has been found to worsen (Palacio et al., 1999a; Das et al., 2015). Eyestalk ablation is also considered as an invasive method and cruel to the shrimp (Treerattrakool et al., 2014) as it may cause physical trauma and stress (Taylor et al., 2004; Bae et al., 2013).

Levels of hormones such as MIH and CHH are found to decline after ablation (Sainz-Hernández et al., 2008; for review see Racotta et al., 2003). Such a drop in MIH forces the female to molt more frequently demanding large amounts of energy to the detriment of reproduction that requires energy at the same time. However the energy requirement may partially be compensated by an increase in food intake and by a more efficient physiological use of it (Racotta et al. 2003). Bae et al., (2013) induced *L. vannamei* to undergo ovarian development by both unilateral and bilateral eyestalk ablation. They observed an increase level of Gonadosomatic index (GSI) and Vitellogenin (Vg mRNA) after 4 days, achieving a peak level at 10 days. However 20 days after ablation these hormones decreased and simultaneously immune related genes started being activated. On the other hand, the reduction of CHH has influence on metabolism of macronutrients, such as carbohydrates and lipid (Racotta et al., 2003; Sainz-Hernández et al., 2008). Biochemical reserves, mostly lipids, increase in ovaries due to eyestalk ablation (Racotta et al., 2003). It means the biochemical pathways of nutrients appear to be altered in response to ablation of shrimp eyestalk. Quackenbursh (1989) observed that *L. vannamei* ablated female had a quick increase in protein synthesis in ovary and hepatopancreas, and during ovarian maturation, yolk proteins were transported through the hemolymph. Ablated females of Pacific white shrimp are reported to lose weight significantly, show decreased hemocyanin levels in hemolymph and of glucose in hepatopancreas during reproduction more than unablated females (Palacio et al., 1999a). Additionally, eggs of ablated females were found to have higher levels of acyglycerides. On the other hand, due to rapid and consecutive maturation and spawning over a short

time, the reproductive performance of ablated female of *L. vannamei* is shown to deteriorate over time because of exhaustion and this is reflected in offspring quality (Palacios et al., 1999b).

Table 1.1: Summary of eyestalk ablation effect in shrimp

Eyestalk ablation impact in shrimp	
Positive	**Negative**
Reduce gonad inhibiting hormone	Physiological imbalance
Induce rapid maturation and spawn	Reproductive exhaustion
	Physical trauma
	Stress
	Reduction of shrimp hyperglicemic and molt inhibiting hormones
	High energy demand
	Activation/reduction of immune related genes
	Influence on metabolism of macronutrients
	Alteration of biochemical pathways
	Weigh loss
	Drop of hemocyanin and glucose in hepatopancreas
	High broodstock mortality
	Compromise offspring quality

1.5 Endocrine Manipulation Alternatives to Eyestalk Ablation

Due to several negative effects associated with ablation explained above and concerning the welfare of shrimp broodstock, predictable maturation and spawning of captive shrimp without the use of eyestalk ablation has been considered as a long-term goal for the industry (Quackenbush, 1991). Different lines of research have been tried to develop alternative techniques to eyestalk ablation. Most attempts have been based on understanding and manipulation of endocrine systems. Biotechnology has been the main tool for this purpose with a focus on the hormone controlling maturation. Potential molecules that may induce maturation and spawning *of L. vannmei* are peptide hormones, steroids hormones, juvenoids hormones, neurotransmitters and neurotransmitter antagonists (Figures 1.5 and 1.6) (Alfaro et al., 2004; Swetha et al., 2011).

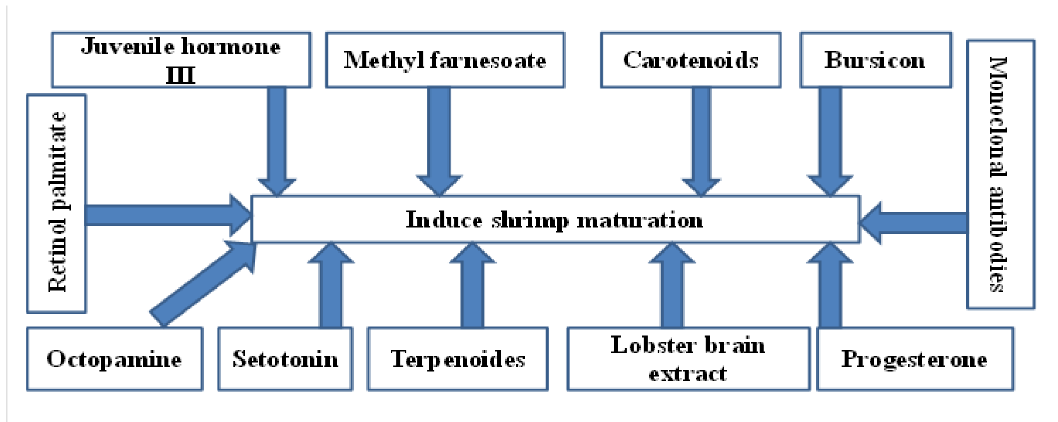

Figure 1.5: Molecules and extract that can induce maturation development of *L. vannamei.*

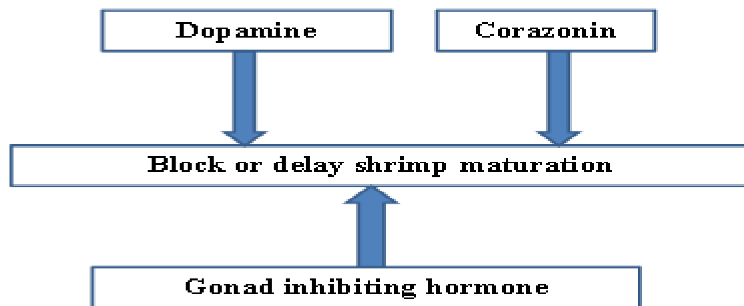

Figure 1.6: Molecules that can inhibit or slow down maturation development of *L. vannamei*.

The primary neurotransmitters that control ovarian development of shrimp are serotonin (5-HT), dopamine (DA), and octopamine (OA) (Sarojini et al., 1995; Tinikul et al., 2014). They regulate the synthesis and liberation of neurohormones from the X-organ sinus gland complex, present in various regions of the central nervous system (CNS) of shrimp, and play key roles in regulating several physiological processes, including reproduction (Tinikul et al., 2014). Serotonin (5-HT) has been reported to stimulate gonadal maturation in the Pacific white shrimp, *L. vannamei* (Vaca and Alfaro, 2000), the black tiger shrimp, *Penaeus monodon* (Wongprasert et al., 2006), the freshwater prawn, *Macrobrachium rosenbergii* (Tinikul et al., 2009; Poljaroen, 2011; Siangcham et al., 2013) and *Penaeus indicus* (Tomy et al., 2009). Dopamine (DA) was observed to have opposite

effect of serotonin in Pacific white shrimp (Tinikul et al., 2014) and fresh water prawn (Tinikul et al., 2009; Poljaroen, 2011; Siangcham et al., 2013). Similar results were obtained by Babu et al. (2013) in *P. monodon* injected with 5-HT and DA. Dopamine delayed ovarian maturation, decreased gonadosomatic indices and oocyte diameters of *L. vannamei* (Tinikul et al., 2014). In the same study, two isoforms of gonadotropin releasing hormone (GnRH), namely octopus GnRH (octGnRH) and lamprey GnRH-III (lGnRH-III), showed the opposite influence of dopamine. Alfaro et al. (2004) combined serotonin and a dopamine antagonist (spiperone) to induce ovarian maturation and spawning of *L. vannamei* and *Litopenaeus stylirostris*. The combination did induce maturation and spawning, and also stimulated the release of maturation promoting hormones into the water. However, the commercial relevance of the technique for commercial facilities remains unvalidated (Alfaro et al., 2004). Another hormone with similar function to dopamine is corazonin (Crz). Poljaroen (2011) and Siangcham et al. (2013) observed induction of ovarian maturation of this hormone on *M. rosenbergii*.

Bursicon is a hormone considered to have different roles in crustacean (Sathapondecha et al. 2015), thus Sathapondecha et al. (2015) investigated a novel function of the hormone in vitellgenin stimulation in reproductive female of *P. monodon*. They found raised Vg mRNA expression in the ovary and stimulation of ovarian development. This hormone has not been investigated in *L. vannamei*. Ovarian maturation of Pacific white shrimp has also been induced by injection of brain extract from vitellogenic female American lobster, *H. americanus* (Sathapondecha et al., 2015 for review). Tsukimura and Kamemoto (1991) tested several mandibular organ compounds and their derivatives in vitro; in four steroids they verified that only 17 cr-hydroxyprogesterone significantly increased oocyte diameter, a key indicator of ovarian maturation, in *L. vannamei*. Progesterone, 17β-estradiol and 17α-dihydroxy progesterone had no effect. Terpenoids, methyl farnesoate (MF) and juvenile hormone III, also significantly increased oocyte diameter (Tsukimura and Kamemoto, 1991). These data suggest that mandibular organ compounds and their derivatives may be potent in stimulating oocyte growth (Tsukimura and Kamemoto, 1991). Methyl farnesoate (MF) has also been proved to be involved in the control of reproduction fresh water prawn. Female and male *Macrobrachium malcomsonii* showed a significant increase in mean oocyte diameter and testicular follicle diameter as well as mean ovarian indices and testicular indices, after

injection of MF (Nagaraju et al., 2003). Alfaro et al.(2008) also reported a better quality of spermatophore of the pacific white shrimp as the sperm count increased and there was a drop in sperm abnormality by using MF. In the same study juvenile hormone III exhibited the opposite function. Carotenoids have also been studied as Liñancabelllo and Jesús (2004) showed that retinol palmitate can enhance ovarian maturation of *L. vannamei*. It is believed that carotenoids are required to obtain other active molecules like retinoids, which contribute to and play a key role in the induction of shrimp maturation.

Serotonin has shown promising results on inducing maturation and spawning, although spawning frequency was higher for eyestalk ablated *L. vannamei* females compared to serotonin treated shrimp, without differences in fecundity, nauplii per spawn and fertilization and hatching rates (Vaca and Alfaro, 2000). And the use of MF appears to be a better option because it can be given directly through the diet and results in a higher spawning frequency, and similar fertilization and hatching rates than untreated controls; when combined with eyestalk-ablation, MF administration in the diet increased hatching and fertilization rate (Racotta et al. 2003).

The influences of gonad inhibiting hormones antibodies for induction of maturation have also been investigated. For example Treerattrakool et al., (2014) found an effective approach for induction of ovarian maturation in female broodstock of *P. monodon* based on the utilization of the monoclonal antibody (anti-GIH mAb) against activity of Pem-GIH, and suggest using combination of this antibody with GIH-dsRNA, as both can interrupt totally the GIH activity. On another hand specific silencing of GIH gene by RNA interference (RNAi) has been used to induce maturation of shrimp females. Treerattrakool et al. (2011) tried to induce *P. monodon* females to mature by injecting anti-GIH double stranded (ds) RNA and Das et al. (2015) by long hairpin RNA to silence GIH expression; but neither study had satisfactory results on inducing maturation. Feijo et al. (2015) demonstrated the potential for RNA interference (RNAi) technology for silencing hormonal transcripts of pond-reared *L. vannamei* in order to stimulate gonadal development. GIH transcript levels in the eyestalks of females injected with dsRNA-GIH were reduced by 64, 73, and 71 % at the 15th, 30th, and 37th dpi (days post-injection), respectively. However, more studies are needed to conclude the effectiveness of this technique. Genes involved in gametogenesis of Pacific whiteleg shrimp are being cloned

and characterized to further understand their roles. Okutsu et al. (2010) did clone and characterize a meiosis related gene (*Dmc1*) and found it to be a potential important indicator of the early stages of germ cell development.

The effort to find alternative hormonal treatments to induce maturation and spawning in penaeid shrimp, mainly for Pacific white shrimp is extensive, suggesting that the level of concern within the shrimp industry about the use of eyestalk ablation on shrimp and sustainable production of shrimp aquaculture is significant. Almost all compounds tested in previous studies still need more investigation before application at a commercial scale. Additionally, new potential hormones with the same role should be further investigated in order to find hormones with best impact on maturation and spawning. It appears that development of gonadal inhibiting hormones antibodies and RNA interference technology to silence GIH expression can be another solution for eyestalk ablation, but more studies are needed to achieve reliable results. However, it is important to highlight some points which need more attention to verify the applicability of these kinds of studies. Firstly, the methods used to administrate the compounds. Almost all studies use injection which on a commercial level is impractical when compared to artificial fish reproduction. Fish morphology, broodstock age and size allow hormones and other products to be easily injected compared to shrimp. In general the number of individual shrimp broodstock required is much higher than fish, so labour costs would be uneconomic unless efficacy and cost effectiveness of the approach are addressed. Reproductive performance and larval quality of females induced by hormones, glands extract, gonadal inhibiting hormones antibodies and RNA interference technology injections have not been evaluated in the most studies mentioned above. Furthermore, the application of injections repeatedly can result in stress of shrimp and other hormone related effect when compared to eyestalk ablation (Racotta et al., 2003).

Therefore, further investigations are needed to find solutions which have promise to improve welfare practices and, specifically obviate the need for unilateral eyestalk ablation in the commercial *L. vannamei* shrimp industry. The commercial shrimp maturation facility sector itself particularly if producing for an organic market that disallows use of unilateral eyestalk ablation, has taken on the challenge to use husbandry-based approaches (Little et al., 2018). For example, Seajoy Seafood, a company which has large shrimp

farming operation in Honduras and Nicaragua, in collaboration with University of Stirling, gathered preliminary evidence indicating the potential for removing unilateral eyestalk ablation from its system with good commercial results, initially to supply organic standard PLs. There are also some regular maturation facilities in Central and Latin America (Brazil, Colombia, Ecuador and México), and Asia (Thailand) which are no longer using eyestalk ablation (Patrick Sorgeloos, 2020. *Pers. Comm*; Robsons McIntosh, 2020. *Pers. Comm.*). These findings and reports do not include injections of molecules, used in most previous studies. It seems that there are some external factors, such as broodstock selective program, nutrition, sex ratio and stocking density, which might have good effect on reproductive performance of non-ablated *L. vannamei* female when they are well managed. For example, some of the regular hatcheries use specific family of breeding animals which are more successful without eyestalk ablation (Pactrick Sergooles, 2020. *Pers. Comm.*). This indicates that more studies are still needed to further understand the contribution of environmental conditions and/or genetic breeding program on effective natural Pacific white shrimp reproduction.

1.6 Broodstock nutrition of Pacific white shrimp (*L. vannamei*)

The requirement for nutrients in shrimp is similar to every animal. They need protein, lipids, carbohydrates, micronutrients (minerals and vitamins) and other supplements. The required levels depend on each stage of their lifecycle and external factors. For instance, the requirement for protein levels of *L. vannamei* is different for larvae, postlarvae, juveniles and adults, and broodstock (Shiau, 1998; Wickins and Lee, 2002). The level of culture technology or systems, and some water quality parameters (such as salinity) also affect the nature of required nutrients and their levels (Shiau, 1998). Hence, besides the role of environmental parameters and feeding management, manipulation of external and internal factors (such as eyestalk ablation) in *L. vannamei* reproduction, nutritional condition must be satisfactory, in order to have successfully sexual maturation and reproduction of the shrimp (Naeseens et al., 1997; Wouters et al., 2001a. In penaeid shrimp, nutritional factors play a critical role in the stimulation of sexual maturation and mating, the enhancement of fertility, and the viability and quality of offspring (Naessens et al., 1997). The Pacific white shrimp broodstock are generally fed a combination of artificial or semi-moist pellets and natural diets. The

nutritional composition of artificial feed is generally high in protein content (Shiau, 1998 and Wouters et al., 2001a)

1.6.1 Use of natural feeds in Pacific white shrimp broodstock

Marine organisms, or derivate of whether marine or terrestrial animals are used as natural feeds in shrimp broodstock. They are essential to support the maturation and reproduction of broodstock shrimp. Marine animals, such as squid, mollusks (mussels, clams, oysters), fish roe, marine polychaetes and crustaceans (shrimp, crab, enriched Artemia biomass) are mostly used worldwide as dietary components of *L. vannamei* broodstock (Wouters et al., 2001a). Beef or pork liver are commonly used in Vietnamese shrimp hatcheries (Hoa et al., 2009). Most of these animals are chosen based on their nutritional value and their availability in local markets.

The role of different natural feed items and feeding practices is not fully understood (Marsden et al., 1992; Wouters et al. 2001a; Hoa et al.2009) and their performance and biochemical properties remain to be clarified. However, it is considered that mollusks, squid and marine worms have been used as crucial dietary components due to their positive effects on penaeid shrimp reproduction and to their nutritional composition. In general they have high levels of polyunsaturated fatty acids, mainly arachidonic acid (ARA, 20:4ω6), eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) (Wouters et al. 2001a; Hoa et al., 2009; Emerenciano et al., 2013). Squid also have been reported to have the highest level of essential amino acids and green mussels the highest level of long chain fatty acids and the highest ω3/ω6 ratio among the natural feed (Marsden et al., 1992). Somehow, Hoa et al. (2009) found on their analysis that squid has higher levels of DHA than EPA and ARA, while oyster has higher levels of EPA than ARA and DHA; marine worms and pork liver has higher levels of ARA than EPA and DHA (Hoa et al., 2009). This difference in nutrient composition of natural feed has influence on the preference and the way hatcheries (maturation facilities) use them as a dietary component. Thus, the natural feed component of diet is generally a combined item of different sources at a general ratio of 2:1:1 for squid, polychaete and mussels respectively. However the combination equation might changes, which means it may differ largely from each maturation facility, between or even within countries (Hao et al., 2009 and personal contact with hatchery managers).

A combination of natural feeds is considered to be key to ensure a balanced inclusion of essential nutrients that closely resemble the ARA/EPA and DHA/EPA ratios of mature ovaries of wild shrimp species (Hoa et al., 2009). The same authors had success in spawning frequency, fecundity, fertilization rate and hatching rate of *P. monodon* broodstock fed with fresh feed ingredients with ARA/EPA and DHA/EPA ratios well balanced. They also had success in stimulating the natural mating of *P. monodon* as well. ARA and EPA are important components of cell membranes and precursors for 2-series and 3-series prostaglandins (PGs), which play a crucial role in vitellogeneses and spawning in decapod crustaceans (Wouters et al., 2001a; Hoa et al., 2009). Essential fatty acids of n-3 series (DHA, EPA and Linolenic acid) are essential for larval development, vitamin C for egg development and hatching, and vitamin E for ovarian maturation and larval development (Wouters et al., 2001b). These nutrients cannot be synthesized *de novo* by shrimp, thus they should be incorporated at high levels in the broodstock diet (Wouters et al., 2001b).

Absence of some micronutrients can also affect sperm quality. *Litopenaeus vannamei* broodsctock fed a diet without supplemental vitamins had significantly lower change in sperm count, with respect to baseline, as compared to broodstock receiving a control diet supplemented with these nutrients, and a diet without supplemental astaxanthin (Perez-velazquez and Gonzaez-feliz, 2003). Supplementations of vitamin C and E in maturation feeds for female shrimp are recommended to obtain higher egg hatchability and better quality larvae (Nguyen et al., 2011).

Marine worms (polychaetes) are important to ensure successful nauplii production in *L. vannamei* (Browdy, 1992) through improved female ovarian development (Bray and Lawrence, 1992). They are not only a source of PUFA but also possibly a source of other hormonally active compounds (Lytle et al., 1990; Hoa et al. 2009). The atokous polychaetes are composed of several types of PGs, of which PGF2α is the most abundant at a concentration of 0.2 µg/g wet weight of the polychaete (Hoa et al. 2009). Injection of an ethanol extract from the polychaete *Perinereis nuntia*, after purification using fast performance liquid chromatography, into domesticated black tiger shrimp resulted in an induction of ovarian maturation and spawning in the eyestalk unablated animals (Hoa et al., 2009). In vitro incubation of *P. monodon* previtellogenesis oocytes with PGE2

extracted from polychaetes and with synthetic PGE2 showed that PGE2 had an effect on oocyte maturation especially during the late maturation and ovulation (Meunpol et al., 2005a). Artemia biomass appears to have similar role as polychatetes. Naessens et al. (1997) have demonstrated that Artemia biomass can be supplemented or replace polychaete in *L. vannamei* maturation diets.

Although natural feeds are essentials in inducing shrimp maturation and spawn, it is important to highlight that when using them there is need to ensure that they are not a biosecurity risk (FAO, 2003). It means, they have to be free of the main diseases causing agents such as virus or bacteria.

1.7 Application of biofloc technology and supplementation of fresh feed before maturation stage

Cardona et al. (2015) showed that a reproductive performance of *L. stylirostris* broodstock from biofloc technology (BFT) was better than those reared extensively in an earthen pond. This improvement is related to complementary dietary lipids from biofloc aggregates and specifically stored phospholipids and essential fatty acids (FAs) in oocytes that are necessary for embryogenesis and pre-feeding larvae development (Racotta et al., 2003; Hargreaves, 2013; Cardona et al., 2015). It may also be associated to bioactive compounds found in biofloc or better water quality created by bioflocs system (Hargreaves, 2013). Hence, the BFT shrimp exhibited higher production of eggs and more frequent spawning than shrimp broodstock reared in clear water. It appears that biofloc can improve nutritional status of shrimp broodstock. Additionally, Braga et al. (2015) found that Pacific white shrimp male reared in biofloc during pre-maturation conditioning exhibited better spermatophore and sperm quality than under clear water system.

Many authors have demonstrated that biofloc is a potential source on nutrients, such as protein, carbohydrate, lipid (mainly essential fatty acids, phospholipids), vitamins, minerals, essential and non-essential amino acids (Khun et al., 2009; Méndez et al., 2009; Crab et al., 2010; 2012; Tidwell, 2012; Hargreaves, 2013; Cardona et al., 2015). Most of them are essential for shrimp reproduction, but phospholipids and essential fatty acids (such as ARA and EPA) are crucial to sustain reproduction, embryonic and larval development of penaeid shrimp (Cardona et al., 2015). ARA and EPA are both precursors

of prostaglandins and are considered essential for the synthesis of these hormones. It seems like biofloc has similar role as marine fresh food used as dietary component of shrimp broodstock and can be used to prepare Pacific white shrimp broodstock during the pre-maturation stage.

Generally, the supplementation with natural feed begins soon after selected shrimp are transferred to broodstock maturation facilities. Studies trying to understand the possible influence of pre-maturation conditioning, like supplementing fresh or frozen food to broodstock before they go to complete maturation conditioning, are rare. Emerenciano et al. (2013) evaluated the effect of short-term natural fresh feed supplementation 20 days prior to ablation in *L. vannamei* broodstock raised under biofloc conditions, and observed that females that received fresh feed supplementation achieved better egg production, spawned more quickly, and presented higher levels of HUFA in eggs as compared to those that did not received fresh food.

Use of biofloc and/or supplementation of fresh-frozen feed for pre-maturation stage should be considered by Pacific white shrimp maturation facilities and certifiers. However, some important information are still required to determine approaches to such feeding at the pre-maturation stage, for instance it should be clear if the shrimp can just be given fresh-frozen feed or combination of fresh-frozen and dry feed, define the fresh-frozen feed combination ratio if it will be the same as for maturation stage, the frequency of applying the food, the age of female and male entering the pre-maturation stage, the period of the stage and economic implications of such approaches. The last and not least, there is need to verify if the application of pre-maturation can improve performance of NAF as in previous studies it was only demonstrated in AF.

1.8 Aims of this study

The general objective of this PhD study was to provide a body of evidence of the potential of using non-ablated female in commercial shrimp hatcheries to support on-going dialogue around the continued use of unilateral eyestalk ablation in modern *L. vannamei* breeding practices.

The specific goals of this thesis included:

1. Evaluate reproductive performance of non-ablated female (NAF) and ablated female (AF) *L. vannamei* and the quality (i.e. growth performance, survival, resistance to stress) of their offspring.
2. Verify the potential of fresh-frozen feeds (squid and polychaete) in pre-maturation on reproductive performance, offspring quality and biochemical composition of NAF *L. vannamei* broodstock.
3. Assess the resilience of postlarvae and juvenile *L. vannamei* produced from NAF and AF broodstock following a disease challenge.
4. Determine the effect of shifting from AF to NAF *L. vannamei* based maturation facility system on production cost and profitability.

This thesis consists of a general introduction (Chapter 1), general material and methods (Chapter 2), four data chapters (Chapter 3-6) and a general discussion and conclusion chapter (Chapter 7).

The data chapters include:

Chapter 3: Reproductive performance and offspring quality of non-ablated Pacific white shrimp *(Litopenaeus vannamei*).

Chapter 4: Effect of pre-maturation conditioning on reproductive performance, offspring quality and biochemical composition of non-ablated Pacific white shrimp *(Litopenaeus vannamei*) female.

Chapter 5: Increased robustness of post-larvae and juveniles from non-ablated Pacific whiteleg shrimp, *Litopenaeus vannamei*, broodstock post-challenged with pathogenic isolates of *Vibrio parahaemolyticus* (VpAHPND) and white spot disease (WSD).

Chapter 6: Economics of non-ablated Pacific white shrimp *(Litopenaeus vannamei*) hatchery based system and their contribution to larviculture and grow-out production costs.

Chapter 2: General Materials and Methods

2.1 Experimental facilities and materials

The trials related to Chapters 3 and 4 were carried out in shrimp commercial facilities of Seajoy Company in Honduras from December 2016 to December 2018. Seajoy, now known as Seajoy Cooke, became part of the multinational company Cooke Aquaculture at the end of 2018, after these experiments had been finalised. The company had a vertically integrated production system. Besides hatchery (maturation and larviculture facilities), it also had production farms based mostly on a semi-intensive system, and its own processing plant. Maturation and larviculture trials were conducted in their hatchery (Larvicultura del Pacífico – LARVIPAC), whilst nursery and grow-out trails were implemented in their farm (Biocultivos Mariños - BIOMAR). The shrimp broodstock of *L. vannamei* were all from Specific Pathogen Resistant (SPR) stocks, produced internally.

A single maturation room with 12 tanks was used to run reproductive performance trials (Chapter 3 and 4). In general the maturation tanks were round geo-membrane tanks, dark-coloured and with an area of 22.9 m^2. All tanks are connected to a single closed recirculating system that allowed 5-10% daily water renewal. The water filtration and treatment system consisted of sand, cellulose, activated carbon and silica filters, and UV light. Photoperiod in the maturation room was maintained naturally by allowing exposure to ambient sunlight through translucent roof windows. As the spawning, hatching and larviculture tanks were different between the two chapters; more details are included in respective chapters. Nursery and grow-out were conducted using semi-intensive commercial ponds and cages. The company provided hatchery and farm economic secondary data used in Chapter 6.

Disease challenge trials were implemented in Thailand from July to December 2019. Experimental animals from non-ablated (NAF) and ablated (AF) *L. vannamei* female were produced and provided by Syaqua Siam Co. hatchery. The challenges were conducted using research facilities of Fish Vet Group Asia Limited (FVGAL). As the tanks and animal size used were different for the two tested disease (AHPND and WSD); more details are included in Chapter 5.

2.2 Experimental design

All animals (i.e. broodstocks, larvae, postlarvae and juveniles) used in experiments were screened for main diseases before starting any trials using qPCR. This procedure was done by technical Seajoy or FVGAL staff. The number of treatments, replication and animals were described in detail in each relevant chapter. However, it is important to highlight that there was limitation on replicating pre-maturation treatments (Chapter 4) as this was done under commercial condition using commercial size tanks which were normally being used by Seajoy. The company had to continue their commercial production, so the number of the experimental units used for the pre-maturation stage was the maximum that could be provided. In addition, replicates under commercial conditions would require large number of broodstock which were unaffordable. Another replicate limitation was found when running the nursery trial at a commercial scale (Chapter 3). There were not enough postlarvae (PL) from each treatment to stock a third replicate as the maturation trial system, although in commercial scale, was not enough to produce what was expected in each treatment. All management protocols used in the trials followed the company practices (i.e. feed type, feeding regime, feeding rate, water exchange, disease screening, and water quality records).

Duration of the trials varied from 96 hours to 4 months depending on the type of the trial, farming process or system considered. *L. vannamei* broodstock, larvae (nauplii), PL or juveniles were used to start an experiment depending on the nature of the trial. Throughout most trials, treatments were randomly allocated to the experimental units (tanks, ponds or cages) (Chapters 3 and 4) and others were semi-randomized block (Chapter 5). All staff who was involved in supporting the trials was properly trained to avoid any misunderstanding on specific trials management such as mixing spawning breeders or eggs from each treatment in same spawning tank, feed inappropriate diet in a pre-maturation tank or contaminate control animals in disease challenge. Furthermore, it is important to highlight that *Vibrio parahaemolyticus* preparation, determination of the WSSV virial titres in the shrimp tissue were carried out by FVGAL staff (Chapter 5).

2.3 Experimental sampling

All sampling were done systematically and the details can be found in each experimental chapter. The shrimp broodstock, PL and juveniles were weighed before starting of each trial and at the end. Eggs, nauplii and PL were sampled for morphometric (Chapter 3) and biochemical (Chapter 4) analyses. Feed, hepatopancreas and ovary were also sampled for biochemical analyses (Chapter 4). Male spermatophore was sampled to check sperm quality (Chapter 4). PL and juveniles samples were taken to confirm disease presence (Chapter 5). Depending on the nature of the analyses, some shrimp were humanely euthanized in pre-iced water (<4˚C) before sampling and others were preserved in 10% formalin solution.

2.4 Shrimp broodstock and offspring performance indicators

As the broodstock from each treatment were collectively managed (Chapter 3 and 4), reproductive performance parameters were estimated per unit time period (days) (Bhujel, 2008) (Table 2.1). Mortality of females per treatment was recorded daily.

Table 2. 1: Formulas used to determine reproductive performance parameters.

Parameters	Calculation method
Mating success (%)*	Number of mated female per day/ number of female per day
Spawning event (%)**	Number of spawned female per day/number of mated female per day
Hatching rate (%)	Number of nauplii per day/ number of eggs per day
Fertilization (%)	Number of fertilized eggs per day/number of total eggs per day
Fecundity (eggs)	Number of eggs per day/ number of spawned female per day
Fecundity (nauplii)	Number of nauplii per day/ number of spawned female per day
Number of eggs/tank/day	Number of eggs per day/ number of tanks per treatment
Number of nauplii/tank/day	Number of nauplii per day/ number of tanks per treatment

*Mating success of female per day is directly related to number of mature female (with developed gonad).

**Spawning event is defined in this study as percentage of mated female that released eggs in spawning tanks.

During larviculture trials, larval stage development was determined daily using larval stage index (LSI) which is a calculated weighted average of stage determination i.e., the number of larvae or PL at each stage were multiplied by the stage number and the sum of the products were divided by the number of larvae sampled as illustrate in equation 1 (Uno and Kwon, 1969). This index was also used to express larval growth (Uno and Kwon, 1969; Manzi et al., 1977). At the end of each trial wet weight and survival was recorded. Survival was determined by taking three 100 mL samples from each 500 L fiberglass tank (used to concentrate PL from each trial tank) and counting all live PLs. Weight was determined by weighing 1g of PLs from each tank and then dividing it with number of PLs observed in that sample.

Postlarval performance was also assessed using a simple salinity stress test which is described in more details in Chapters 3, 4 and 5. In addition survival of PLs and juveniles were recorded during the disease challenge (Chapter 5). Performance of PLs and juveniles in nursery and grow-out were evaluated using parameters described in Table 2.2.

Equation 1: $\boldsymbol{LSI = (\sum niEi)/n}$ in which: $\boldsymbol{LSI}$ = Larval stage index; $\boldsymbol{ni}$ = number of larvae at stage $\boldsymbol{Ei}$; n = number of larvae analysed; $\boldsymbol{Ei}$ = larval or postlarval stage.

Table 2. 2: Formulas used to determine growth performance and survival.

Parameters	**Calculation method**
Final survival	100 x (final shrimp number/initial shrimp number)
Final weight	
Weekly growth	Final weight/time in weeks
Yield	(Final shrimp number x final weight)/area
Feed conversion rate	Consumed feed (dry weight)/total weight gain
Total weight gain	Final weight – initial weight
Specific growth rate	100 x (ln final weight – ln initial weight)/time in days

2.5 Spermatophore and sperm quality

Following Leung-Trujillo and Lawrence (1987) method, spermatophore mass was ejaculated from live male, weighed then homogenized in calcium free saline solution before adding 1% stock solution of trypan blue dye. The spermatophore was first added to a glass tissue grinder with 5 ml of calcium solution, and then the sperm was released by

grinding and then mixed several times to ensure homogeneity. After that, 0.9 ml of the sperm suspension and 0.1 ml of stock solution of trypan blue dye were mixed and incubated for 15 mins before counting the number of live (unstained) and dead (stained) sperm cells using an hemacytometer. Calculation related to sperm cells were done as follow: Total sperm count (Live cells + dead cells) and percentage of dead cells [100 (dead cells/total cells)].

2.6 Biochemical analyses

Biochemical contents of the feed, hepatopancreas, ovary, eggs, nauplii and postlarvae were analysed at the University of Stirling using the methods described below. Each sample was analysed in triplicate.

2.6.1 Crude Protein

The protein was determined from the Nitrogen (N) content of each sample which assumes that protein contains 16% nitrogen, using automated Tecator Kjeltec TM 2300 analyser (Foss, Warrington, UK) following the standard method (Person, 2008) and manufacture's instructions. In summary, nearly 250 mg of each sample was placed in a Kjeldahl digestion tube, then added two mercury Kjetabs and 5 ml of concentrated sulphuric acid and boiled at 420ºC for 1 hour. After cooling to room temperature, distillation was carried out using the Tecator Kjeltec TM 2300 and the crude protein values recorded.

2.6.2 Total Lipid

Folch method was used to extract total lipid. Briefly, the extraction was done from 0.5 g of sample, by homegenising in 20 volumes of ice-cold chloroform/methanol (2:1 v/v) using an UltraTurrax tissue disruptor (Fisher Scientific, Loughborugh, UK) according to Folch et al. (1957) and determined gravimetrically after an overnight desiccation under vacuum.

2.6.3 Fatty acids

After extraction of total lipid, the concentration of Fatty Acid Methyl Esters (FAME) was determined. FAME was prepared from total lipid re-dissolved in chloroform/methanol (2:1 v/v) at a concentration of 10 mg/ml by acid-catalysed transesterification at 50ºC for 16 hours (Christie, 1993). Extraction and purification of FAME were performed as described by Tocher and Harvie (1988) and separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with 30mx0.32mm i.d. x 0.25 µm ZB-Was column (Phenomenex, Cheshire, UK) ‘on column’ injection and flame ionisation detection. Hydrogen was used as carrier gas with initial oven thermal gradient of 50ºC to 150ºC at 40ºCm^{-1} to a final temperature of 230ºC at 2ºCm^{-1}. Individual FAME were indentified by comparison to known standards (Supelco™ 37-FAME mix; Sigma-Aldrich Lts., Poole, UK). Data were collected and processed using Chromocard for Windows (Version 1.19; Thermoquest Italia S.p.A., Milan, Italy). Fatty acid content (g/100g of sample) was calculated using heptadecanoic acid (17:0) as internal standard.

2.6.4 Total carbohydrate

Modified Dubois phenol-sulphuric method was used to determine total carbohydrate in samples following the standard method (Dubois, et al., 1959). Briefly, nearly 5 mg of each sample was placed in Pyrex test tube, then added 2.5 ml of deionised water, 1 ml of phenol solution and 8 ml conc. Sulphuric acid. After the final solution stabilized, absorbance of each tube was read using spectrophotometer at 520 nm. From a previously plotted calibration curve of absorbance versus glucose concentration, the samples values were read as mg of glucose which was then used to determine % of total carbohydrate [(mg of glucose in sample/sample weight) x 100].

2.7 Statistical analyses

All statistics were run using STATISTICA software (version 7) and EXCEL (2016). Treatments were compared at significance level of 0.05. Most of the data were presented as the mean together with standard error (mean ± SE). Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. One-way ANOVA or multifactorial ANOVA followed by a Tukey test (Zar, 2010) was used to

compare the treatments. Data indicating non-normal distribution or homogeneity were transformed using either square-root or Log/Ln. Regression analysis was carried out to verify the relationship between total lipid in the hepatopancreas and ovary in specific case. Pairwise Kaplan-Meier survival analyses with subsequent *post-hoc* Mantel-Cox log-rank tests were performed to calculate the survival probabilities in disease challenge trials.

Chapter 3: Reproductive performance and offspring quality of non-ablated Pacific white shrimp *(Litopenaeus vannamei*)

3.1 Introduction

Until currently standard hatchery methods worldwide to control the maturation and reproduction of *L. vannamei* are based on the use of eyestalk ablation (Chamberlain and Lawrence, 1981; Zhang et al., 1997; Palacios et al., 1999a; FAO, 2003; Sainz-Hernández et al., 2008; Das et al., 2015). Ablation can increase and improve predictability of commercial hatchery output (Treece and Fox, 1993; Palacios et al., 1999a) but is also known to have disadvantages. Removal of the optic ganglia is known to severely impact the animal's welfare. In addition to causing trauma and stress associated with the procedure itself, physiological imbalance and a compromised immunological defense that lead to accelerated reproductive exhaustion and increased broodstock mortality are possible consequences (Palacios et al., 1999ab; Bae et al., 2013; Treerattrakool et al., 2014; Das et al., 2015). Additionally growing consumer awareness of animal welfare and ethics in food production and the related interest of choice editors to reduce commercial risk (Little et al., 2018) have made a reassessment of the use of eyestalk ablation in hatcheries a priority for producers, retailers and seafood certifiers.

The use of non-ablated females (NAF) has attracted interest for some time especially as hatchery managers report NAF have longer reproductive lifetime than ablated individuals under intensive maturation conditions (FAO, 2003). However NAF suffer from less predictable maturation peaks and spawning synchronization, making the establishment of production schedules more difficult (Palacios et al., 1999b). Few studies have comprehensively evaluated differences in productivity between systems based on eyestalk ablated (AF) and non-ablated (NAF) shrimp. Mating success, spawns, hatching rate, female mortality rate and fecundity (number of eggs and nauplii per spawn) of NAF and AF of *L. vannamei* have been assessed by Chamberlain and Lawrence (1981) and Palacios et al (1999b), and in other species (Santiago Jr, 1977; Emmerson, 1980; Chamberlain and Lawrence, 1981; Yano, 1984; Choy, 1987; Makinouchi and Primavera, 1987; Gendrop-Funes and Valenzuela-Espinoza, 1995). Zhang et al. (1997) only assessed spawning and molt cycle of *Stenopus hispidus*.

Non-ablation of *L. vannamei* female can affect mating success but not egg hatching rate, female mortality rate, number of eggs and nauplii per spawn (Chamberlain and Lawrence 1981; Palacios et al. 1999b). In addition, non-ablation did not have a significant

effect on larval survival in zoea, mysis and postlarvae 1-day-old (PL1) stages (Palacios et al. 1999b). However, Chamberlain and Lawrence (1981) evaluated only reproductive parameters under non-commercial conditions and some of their results might have been affected because NAF and AF animals were maintained in the same tanks. Furthermore the authors assumed that some of their AF animals might have had regenerated eyestalk during the trial. This may have led to some animals being classified as NAF whilst actually being AF. Palacios et al (1999b) are the only authors to date who have compared reproductive performance between both groups under intensive reproduction conditions, but their experimental design was unbalanced with respect to the numbers of AF and NAF used which might have influenced their results. Additionally, they also compared larval growth and survival to PL1 from eggs derived from both NAF and AF but not under commercial conditions. Moreover there is an urgent need for more contemporary assessments of the effects of the impact of non-ablation on broodstock performance in terms of reproductive outputs and offspring quality in the context of shrimp strains improved through selective breeding (Castillo-Juarez et al, 2015) and based on modern production practices.

This study aimed to evaluate reproductive performance of NAF and AF *L. vannamei* and the quality of their offspring in terms of growth performance and survival during larviculture, nursery and grow-out under commercial conditions.

3.2 Material and methods

Two independent studies (Study I and II) were conducted in the commercial hatchery (Larvicultura del Pacífico - LARVIPAC) and farm (Biocultivos Mariños - BIOMAR) of Seajoy company in Honduras. The main difference between Study I and Study II is that broodstock restocking (replacement of deceased animals) in maturation and reproduction tanks over the experimental period was practiced in Study 1,whilst no restocking (no replacement of deceased animals) was practiced in Study 2. The main idea was to verify how non-restocked population would affect reproductive performance in both NAF and AF, and their offspring. Furthermore, larviculture, nursery and grow-out on the first study were conducted under commercial conditions.

Study I

3.2.1 Broodstock and experimental rearing conditions

L. vannamei broodstock with mean male and female weight, 39.7 ± 2.6 and 49.7 ± 4.3 g respectively, were used in this experiment. Prior to the experiment, shrimp entered pre-maturation conditioning of approximately 36 days where males and female were reared in separate systems consisting of two 41.8 m^2 geomembrane tanks. Both male and females were fed daily with chopped defrosted squid (*Loligo opalescent*) (12:00 pm and 3:00 am) at 5% of biomass (ww) and polychaete worms (*Nereis virens*) (7:00 am) at 2.5% of biomass (ww). All fresh feeds were defrosted and chopped to a particle size of approximately 0.6 g. These were supplementary to the feeding of 2 commercial maturation diets: a shrimp broodstock diet (40% Crude protein, Zeigler, USA) was used at 5% of biomass (dw) daily and split between three feeds at 9:00 am; 3:00 pm and 10:00 pm, and EZ-mate (55% Protein, Zeigler ,USA) was supplied at 3.5% of biomass (dw) daily and split between two feeds at 8:00 pm and 5:00 am.

Three days before starting of the experiment, unilateral eyestalk ablation was performed on control females by cauterization. Ten maturation tanks (round geomembrane tanks, dark-coloured; 22.9 m^2 area) were used in total; five were stocked with AF (control group) and five with NAF (experimental group). Water depth was maintained at 0.5 m using a central stand pipe and tanks were aerated constantly using two air stones per tank. Males were stocked in all tanks one day before starting the experiment. Each tank was stocked with 300 animals (13/m^2) with a sex ratio of 1:2 (male to female). All experimental units were connected to a single recirculating system that allowed 5-10% daily water exchange. The water filtration and treatment system consisted of sand, cellulose, activated carbon and silica filters, and UV light. Photoperiod was maintained naturally by allowing exposure to ambient sunlight through translucent roof windows in the maturation room. Water temperature, salinity, dissolved oxygen, pH, alkalinity and ammonia were maintained at 28.9 ± 0.1 ºC, 32.0 ±1.0 ppt, 4.3 ± 0.0 mg/L, 7.0 ± 1.0, ≥ 100 mg/L $CaCO_3$ and < 1 mg/L respectively. The broodstock remained in maturation tanks for 62 days and were fed daily with polychaete worms twice a day (07:00 am and 10:00 pm) at 5% of biomass (ww), squid three times a day (9:00 am, 2:00 pm and 3:00 am) at 12% of biomass (ww), and mussels twice a day (12:00 pm and 5:00 am) at 6% of biomass (ww). All fresh

feed were defrosted and chopped to approximately 0.6 g. In addition a commercial maturation diet EZ-mate (55% Protein, Zeigler, USA), was also applied once a day (8:00 pm) at 2% of biomass (dw) according to commercial protocol. The common practice of commercial hatcheries to replace deceased individuals in order to maintain the same stocking density throughout the experiment was followed. Females (509) and males (242) of similar conditions described above were used to restock non-ablated treatment while ablated group used 738 females and 220 males.

3.2.2 Reproductive performance and broodstock mortality

Reproductive performance was evaluated a minimum of three times per week over the 62-day study based on standard commercial hatchery practices and previously published recommended protocols (Crocos and Kerr, 1986; Coman and Crocos, 2003). Gonad development was monitored three times daily with a lamp, from 4:00 to 7:00 pm, by observing the size and colour of the gonad through the exoskeleton. Mature females from each treatment with an attached spermatophore were collected, transferred and pooled in three 5000 L spawning tanks per treatment. Spawning tanks were maintained at a temperature of 28.0 ± 1 ºC. Females from each treatment were then returned to their respective maturation tanks at midnight, and eggs were collected from the spawning tanks and pooled in a 25L bucket. After stirring, three 5 mL subsamples of water per treatment were taken for volumetric assessment of number of eggs in each treatment. Each group of eggs were subsequently transferred to an illuminated 1000 L fibreglass hatching tank and kept for 13 h. Each hatching tank was aerated constantly using one air stone per tank and temperature and salinity were maintained around 32.0 ± 0.5 ºC and 30.0 ± 0.0 ppt. Nauplii were then collected exploiting their positive phototactic behaviour, transferred to a 25.0 L bucket, where volumetric assessment of numbers was undertaken for each treatment using the same method as for eggs.

As the females from each treatment were collectively managed, reproductive performance parameters were estimated per unit time period (days) (Bhujel, 2008): mating success per day, spawning event per day, hatching rate per day, fecundity in term of eggs per spawned female per day and nauplii per spawned female per day, and tank production parameters in term of eggs per tank per day and nauplii per tank per day (Calculation methods are shown in Chapter 2). Spawning event is defined in this study as percentage of

mated female that released eggs in spawning tanks. Mortality of females per treatment was recorded daily.

3.2.3 Larval rearing

Two different larval rearing trials (Larviculture I - LI and larviculture II - LII) of 16 days each were conducted using larvae (nauplii 5) produced at the beginning (day 12) and middle (day 38) of the observation period as the productivity of eyestalk ablated animals are reported to deteriorate over time (typically two to three months) because of exhaustion (Palacios et al., 1999a) and this might affect the quality of the offspring. In LI, six rectangular geomembrane tanks (16 000L) with initial 10 000L water volume (8000 L seawater + 2000 L algae) were stocked with 2 500 000 nauplii each at a density of 250/L on the first day. Both treatments were triplicated and randomly distributed in a greenhouse. Algae (*Thalassiosira* sp.) were added daily to the tanks at concentration of 100 000 – 130 000 cells/mL until the shrimp reached PL2, and then 25% of water was renewed daily until the end of the trial. The diet was also composed of *artemia* sp. (from Mysis 1 to PL6) and artificial liquid and dry diets (Frippak – Inve Aquaculture; liqualife ZM and MPL – Cargil; liquid hatchery feed – Epicore; and brine shrimp flake – Mackey Marine (from Zoea 1 to PL12-PL14). The amount of feed applied in the trials followed manufacture recommendations. Water temperature, salinity, dissolved oxygen, pH, ammonia, nitrate, total suspended solids and alkalinity were kept in 32.7 ± 0.5 ºC, 32.9 ± 0.0 ppt, 4.4 ± 0.1 mg/L, 8.0 ± 0.0, < 1 mg/L, 1.5 ± 0.2, 34.3 ± 2.3 and 140 ± 2.6 mg/L $CaCO_3$ respectively. The procedures used in trial II were similar to the first trial except for the following details: ten rectangular geomembrane tanks (5 for each treatment) of 24 000 L each, in addition each unit had initial 12 000L water volume (10000 L seawater + 2000L algae) stocked with 1 950 000 nauplii each at a density of 162.5/L on the first day.

During both trials larval stage development was determined daily using larval stage index (LSI) (Uno and Kwon, 1969). This index was also used to express larval growth (Uno and Kwon, 1969; Manzi et al., 1977). At the end of the trials wet weight and survival were recorded. Survival was determined by taking three 100 mL samples from each 500 L fiberglass tank (used to concentrate PL from each trial tank) and counting all live PLs. Weight was determined by weighing 1g of PLs from each tank and then dividing it with number of PLs observed in that sample. Postlarval quality was also assessed using a simple

salinity stress test. 100 PLs11-12 (for LI), PLs13-14 (for LII) per tank were transferred from full strength seawater (32 ppt) to a 1 L beaker of fresh water (0 ppt) for a period of 30 minutes before being returned to a beaker of seawater. After a further 30 minutes, survival (%) was evaluated based on immobility/reaction after physically stimulating with a pipette (Palacios et al., 1999b; Racotta et al., 2004).

3.2.4 Nursery and grow-out

PLs from larviculture II were used for nursery and grow-out. Four rectangular earthen ponds of 1.50 ha each were stocked with PLs13-14 from the two treatments (two ponds per treatment) at a density of 123/m^2 and reared for 20 days. During this stage the animals were fed only artificial feed Diamassa (35% Protein, Gisis, Ecuador) 7 times a day and at a rate of 15 – 20% of total biomass. Two feed trays of 50 cm^2 each were placed 1 m depth in the water column. Water temperature, dissolved oxygen, salinity, pH and ammonia were monitored and no significant difference was observed between the treatments (Table 1). Final survival, final weight (g), weekly growth and yield and feed conversion rate (Calculation methods are presented in Chapter 2, Table 2.2) were used to assess the culture performance of PLs generated by NAF and AF broodstock.

After the nursery period, juveniles from each treatment were transferred to triplicate rectangular, semi-intensive earthen ponds of 1.82 ha each and stocked at 25 juveniles/m^2 for final grow-out (86 days). Artificial feed (Diamassa, 35% Protein, Gisis, Ecuador) was offered three times a day at rate of 2 – 10% of total biomass. The amount of feed offered at the beginning of the trial was equal in all replicates, but subsequently adjusted based on weekly animal growth and biomass. Two feed trays of 50 cm^2 each were placed 1 m depth in the water column. Final survival, final weight (g), weekly growth total weight gain, yield, feed conversion rate and specific growth rate (Calculation methods are presented in Chapter 2, Table 2.2) were determined to evaluate their performance. Water temperature, salinity, dissolved oxygen, pH, ammonia and alkalinity in nursery were 31.4 ± 0.6 ºC, 43.0 ± 1.7 ppt, 4.2 ± 0.7 mg/L, 7.0 ± 1.0, < 1 mg/L, 1 and 31.3 ± 0.9 mg/L $CaCO_3$ respectively. Water temperature, salinity, dissolved oxygen, pH, ammonia, nitrate, silicate, phosphate and alkalinity in grow-out were 31.1 ± 1.1 ºC, 31.9 ± 8.0 ppt, 3.9 ± 1.2 mg/L, 7.0 ± 1.0, < 1

mg/L, 5.7 ± 0.9 mg/L, 3.6 ± 1.5 mg/L, 1.8 ± 0.8 mg/L and 98.8 ± 7.4 mg/L $CaCO_3$ respectively.

Study II

3.2.5 Broodstock and experimental rearing conditions

L. vannamei broodstock with mean male and female weight, 37.5 ± 0.4 and 44.8 ± 0.6 g respectively, were used in this study. Prior to the experiment, shrimp entered pre-maturation following the protocol used in Study I. However, in Study II there was used different family. In addition, Study II was conducted 1 year after Study I.

The maturation tank system was the same used in Study I. Three days before starting the experiment, unilateral eyestalk ablation was performed on control females by cauterization. Six maturation tanks were used in total; three were stocked with AF (control group) and other with NAF individuals (experimental group). Males were stocked in all tanks one day before starting the experiment. Each tank was stocked with 300 animals ($13/m^2$) with a sex ratio of 1:2 (male to female). Water temperature, salinity, dissolved oxygen, pH, alkalinity and ammonia were at 29.8 ± 0.0 ºC, 32.0 ± 0.5 ppt, 4.4 ± 0.0 mg/L, 7.9 ± 0.0, ≥ 100 mg/L $CaCO_3$ and < 1 mg/L respectively. The broodstock remained in maturation tanks for 68 days and were fed daily with mussels three times a day (07:00 am, 12:00 pm and 3:00 am) at 8% of biomass, squid four times a day (9:00 am, 2:00 pm, 8:00 pm and 5:00 am) at 15% of biomass, and commercial maturation diet EZ-mate (55% Protein, Zeigler, USA) once a day (8:00 pm) at 2% of biomass according to commercial protocol. All fresh feed were defrosted and chopped to approximately 0.6 g. Deceased individuals were not replaced over the experimental period as was done in previous study.

3.2.6 Reproductive performance, broodstock mortality and larval rearing

Reproductive performance was evaluated during 68 days and following the methodology used in Study I. Two different larval rearing trials (Larviculture III - L III and larviculture IV – L IV) of 16 days each were conducted using larvae (nauplii 5) produced at the beginning (day 12) and almost at the end (day 47) of the observation period. In both

trials (L III and L IV), six fiberglass tanks (500L) with initial 250L water volume (220L seawater + 30L algae) were stocked with 53 500 nauplii each at a density of 214/L on the first day. Each treatment was triplicated and randomly distributed in a greenhouse. Algae (*Thalassiosira sp*) were added daily to the tanks at concentration of 100 000 – 130 000 cells/mL until the shrimp reached 2-day-old post-larval stage (PL2), and then 25% of water was renewed daily until the end of the trial. The diet was also composed of artemia sp. (from Mysis 1 to PL6) and artificial liquid and dry diets (Frippak – Inve Aquaculture; liqualife ZM and MPL – Cargil; liquid hatchery feed – Epicore; and brine shrimp flake – Mackey Marine (from Zoea 1 to PL12-PL14). Amounts fed in the trial followed manufacture recommendations. Water temperature, salinity, dissolved oxygen, pH, ammonia and alkalinity were at 31.7 ± 0.2 ºC, 32.0 ± 0.0 ppt, 6.2 ± 0.0 mg/L, 7.5 ± 0.0, < 1 mg/L and 100.8 ± 2.0 mg/L $CaCO_3$ respectively. At the end of the trial wet weight, final survival and survival to salinity stress test of PLs10-11 were recorded following similar method from Study I.

3.2.7 Eggs, nauplii and postlarvae sampling for morphometric assessments

For morphometric analyses, 30 eggs at first-second cleavage and 30 nauplii at stage 5 used for larviculture trials were collected and preserved in 4% formaldehyde, then used to measure egg diameter and nauplii rostrocaudal length with a light microscopy and micrometer. At the end of each larviculture trials, triplicate samples of 30 PLs10-11 per replicate were collected and preserved in 4% formaldehyde, then used to measure postlarvae rostrocaudal length with a light microscopy and micrometer.

3.2.8 Nursery and grow-out

PLs from larviculture trial IV were used for nursery and grow-out. Six concrete tanks (covered with dark-coloured geomembrane; 14 m^3 cubic each) were stocked with PLs13-14 from the two treatments (three tanks per treatment) at a density of 230/m^3 and reared for 26 days. During this stage the animals were fed only artificial feed Nicovita (45% Protein, Nicovita, Ecuador) eight times a day and at a rate of 15 – 20% of total biomass. Two feed trays of 50 cm^2 each were placed 0.8 m in the water column. The culture performance was evaluated following same methods in Study I.

After the nursery period, juveniles from each treatment were transferred to triplicate rectangular cages (9 m^2 each) which were previously installed in an earthen pond of 1.9 ha. Each cage was stocked with $44/m^2$ shrimp for final grow-out (32 days). Artificial feed (Nicovita, 35% Protein, Gisis, Ecuador) was offered six to eight times a day at rate of 2 – 10% of total biomass. The amount of feed offered at the beginning of the trial was equal in all replicates, but subsequently adjusted based on observed consumption rate and weekly animal growth and biomass. Two feed trays of 50 cm^2 each were placed at a depth of 0.8 m in the water column. The culture performance was evaluated following same methods as in Study I.

Water temperature, salinity, dissolved oxygen, pH, ammonia and alkalinity in nursery were 30.1 ± 0.1 ºC, 18.0 ± 1.0 ppt, 5.2 ± 0.1 mg/L, 8.2 ± 0.0, < 1 mg/L, and 92.2 ± 1.0 mg/L $CaCO_3$, respectively. Water temperature, salinity, dissolved oxygen, pH, ammonia, nitrate and alkalinity in grow-out were 32.2 ± 0.3 ºC, 19.4 ± 0.8 ppt, 6.6 ± 0.3 mg/L, 8.3 ± 0.0, < 1 mg/L, 3.8 ± 0.0 mg/L, and 90.7 ± 2.4 mg/L $CaCO_3$, respectively.

3.2.9 Statistical analysis

In both experiments One-way ANOVA followed by a Tukey test (Zar, 2010) was used to compare the treatments in significance level of 0.05. Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. Reproductive outputs such as number of eggs and nauplii per female per day were analysed by two-way ANOVA using first variable as time (days) and second one as non-ablation and ablation. All statistics were run using STATISTICA 7 software.

3.3 Results

3.3.1 Reproductive performance

Spawning event per day (percentage of mated female that released eggs in spawning tanks) and hatching rates were similar for both treatments in both studies (Table 3.1). The mating success, mortality, number of eggs and nauplii production per tank of NAF per day were significantly lower than AF (Table 3.1). The fecundity (number of eggs or nauplii per female per day) from NAF was significantly higher than AF (Table 3.1).

Table 3. 1: Reproductive performance (mean ± SE) of non-ablated (NAF) and ablated (AF) *L. vannamei* female from two independent studies. (Study I: n = 35*; Study II: n=3**).

Reproductive Performance		
Study I	**NAF**	**AF**
Mating success per day (%)	3.2 ± 0.0[b]	7.6 ± 0.0[a]
Spawning event day (%)	90.1± 0.1 [a]	95.5 ± 0.0 [a]
Hatching rate per day (%)	78.0± 0.0 [a]	81.7 ± 0.0 [a]
Mortality of female per day (%)***	1.3 ± 0.0 [b]	2.3 ± 0.0 [a]
Number of eggs/spawned female/day	142 413 ± 4558 [a]	116 752 ± 3568 [b]
Number of nauplii/spawned female/day	112 610 ± 4923 [a]	95 127 ± 2954 [b]
Number of eggs/tank/day	811 004 ± 86 858 [b]	1440 285.7 ± 116 344 [a]
Number of nauplii/tank/day	653 004 ± 73 466 [b]	1186 450 ± 103 853 [a]
Study II	**NAF**	**AF**
Mating success per day (%)	4.2 ± 0.5 [b]	10.9 ± 0.0 [a]
Spawning event per day (%)	82.3± 1.2 [a]	84.1 ± 2.6 [a]
Hatching rate per day (%)	55.5 ± 4.8 [a]	56.2 ± 3.5 [a]
Fertilization rate per day (%)	66.3 ± 3.4 [a]	71.9 ± 3.0 [a]
Mortality of female per day (%)	4.0 ± 0.1 [b]	8.2 ± 2.0 [a]
Number of eggs/spawned female/day	177 239 ± 10 821 [a]	145 729 ± 6325 [b]
Number of nauplii/spawnedfemale/day	89 406 ± 4674 [a]	73 493 ± 4718 [b]
Number of eggs/tank/day	619 938 ± 32 595 [b]	905 900 ± 79 601 [a]
Number of nauplii/tank/day	332 684 ± 21 366 [b]	511 382 ± 62 969[a]

Rows with different superscript letters are significantly different ($p < 0.05$, Tukey test).
*n value is based on number of daily observation as tanks from each treatment were collectively pulled. **n value was based on triplicate tanks. ***n = 62

There was an interactive effect of ablation and time on the number of eggs and nauplii per female per day in study I. A significant difference in egg production per female per day between treatments became apparent two weeks after the onset of the trial (Figure 3.1). After four weeks a significant difference was observed for nauplii production per female per day in study I (Figure 3.2). Within each treatment no significant difference was observed in fecundity (eggs or nauplii) per spawned female per day) over time (Figures 3.1

and 3.2). There was no interaction effect of ablation and time on the number of eggs and nauplii per per day in study II but effect of ablation only was observed and the data are already shown in Table 3.1.

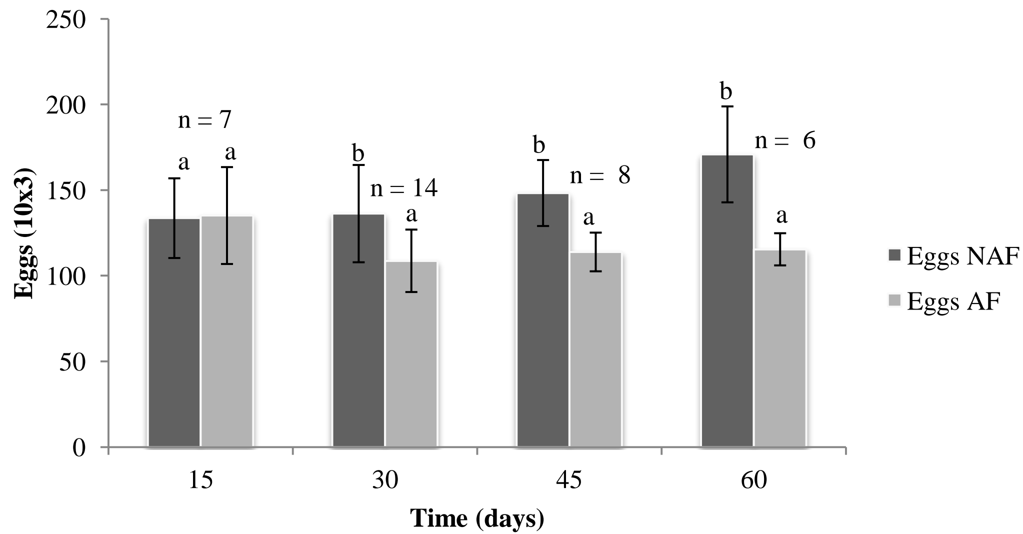

Figure 3. 1 Egg production (mean ± SE) per non- ablated (NAF) and ablated spawned female per day (AF) over time (Study I). Different superscript letters mean the graphs are significantly different at these time periods ($p < 0.05$, Tuckey test).

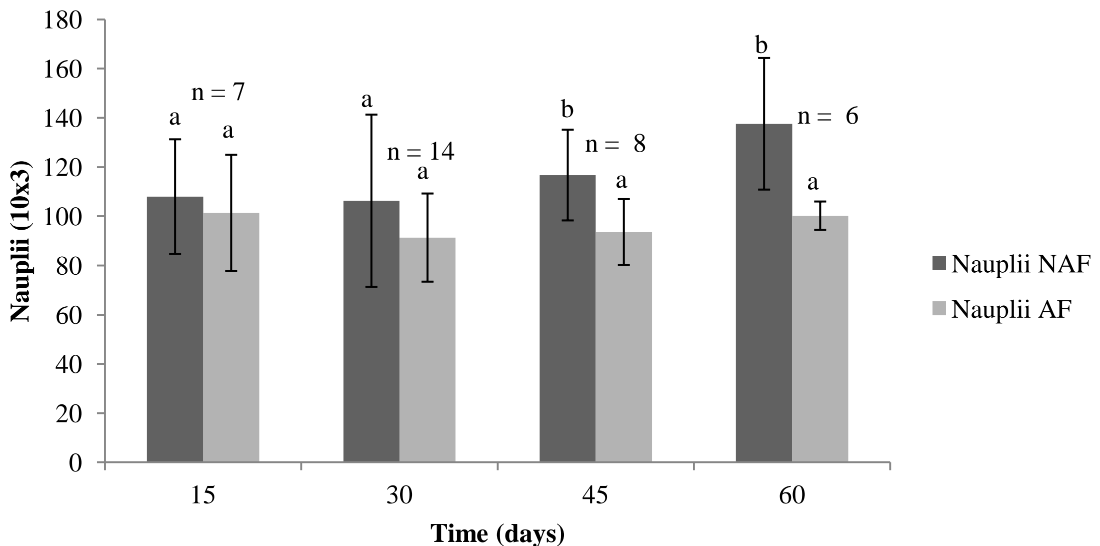

Figure 3. 2 Nauplii production (mean ± SE) per non-ablated (NAF) and ablated spawned female per day (AF) over time (Study I). Different superscript letters mean the graphs are significantly different at these periods ($p < 0.05$, Tuckey test).

3.3.2 Eggs, nauplii and postlarvae morphometric, larval rearing, nursery and grow-out

In both larviculture trials from study I (Figure 3.3) there was no significant difference in the daily larval stage index between the treatments. Similarly there was no significant difference between the treatments in response to a salinity stress test, and final survival and weight in Trial 1 (Table 3.2). In contrast, in Trial 2, postlarvae derived from NAF had significantly higher survival to salinity stress test than AF.

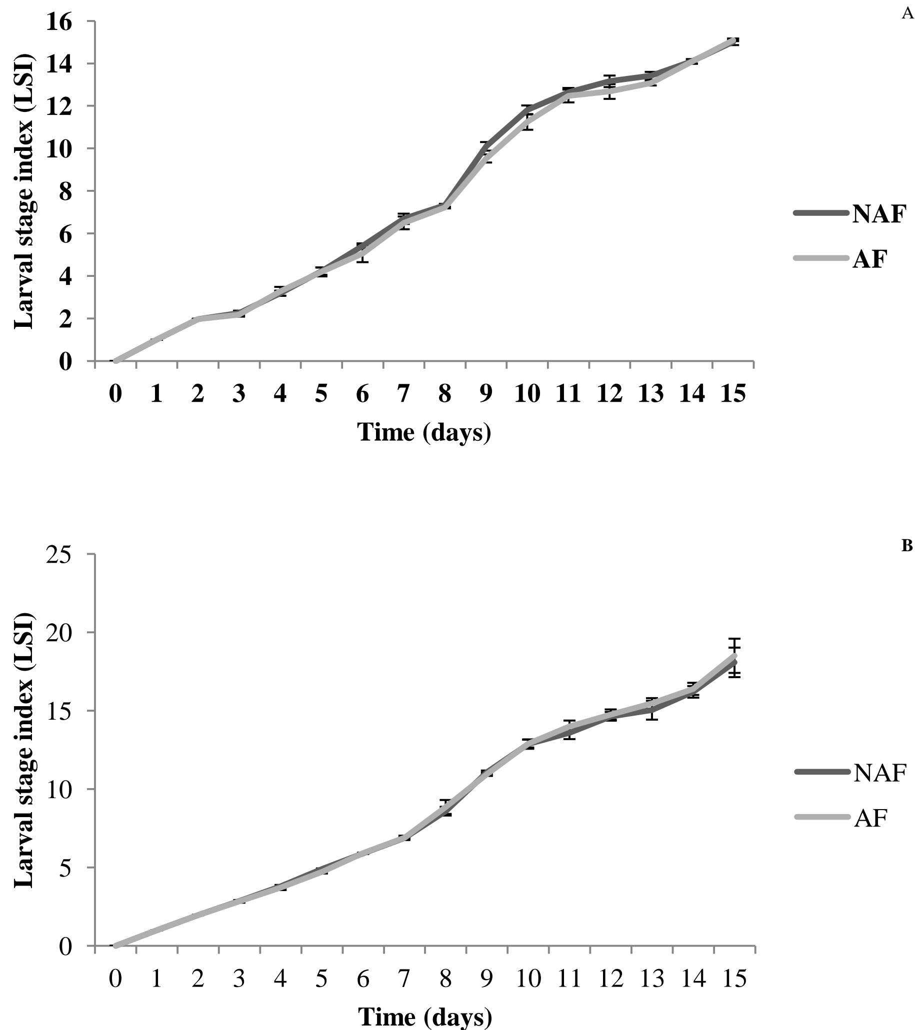

Figure 3. 3: Larval stage index (LSI) (mean ± SE) of *L. vannamei* from non-ablated (NAF) and ablated female broodstock (AF) during larviculture trials from study I (A – L I; B – L II) (n = 3 and n = 5, respectively). There was no significant difference at each time point between LSI from LI and LII ($p < 0.05$, Tuckey test).

Eggs of NAF in study II had similar diameters to the ones from AF (Table 3.2). Furthermore, nauplii and postlarvae lengths were not significantly different (Table 3.2). There was no significant difference between treatments in both trials (L III and IV) in final survival and weight; however postlarvae from NAF had significantly higher survival to salinity stress test than AF in both trials (Table 3.2).

Table 3. 2: Growth performance, final survival and survival to SST (salinity stress test) (mean ± SE) of *L. vannamei* PLs in larviculture trials conducted in study I (L I: n = 3; L II: n = 5) and II (L III: n = 3; L IV: n = 5),

Study I	**Larviculture I**		**Larviculture II**	
Parameters	**NAF**	**AF**	**NAF**	**AF**
Survival to SST (%)	94.7 ± 2.6 [a]	85.7 ± 6.1 [a]	97.4 ± 0.2 [a]	94.9 ± 0.5 [b]
Final survival (%)	48.0 ± 8.7 [a]	41.7 ± 7.0 [a]	48.8 ± 2.6 [a]	43.9 ± 5.6 [a]
Final weight (mg)	3.4 ± 0.3 [a]	3.7 ± 0.2 [a]	6.5 ± 0.6 [a]	6.7 ± 0.8 [a]
Study II	**Larviculture III**		**Larviculture IV**	
Parameters	**NAF**	**AF**	**NAF**	**AF**
Eggs diameter (µm)*	269.6 ± 8.5 [a]	264.3 ± 1.3 [a]	282.4 ± 1.1 [a]	282.9 ± 2.0 [a]
Nauplii length (µm)*	437.7 ± 5.4 [a]	451.0 ± 2.8 [a]	449.4 ± 3.1 [a]	445.2 ± 2.9 [a]
Survival to SST (%)	99.0 ± 0.0 [a]	96.0 ± 1.0 [b]	90.0 ± 0.6 [a]	87.7 ± 0.9 [b]
Final survival (%)	42.6 ± 3.7 [a]	42.1 ± 3.8 [a]	43.8 ± 3.3 [a]	41.0 ± 6.0 [a]
Final weight (mg)	6.7 ± 0.4 [a]	6.2 ± 0.1 [a]	5.0 ± 0.6 [a]	5.3 ± 0.9 [a]
PLs length (mm)	8.9 ± 0.3 [a]	8.7 ± 0.0 [a]	8.6 ± 0.4 [a]	8.6 ± 0.4 [a]

Rows with different superscript letters in each larviculture trial are significantly different ($p < 0.05$, Tuckey test). * n= 25

At the end of the nursery period from both studies, juvenile survivals were similar between the two treatments and also in final weight, weekly growth, feed conversion rate (FCR) and yield (Table 3.3). No significant differences between treatments were observed for all considered parameters during grow-out trials in both studies, including weight gain and specific growth rate (Table 3.3).

Table 3. 3: Growth performance and final survival (mean ± SE) of *L. vannamei* offspring from non-ablated (NAF) and ablated female (AF) in nursery and grow-out. (n = 3, except for nursery in study I which n = 2).

Nursery	**Study I**		**Study II**	
Parameters	**NAF**	**AF**	**NAF**	**AF**
Final Weight (g)	0.5 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.8 ± 0.1
Weekly Growth (g)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Final survival (%)	40.2 ± 7.5	45.4 ± 3.0	89.2 ± 2.3	92.2 ± 1.8
FCR*	0.9 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Yield (Kg/ha)/ (g/m3)	273.7 ± 32.8	340.6 ± 41.1	165.4 ± 14.6	188.4 ± 22.0
Grow-out				
Parameters	**NAF**	**AF**	**NAF**	**AF**
Initial weight (g)	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Final weight (g)	14.7 ± 0.5	14.9 ± 0.2	14.3 ± 0.0	14.5 ± 0.3
weight gain (g)	14.1 ± 0.5	14.2 ± 0.2	13.5 ± 0.0	13.7 ± 0.3
Weekly Growth (g)	1.2 ± 0.0	1.2 ± 0.0	3.1 ± 0.0	3.2 ± 0.01
SGR (%)**	3.7 ± 0.0	3.7 ± 0.0	8.9 ± 0.0	8.9 ± 0.1
Final survival (%)	51.7 ± 0.9	47.7 ± 2.5	93.0 ± 1.4	95.9 ± 0.8
FCR	1.3 ± 0.1	1.3 ± 0.1	0.7 ± 0.0	0.8 ± 0.0
Yield (Kg/ha)/ (g/m^3)***	1875.2 ± 27.6	1776.6 ± 82.8	591.6 ± 8.6	617.7 ± 7.5

*FCR – Feed conversion rate. ** SGR – Specific growth rate. Rows without superscript letters are not significantly different ($p > 0.05$, Tuckey test). *** Yield units are different between the two studies (Kg/ha is for Study I and g/m^3 for Study II).

3.4 Discussion

This is the first study to compare reproductive performance between NAF and AF under intensive commercial conditions, and to follow the impact of non-ablation on offspring development, growth and survival in larviculture, nursery and grow-out at commercial scale. Non-ablation of *L. vannamei* female eyestalk did not affect spawning frequency or hatching rate. It has previously been suggested that mated *L. vannamei* females did not or only partially release the eggs under typical hatchery conditions, possibly due to stress from a range of factors, such as inappropriate handling from maturation to the spawning tanks, noise in spawning rooms and poor water quality (FAO, 2003; Treece and Fox, 1993). The present study suggests that mated NAF *L. vannamei*

(with attached spermatophore) will spawn (release the eggs in spawning tanks) in the same way as AF individuals. Hatching success of eggs from NAF and AF *L. vannamei* were not significantly different which corroborated the earlier results reported by Chamberlain and Lawrence (1981) and Palacios et al. (1999b) with the same species, and in *Penaeus stylirostris* (Gendrop-Funes and Valenzuela-Espinoza, 1995), and *Penaeus canaliculatus* (Choy, 1987). This indicates that hatching rate may not be related to non-ablation of female shrimp but depend on other factors like the spawning tank environment (Ogle, 1995; FAO, 2003; Primavera, 1988; Treece and Fox, 1993), variable fertilization rate, and deterioration of the female during breeding time (reproductive exhaustion). The impact of broodstock ablation on subsequent hatching rate may also depend on the method used for ablation. The hatching rate of eggs from non-ablated *Penaeus indicus* were significantly higher than from females ablated by pinching the eyestalk but not from those ablated by tying and cauterizing (Makinouchi and Primavera, 1987). Choy (1987) also observed lower hatching rates of eggs from female *Penaeus canaculatus* ablated by pinching. This method is stressful for broodstock as pinched eyestalk takes longer to heal and hemolymph loss is greater than by cauterization and tying (Makinouchi and Primavera, 1987). In the present studies, where no difference in egg hatching was observed, cauterization was used on AF. However, hatching rates observed in Study II were lower than Study I. This may be associated to the fact that different families were used in each study. Broodstock performance can be affected by the genetic line used in maturation tanks (FAO, 2003; Racotta et al., 2003).

Eyestalk ablation acts to reduce the level of gonad inhibiting hormone which is responsible for limiting the reproduction process of shrimp in captivity (Alava and Primavera, 1979; Bae et al., 2013; Das et al., 2015; Raviv et al., 2006; Treerattrakool et al., 2014). This leads the *L. vannamei* female to mature and spawn frequently within a short time interval, thus the mating success in the current studies were more than double the level for AF compared to NAF, as was previously demonstrated by Chamberlain and Lawrence (1981) and Palacios et al. (1999b) with similar species and others (*P. canaculatus* and *P. stylirostris*) (Choy, 1987; Gendrop-Funes and Valenzuela-Espinoza, 1995). In contrast, mortality rates of AF females were almost double that of NAF confirming the trauma and stress associated with the technique (Bae et al., 2013; Choy, 1987; Taylor et al., 2004). Ablation has been linked to causing physiological imbalance,

reducing immunological defense, and to reduce reproductive productivity of female shrimps over time (Das et al., 2015; Palacios et al., 1999b; Treerattrakool et al., 2014; Lee et al., 21017). Depleted nutrient stores have been observed in AF which could explain lower survival rates of AF to NAF (Treece and Fox, 1993). The higher mortality in the current studies has also been observed in other research on AF. Mean mortality of NAF was considerably lower (3.7%) than AF (33.3%) of *Penaeus stylirostris* after 97 days. Santiago Jr. (1977) also observed lower mortality for NAF (61%) than unilateral (73%) and bilateral (100%) AF *P. monodom* over 265 days. Chamberlain and Lawrence (1981) and Palacios et al., (1999a) found similar mean and overall mortality rate on their study with *L. vannamei*, 41.7% and 48.3%, and 13% and 17% to NAF and AF after 97 and 90 days respectively.

In the present studies, NAF produced more eggs and nauplii per spawned female per day (>20% and >16% for study I, and >18% and >18% for study II, respectively) than AF which is in agreement with Choy (1987) and Emmerson (1980) with *P. canaliculatus* and *P. indicus*. In contrast, Makinouchi and Primavera (1987), Gendrop-Funes and Valenzuela-Espinoza (1995), Chamberlain and Lawrence (1981) and Palacios et al. (1999b) observed no significant difference in the number of eggs per spawn in *P. indicus, P. stylirostirs* and *L. vanammei*. Furthermore Gendrop-Funes and Valenzuela-Espinoza (1995) and Palacios et al. (1999b) reported no difference in nauplii per spawn between NAF AF of *P. stylirostirs* and *L. vanammei*.

Eyestalk ablation accelerates maturation and spawning process in shrimp, but it also affects many other aspects of shrimp physiology that are regulated by the X-organ Sinus Gland Complex (Choy, 1987; Das et al., 2015; Palacios et al., 1999a). For instance, levels of hormones such as molt inhibitory hormone (MIH) and crustacean hyperglycemic hormone (CHH) are found to be reduced (Sainz-Hernández et al., 2008; Racotta et al., 2003). Hence, a drop of MIH forces the female to molt more frequently, demanding additional energy, at the same time as nutritional demands associated with reproduction are elevated (Emmerson, 1983; Racotta et al., 2003). This will influence the reproductive performance of ablated female, especially under intensive commercial conditions, over time as nutritional resources required to support reproduction are likely to become limiting. This undoubtedly explains the reduced fecundity (lower amount of eggs and nauplii per

female per day or per spawn) and lower reproductive productivity associated with ablation observed here as time progressed and in other studies.

Tank production parameters of NAF were 44% and 45% lower than AF for eggs and nauplii in Study I, respectively. Similarly, tank production parameters of NAF were 31% and 35% lower than AF for eggs and nauplii in Study II, respectively. This was directly affected by higher mating success observed in AF animals and suggests that timing to meet a hypothetical daily production target for larvae would require either more broodstock or tanks or require rescheduling because of the lower level of breeding intensity in NAF than AF. For example, to produce 10 million nauplii using 1000 broodstock, AF would require 2 days and NAF 3 days. However, an interesting phenomenon has been observed in the industry; specifically the company where this study was conducted is the trend of increasing % of mating success over increasing generations of NAF. This might be a result of domestication and selective breeding program which should be considered when using NAF in maturation facilities.

Indeed, the rapid, consecutive maturation and spawning characterizing the reproductive performance of AF *P. indicus* (Emmerson, 1980) and *L. vannamei* (Browdy and Samocha, 1985; Palacios et al., 1999a) was shown to lead to exhaustion-related deterioratation, over 75 days in *L. vannamei*. Data from current studies did not find significant deterioration in fecundity of AF over our experimental period.

When comparing both groups at each time point, NAF produced more eggs per spawned female per day than AF from the third week onwards and more nauplii per spawned female per day from fifth week onwards on study I. This has also important implications for broodstock management and costs. Increasing productivity over time and improved longevity for NAF might compensate for the higher short-term productivity of AF. Such tradeoffs in reproductive performance are well known in the terrestrial livestock breeding literature e.g. De Vries (2017).

Shrimp broodstock condition is considered to be crucial for production of good quality of offspring (Racotta et al., 2003). Broodstock condition has both nutritional (Braga et al. 2015; Cardona et al. 2016; Emerenciano et al. 2013; Emerenciano et al., 2014; Hoa et al., 2009; Naessens et al., 1997; Perez-velazquez and Gonzaez-feliz, 2003; Racotta et al.,

2003; Wouters et al., 2001a; Wouters et al., 2001b;) and physiological components (Chamberlain and Lawrence, 1981; Lawrence, 1983; Palacios et al., 1999ab; Racotta et al., 2003). Theoretically, NAF broodstock would demonstrate better overall condition than AF and this would be reflected in differential quality of their offspring. However, four larviculture trials presented little evidence that larval growth and development over time was affected by broodstock ablation, based on larval stage index (LSI), final survival and length and weight of PLs. Palacios et al. (1999b) also did not find any difference when comparing larval survival from NAF and AF *L. vannamei* females in Zoea (from stage 1 to 3), Mysis (from stage 1 to 3) and PL1. Furthermore, these authors did not find difference in egg diameter from same spawn and nauplii length from NAF and AF. This was corroborated in Study II as no difference in egg diameter and nauplii length was observed. Lack of difference from early development stages (egg and nauplii) support the similarity on growth and final survival observed in four larviculture trials. However, there was some evidence for improved tolerance to salinity stress test in NAF derived PLs with a significant improvement in three of four independent tests. However, a better evaluation of PLs stress resistance would require multi-assessment approach in the future (i.e. using disease challenge as shown in Chapter 5 or use higher salinity values than the salinity of seawater) as the difference in salinity stress survival (2.3 – 3%) would not be relevant for the industry. Final weight of postlarvae from Study I were lower than in Study II because in the second study there was used lower stocking density which may have contributed to faster growth of the animals (Wickins, et al., 2002).

Nursery and grow-out trials were conducted using PLs of the second larviculture trials (study I and II), where the results of salinity stress suggested NAF produced larvae were more robust. Salinity stress tests are commonly used to date by farmers to evaluate postlarvae quality (Álvarez et al., 2004; Palacios and Racotta, 2007) as it is assumed that higher survival from this test would mean better performance during nursery and grow-out (Racotta et al., 2004; Palacios and Racotta, 2007). However, performance during nursery and grow-out phases demonstrated no differences between PLs from NAF and AF. This is consistent with the findings of Alvarez et al. (2004) who found no relationship between survival to a salinity stress test of PL15 and survival during grow-out. Apparently the effect of non-ablation is reflected in early shrimp development stage but not at the nursery and grow-out stage. Clearly other factors (environment, nutrition and management) affect

survival (Van Wyk et al., 1999; Alvarez et al., 2004) and growth (Van Wyk et al., 1999; Wickins and Lee, 2002) of shrimp during nursery and grow-out more than broodstock condition alone. This study finding also support the idea that a better evaluation of PLs stress resistance would require multi-assessment approach in the future as the difference in salinity stress survival (2.3 – 3%) was not reflected in further farming stages. The difference between Study I and II on grow-out yield is associated to difference in experimental set up. In Study I there were used commercial semi-intensive earthen ponds stocked with more animals (455000 shrimp each) whilst in Study II there were used small cages stocked with only 396 shrimp each.

Broodstock restocking in maturation and reproduction tanks is a common hatchery practice to refresh the broodstock performance or increase production. At first it was hypothesized that the restocking made in study I might have affected the reproductive performance and offspring quality results. Therefore, there was needed to run a second study without restocking. Although no restocking was done in study II, the findings were similar to study I.

Overall, the results of this indicate that a comparable level of productivity (eggs and nauplii per tank) can be realised by an intensive commercial maturation facility based on NAF system. However, the calculation has to take into account changes in productivity as well as mating success and mortality of female. The managers should also consider other factors such as the sex ratio used in their production tanks. Normally, hatcheries based on AF system use 1:1 male to female ratio and if they switch to NAF system would require increasing sex ratio to 1:2 male to female in order to get same productivity observed in 1:1 sex ratio. It might be expected that hatchery scale would determine the impact of a switch from AF to NAF with larger scale hatcheries most likely to invest in more capacity. A major advantage of using NAF would be the extended productive lifespan of these expensive animals but a full analysis of this potential advantage would require commercial level data over several years of production. Furthermore, a trend of increasing percentage of mating success of NAF over increasing generations of closed-cycle breeding has been observed in the industry. This demonstrates the potential of selective breeding program for rapid maturation of NAF. The best evidence for the commercial viability of the approach is the wholesale switch of Seajoy to using NAF. In addition, there are also some regular

maturation facilities using NAF in Central and Latin America (P. Sorgeloos, 2020. *pers. commun.*)

In conclusion, commercial intensive hatcheries can use a NAF production based system as long as some hatchery management practices are changed, i.e. on sex ratio, selective breeding program. The hatchery managers and farmers should be confident that offspring derived from non-ablated female of *L. vannamei* perform comparably in all culture stages and in addition there is in fact evidence of greater resilience to stress in NAF produced PLs.

Chapter 4: Effect of pre-maturation conditioning on reproductive performance, offspring quality and biochemical composition of non-ablated Pacific white shrimp (*Litopenaeus vannamei*) female

4.1 Introduction

Several researchers have evaluated alternatives to unilateral eyestalk ablation, based on environmental control (Chamberlain and Lawrence, 1981b; Emerson et al., 1983; Wickins and Lee, 2002) and manipulation of the endocrine systems (Siangcham et al., 2013; Tinikul et al., 2014; Li, et al., 2015; Sathapondecha et al., 2015; Tomy et al., 2016) Nevertheless, environmental parameters have been considered inadequate in inducing sufficient spawning to meet commercial production schedules for *L. vannamei* (Chamberlain and Lawrence, 1981b; Wickins and Lee, 2002). Therefore, the industry has so far preferred to rely on the manipulation of the endocrine system, using unilateral eyestalk ablation, to improve reproductive performance (Lawrence, 1983; Palacio et al., 1999a; Wickins and Lee, 2002; Little et al., 2018).

Potential molecules that have also been assessed to date as alternatives to induce maturation and spawning in shrimp including *L. vannamei* are peptides, steroids and juvenoid hormones, neurotransmitters and neurotransmitter antagonists (Alfaro et al., 2004). In addition gonadal inhibiting hormones antibodies and RNA interference technology to silence GIH expression have been developed to replace unilateral eyestalk ablation (Treerattrakool et al. 2011; Treerattrakool et al., 2014; Das et al., 2015; Feijó et al., 2016). However, none of these methods have been validated and adopted on a commercial scale. This might partly be explained by the need to deliver the test compounds into broodstock shrimp by injection, which is both impractical for commercial scale production and likely to cause more stress for shrimp compared to unilateral eyestalk ablation (Racotta et al., 2003). Reproductive performance and larval quality of females induced by hormones, endocrine glands extract, gonadal inhibiting hormones antibodies and RNA interference technology injections have not been evaluated in the previous studies, so their effects on the resulting offspring is still unclear.

In Chapter 3 it was demonstrated that a comparable level of productivity (eggs and nauplii per tank) could be realised by an intensive commercial hatchery based on NAF broodstock. However, some hatchery management practices, such as sex ratio of broodstock, would have to be changed, i.e. sex ratio (from 1:1 to 1:2, male and female, respectively). Recent development in some commercial hatcheries in Central America, including Seajoy, have suggested that feed management and nutrition can accelerate

maturation and natural reproduction of *L. vannamei* in captivity, at commercial scale and sex ratio management would not be required. Nutritional factors play a critical role in the stimulation of sexual maturation and mating, the enhancement of fertility, and the viability and quality of offspring (Yano, 1995; Naessens et al., 1997; Wouters et al., 2001a; Du et al., 2004; Cardona et al., 2016; Emerenciano et al., 2013; Xu et al., 2017). Natural feeds such as squid, mollusks (mussels, clams, and oysters), fish roe, marine polychaetes and crustaceans (shrimp, crab, enriched Artemia biomass) provide essential nutrients for sexual maturation and reproduction in shrimp (Wouters et al., 2001a; Hoa et al., 2009; Emerenciano et al., 2013). In general, as a commercial practice, the supplementation of fresh-frozen natural feeds occurs when the selected shrimp broodstock are transferred to maturation facilities.

In contrast pre-maturation conditioning (PMC) occurs in between the grow-out and maturation phase, when shrimp diets are supplemented with fresh-frozen natural feeds (Emerenciano et al., 2013; Braga et al., 2015). It improves reproductive performance of unilateral AF *L. vannamei* in terms of better egg and nauplii production, faster onset of spawning, and increases the level of highly unsaturated faty acids (HUFA) in eggs compared to controls (Emerenciano et al., 2013). Additionally, Braga et al. (2015) found that *L. vannamei* males reared in bioflocs as pre-maturation conditioning exhibited better spermatophore and sperm quality than under clear water system.

The potential role of PMC for improving productivity of NAF Pacific white shrimp to offset the lower mating success compared to ablation on maturation facility performance has yet to be determined. This research was set out to evaluate the impact of fresh-frozen natural feeds prior to maturation and reproduction conditioning on reproductive performance, offspring quality and biochemical composition of non-ablated *L. vannamei* broodstock. A key objective of this work is to provide evidence to commercial hatcheries of PMC methods that improve reproductive success of NAF at commercial scale.

4.2 Material and Methods

4.2.1. Experimental design and rearing conditions

4.2.1.1. Pre-maturation

This study was conducted in the Larvicultura del Pacífico (LARVIPAC), commercial hatchery of Seajoy company in Honduras. *L. vannamei,* mixed sex, broodstock with average male and female individual weight, 34.4 ± 3.6 and 35.2 ± 1.2 g respectively, previously reared in earthen ponds were used in the trial. One treatment (Fed dry feed, squid and polychaetes – DF&NF) and control (Fed only dry feed following the normal approach – DF) were considered at this stage (Figure 4.1).

PMC was carried out in geomembrane tanks (41.8 m^2), in which 2096 males and 2109 females were reared separately in single tanks and fed chopped defrosted squid *Loligo opalescent* (approximately 0.6g per piece) three times a day (at 12:00 pm, 10:00 pm and 5:00 am) at 5.4% of biomass (ww) split between three feeds, polychaete *Nereis virens* (9:00 am and 3:00 pm) at 2.7% of biomass (ww) split between two feeds, and dry feed - Maturation, Zeigler, USA (7:00 am, 8:00 pm and 3:00 am) at 4% of biomass (dw) split between three feeds, - DF&NF (Dry feed and fresh-frozen natural feed). The control treatment was conducted in 2 separate tanks, at the same stocking density but animals were fed dry feed (DF) only (Maturation, Zeigler, USA) at 7:00 am, 9:00 am, 12:00 pm, 3:00 pm, 8:00 pm, 10:00 pm, 3:00 am and 5:00 am at 12% of biomass (dw) daily and split between eight feeds. Throughout the course of the trial feeding rate was adjusted weekly based on observed consumption rate and maturation index. Feeding rate of fresh-frozen natural feeds was increased significantly on days 29 to 42 post start of pre-maturation compared to the first days (from 5.4% and 2.7% of squid and polychaete to 24% and 15% of biomass respectively), whilst the dry feed was only increased by 1%. The feed in the control treatment was not increased.

Water temperature, salinity, dissolved oxygen, pH, alkalinity and ammonia were maintained at 31.3 ± 0.0 ºC, 32.0 ±1.0 ppt, 4.4 ± 0.0 mg/L, 7.4 ± 0.0, ≥ 100 mg/L $CaCO_3$ and < 1 mg/L respectively. The animals remained in pre-maturation conditioning for 60 days before being transfered to the maturation stage. Replacement animals were selected from earthen ponds and stocked into the pre-maturation tanks as in the flow diagram

(Figure 4.1) (275 males per male's tank; 600 females per female's tank) ten days later. Mortalities were recorded daily and removed from the tanks.

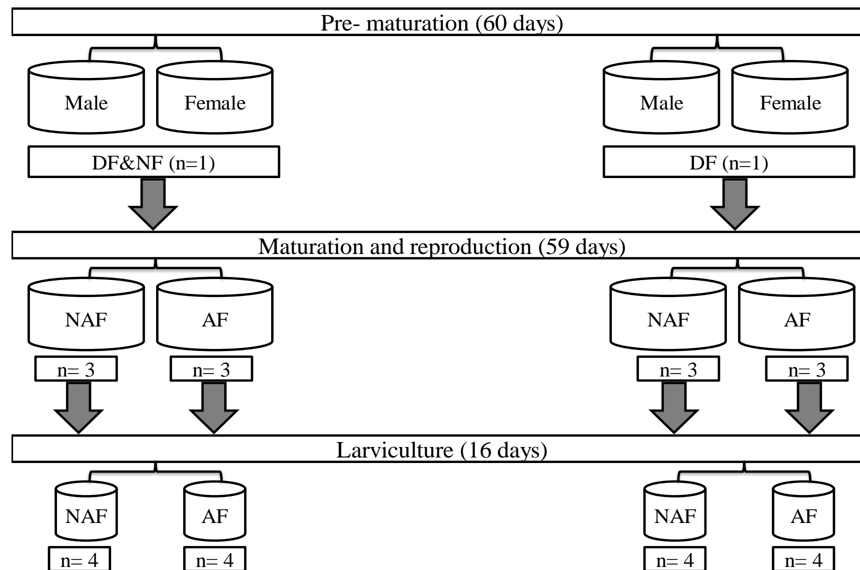

Figure 4. 1: Schematic diagram of experimental design. DF&NF - Dry feed and supplementary feed treatment; DF – Dry feed treatment; NAF – Non-ablated female; AF – Ablated female.

4.2.1.1.1 Male, female and feed sampling

On day 1, 22 and 45 post PMC, 10 males from each treatment were randomly sampled using hand net, weighed, their spermatophores ejaculated and then weighed according to Perez-Velazquez (2001). Each spermatophore was then individually homogenized following the methods previously described (Leung-Trujillo and Lawrence, 1987) to evaluate sperm quality (sperm counting and percentage of dead cell) per compound spermatophore.

During the same sampling period, 10 female from each treatment were randomly sampled, weighed and checked for their maturation stage based on the external observation of ovarian size and colour as described by Alfaro et al. (2004). Subsequently, their hepatopancreas and ovaries were dissected, weighed, placed in a 1.5 ml Eppendorf tube and immediately frozen at -23 ºC for biochemical analysis. Tissues were removed through

an incision on the back of the cephalothorax and dorsal region of the entire abdomen length. Sampled animals were killed by thermal shock which consisted of putting the animals in iced water with temperature of 4 ◦C for 30 to 60 seconds. Female maturation stage was expressed as a Maturation Index (M.I) based on the Equation 4.1 first reported by Alfaro et al. (2004). Hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as the percentage of the hepatopancreas and ovary weight relative to each individual body weight (Tahara and Yano 2004). Triplicate samples of squid, polychaete and dry feed of approximately 4 g fresh basis each were also collected during the pre-maturation stage, placed in 1.5 Eppendorf tube and immediately preserved at -23 ºC for biochemical analysis.

$$\text{Equation 4.1: MaturationIndex} = \frac{\text{Females in stage III and IV}}{\text{Total number of females}}$$

It is important to highlight that no male or female sampling was done between days 46 to 60 as it was originally planned to end PMC on day 45. However due to higher production demand from the Seajoy in that period, the maturation and reproduction facilities were not available as planned. Therefore there was a need to wait (up to 15 days) until facilities became available. Only maturation stage could be recorded.

4.2.1.2. Maturation and reproduction

The maturation tanks system is the same used in Chapter 3 (section 3.2.1) which is described in Chapter 2 (section 2.1). Males and females from DF&NF and DF were used for reproductive performance assessment. Twelve maturation tanks (round geomembrane tanks, dark-colored; 22.9 m^2 area) were used in total; six were used to stock females from the DF&NF group. Of these, 3 tanks were used for non-ablated (DF&NF/NAF) and three were used for ablated (DF&NF/AF) animals. The remaining six tanks were stocked with females from the DF treatment, also equally distributed between DF/NAF and DF/AF (Figure 4.1). Three days before starting the maturation experiment, unilateral eyestalk

ablation was performed on control females by cauterization. DF&NF and DF males were stocked in respective tanks one day prior commencement of the experiment. Each tank was stocked with 225 animals ($9.8/m^2$) with a sex ratio of 1:2 (male to female). The animals were randomly allocated to maturation tanks.

Water temperature, salinity, dissolved oxygen, pH, alkalinity and ammonia were maintained at 30.4 ± 0.2 ºC, 32.0 ±1.0 ppt, 4.3 ± 0.0 mg/L, 7.3 ± 1.0, ≥ 100 mg/L $CaCO_3$ and < 1 mg/L respectively. The broodstock remained in the maturation tanks for 59 days and were fed daily with squid four times a day (9:00 am, 2:00 pm, 8:00 pm and 5:00 am) at 15% of biomass (ww), and mussels three times a day (7:00 am, 12:00 pm and 3:00 pm) at 8% of biomass (ww). All fresh feeds were defrosted and chopped to individual pieces weighing approximately 0.6 g. In addition, a commercial maturation diet EZ-mate (55% Protein, Zeigler, USA), was also applied once a day (10:00 pm) at 4% of biomass (ww) according to manufacturer's recommendations. Each tank was restocked with other animals 26 days after beginning of the experiment. Each tank was restocked (replace deceased animals) to ensure a stocking density of 11.8 m^{-2} and sex ratio of 1:2 (male to female). 265 and 281 DF&NF females were restocked in triplicate DF&NF/NAF and DF&NF/AF tanks respectively. In addition DF&NF/NAF and DF&NF/AF groups received 102 and 96 males respectively. Regarding DF treatments, DF/NAF and DF/AF group were restocked with 299 and 353 females, and 99 and 88 males respectively.

4.2.2. Reproductive performance and broodstock mortality

Reproductive performance was evaluated daily over the 59 day study. Gonad development was monitored by visual assessment three times daily with a lamp, between 4:00 to 7:00 pm, by observing the size and colour of the gonad through the exoskeleton. Mature females with an attached spermatophore were taken from each maturation tank and transferred to respective 500 L spawning tank. After egg release, females were returned to their respective maturation tank at midnight, and eggs were collected from each spawning tank and transferred to a 25L bucket. Three 5mL subsamples of water per tank were taken for volumetric assessment of number of eggs in each tank. Groups of eggs from each spawning tank were subsequently transferred to an illuminated 500 L fibreglass hatching

tank and kept for 13 h. Each hatching tank was aerated constantly using one air stone per tank and temperature and salinity were maintained at 32 ± 0.5ºC and 30 ± 0 ppt. Nauplii were then collected exploiting their positive phototropic behaviour, transferred to a 25L bucket, where volumetric assessment of numbers was undertaken for each replicate using the same method as for eggs.

As the females from each tank were collectively managed, reproductive performance parameters were estimated per unit time period (days) (Bhujel, 2008) as described in Chapter 2.

4.2.2.1 Hepatopancreas, ovaries, eggs and nauplii sampling

26 days after the beginning of maturation and reproduction stage, six mature female and with attached spermatophore were randomly selected from each tank, weighed and their hepatopancreas and ovaries collected, weighed, placed in a 1.5 ml Eppendorf tube and immediately preserved at -23 ºC for biochemical analysis. Similar procedure was used at the end of the trial (59 days post reproduction start). The first sampling point was chosen because it was the last day before restocking the tanks with other animals to compensate for mortality. So, there was only one restocking. Hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as previously described.

Triplicate samples of eggs and nauplii mass of nearly 100 mg each per treatment were collected directly from spawning and hatching tanks, carefully dried with a paper towel and then placed in a 1.5 ml Eppendorf tube and immediately preserved at -23 ºC for biochemical analysis. Eggs and nauplii were collected from each tank at 7, 14, 35, 42 and 59 days after the start of the study. Eggs and nauplii from days 7 and 14, and 35 and 42 were each pooled to represent only two points and also to have enough egg and nauplii mass for the biochemical analyses.

4.2.3. Larval rearing

One larval rearing trial was conducted using larvae (nauplii 5) from four treatments (Figure 4.1) produced at almost the end (day 50) of the observation period as eyestalk

ablated animals are reported to deteriorate over time because of exhaustion (Palacios et al., 1999a) and this might affect the quality of the offspring. Restocking at 25 days post study inception did not have a significant impact on the broodstock fecundity response as was demonstrated in Chapter 3. Sixteen fibreglass tanks (500L) with an initial volume of 250L (220L seawater + 30L algae) were stocked with 30 000 nauplii each at a density of 120/L on the first day. Each treatment was quadruplicated, randomly distributed in a greenhouse and stocked with larvae from a collective pool originated from triplicate maturation tanks. Algae (*Thalassiosira* sp) were added daily to the tanks at a concentration of 100 000 – 130 000 cells/mL until the shrimp reached 2-day-old post-larval stage (PL2), and then 25% of water was renewed daily until the end of the trial following the same protocol from Chapter 3. The diet was also composed of *artemia sp*. (from Mysis 1 to PL6) and artificial liquid and dry diets (Frippak – Inve Aquaculture; liqualife ZM and MPL – Cargill; liquid hatchery feed – Epicore; and brine shrimp flake – Mackey Marine (from Zoea 1 to PL12-PL14) in accordance with manufacture recommendations. Water temperature, salinity, dissolved oxygen, pH, ammonia, nitrate, total suspended solids and alkalinity were maintained at 29. ± 1.0 ºC, 28.0 ± 1.0 ppt, 4.5 ± 0.5 mg/L, 7.3 ± 0.0, < 1 mg/L, 1.8 ± 0.1, 38.2 ± 1.8 and 100 ± 1.1 mg/L $CaCO_3$ respectively.

During the trial, larval stage development was determined at protozoea 1, mysis 1, postlarvae 1 and 10 using a larval stage index (LSI) (Uno and Kwon, 1969). At the end of the trial individual mean wet weight and survival were estimated following the procedure from Chapter 3. Postlarval quality was also assessed using a salinity stress test at the end of the trial also following the protocol from Chapter 3. Triplicate samples of PLs10-11 of nearly 0.8 g each per treatment were collected at the end of the trial, carefully dried with a paper towel and then placed in a 1.5 ml Eppendorf tube and immediately preserved at -23 ºC for biochemical analysis.

4.2.4. Biochemical analyses

Total lipid and fatty acids content of feed (squid, polychaete and dry feed-Maturation, Zeigler) and tissue samples (hepatopancreas, ovaries, eggs, nauplii and postlarvae) was analyzed. Total lipids were extracted using methods described by Folch et al. (1957) and Christie (2003), whereas fatty acids compositions were determined by gas

chromatography of fatty acid methyl esters (FAME) prepared using acid-catalyzed transesterification of total lipid (Christie, 2003). Due to the lower amount of eggs and nauplii samples, protein and carbohydrate analyses were only done on feed, hepatopancreas and ovaries. Crude protein was determined by the Kjeldahl method using the Tecator Kjeltec system (Person, 2008) while total carbohydrate were analyzed following a modified Dubois phenol-sulphuric method (Dubois et al., 1956).

Dry feed had a higher content of carbohydrate, total monounsaturated fatty acids, total n-6 PUFA and linoleic acid (LA) than squid and polychaetes (Table 4.1). On the other hand, dry feed had significantly lower levels of protein, total n-3 PUFA, eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA) than squid and polychaete. Squid had higher protein, total monosaturated fatty acids, total n-6 and n-3 PUFAs, and DHA than polychaete. Furthermore it had less linoleic acid than polychaetes.

Table 4. 1: Biochemical content of natural feed (squid and polychaete) and dry feed (Maturation, Zeigler, USA) used during pre-maturation of 60 days under commercial scale. (Mean ± SE) (n=3).

Parameters (mg/g)*	**Squid**	**Polychaete**	**Dry Feed**
Proteins	907.2 ± 23.8^{a}	414.0 ± 10.3^{b}	357.7 ± 1.5^{c}
Carbohydrates	30.3 ± 4.4^{b}	46.6 ± 3.7^{b}	274.4 ± 11.0^{a}
Total lipids	121.9 ± 1.3^{b}	137.7 ± 1.8^{a}	117.4 ± 0.1^{b}
Total saturated	16.6 ± 0.4^{a}	15.3 ± 1.3^{a}	17.7 ± 0.3^{a}
Total monounsaturated	6.3 ± 0.0^{b}	3.6 ± 0.4^{c}	23.5 ± 0.3^{a}
Total n-6 PUFA	1.3 ± 0.0^{b}	0.9 ± 0.0^{c}	25.5 ± 0.4^{a}
Total n-3 PUFA	31.5 ± 1.3^{a}	20.2 ± 0.4^{b}	9.5 ± 0.1^{c}
18:2n-6	0.2 ± 0.0^{c}	0.9 ± 0.0^{b}	24.8 ± 0.4^{a}
20:4n-6	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.3 ± 0.0^{a}
20:5n-3	8.1 ± 0.4^{a}	8.1 ± 0.4^{a}	3.7 ± 0.1^{b}
22:6n-3	22.5 ± 0.9^{a}	3.1 ± 0.4^{b}	0.2 ± 0.1^{c}

*Data are shown on dry matter basis. Rows with different superscript letter are significantly different ($p < 0.05$, Tuckey test).

4.2.5. Statistical analyses

All data were analysed using Statisica 7.0. Two-way ANOVA followed by a Tukey test (Zar, 2010) was used to compare the data in pre-maturation stage. Regression analysis was used to verify the relationship between total lipids in the hepatopancreas and ovaries in

the pre-maturation stage as it has been reported that some of *L. vannamei* ovaries lipid does not derivate from hepatopancreas storage. Data from reproductive performance, larviculture and biochemical contents of postlarvae were analyzed by two-way ANOVA using feed as the first variable (DF&SF and DF) and ablation as the second variable. Female broodstock hepatopancreas during reproduction, eggs and nauplii biochemical contents were analyzed by factorial ANOVA with the first variable as feed, second as ablation and third as time. Normality and homogeneity of variance of the data were tested using Shapiro-Wilk and Levene tests, respectively. Percentage data were transformed to arcsine square-root prior to statistical analyzes. Data indicating non-normal distribution or homogeneity were transformed using either square-root or Log/Ln. All comparisons were conducted at a significance level of 0.05. All data are presented as mean ± standard error.

4.3 Results

Some results presented in tables were not significantly affected by factors interaction (i.e. feed and time, feed and ablation, ablation and time, or feed, ablation and time). Therefore, where the interactions were not observed, the difference was reported in the text to consolidated values purely in relation to a specific factor. However, the original data where factor interaction was not observed are presented in the tables.

4.3.1. Pre-maturation

Overall male and female survival in the treatment (DF&NF) and control (DF) over the 60 days of pre-maturation were 74.2% and 72.7%, and 81.2% and 86.7% respectively. The mean maturation index averaged over the length of the pre-maturation study was lower for experimental treatments (40.0 ± 13.2%) than control (75.0 ± 8.2%). Figure 4.2 shows that control females advanced to maturation by day 22, whilst for DF&NF females only by day 45; by day 60, both groups had a similar maturation index. There was a significant interaction between feeding and time on the GSI levels observed with the greatest difference being apparent at day 22 where GSI was significantly higher in the DF compared to the DF&NF treatment (Table 4.2). HSI was significantly influenced only by

time and was significantly lower at the end (3.4 ± 0.1, 3.8 ± 0.2 and 3.1 ± 0.1 for days 1, 22 and 45, respectively) (Table 4.2). Similarly, spermatophore weight (32.8 ± 0.0, 36.9 ± 0.0 and 46.8 ± 0.0 mg for days 1, 22 and 45, respectively) and sperm count (14.7 ± 1.7, 26.5 ± 3.4 and 29.8 ± 2.9 for days 1, 22 and 45, respectively) were only affected by time (Table 4.2). Spermatophore weight and sperm count were significantly higher on day 45.

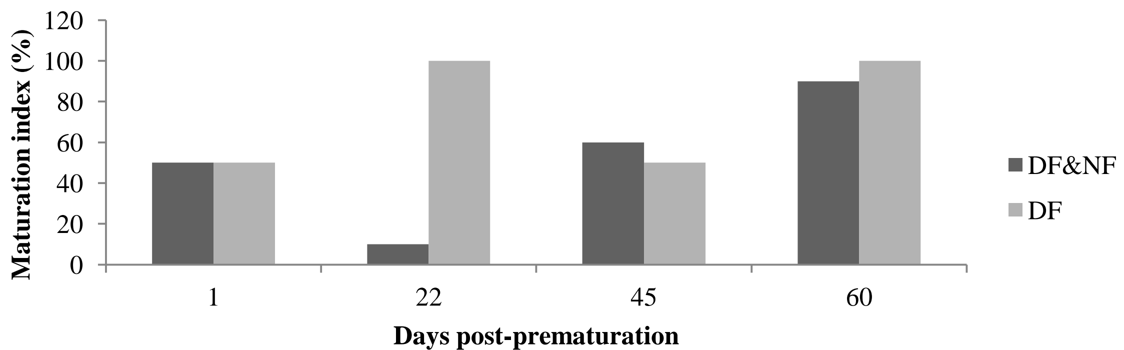

Figure 4. 2: Maturation development over time of female fed dry feed and fresh-frozen natural feed (squid and polychaete) (DF&NF), and only fed dry feed (DF).

Table 4. 2: Hepatosomatic index (HSI), gonadosomatic index (GSI) and sperm quality of female and male fed dry feed and natural feed (squid and polychaete) (DF&NF) and only dry feed (DF) during pre-maturation.

Time (Days)	1		22		45		Signficance		
Parameters	DF&NF	DF	DF&NF	DF	DF&NF	DF	F	T	FxT
GSI (%) (n= 10)	1.5 ± 0.3^{ab}	1.7 ± 0.6^{ab}	0.9 ± 0.1^{b}	2.4 ± 0.2^{a}	1.7 ± 0.4^{ab}	1.5 ± 0.3^{ab}	ns	ns	*
HPSI (%) (n= 10)	3.5 ± 0.2	3.5 ± 0.3	3.4± 0.1	4.2 ± 0.2	3.1 ± 0.3	3.1 ± 0.3	ns	*	ns
Spermatophore weight (mg) (n= 7)	35.2 ± 2.5	30.4 ± 2.9	38.5 ± 4.6	46.0 ± 4.6	43.0 ± 3.3	50.6 ± 5.9	ns	*	ns
Sperm Count (10^6) (n= 7)	17.5 ± 2.5	11.8 ± 1.9	24.1 ± 4.5	28.9 ± 5.2	35.6 ± 3.2	24.1 ± 4.0	ns	*	ns
Dead Sperm (%) (n= 7)	21.1 ± 7.4	22.8 ± 7.4	28.2 ± 8.7	17.2 ± 3.8	28.3 ± 6.0	32.2 ± 2.7	ns	ns	ns

ns – not significant. Rows with different supscript letter are significantly different ($p < 0.05$, Tuckey test)

Hepatopancreas carbohydrate was similar over time and was not affected by the feeding regime in females, whilst protein was affected only by feed (156.2 ± 7.1 and 137.6 ± 5.6 mg/g for DF&NF and DF, respectively) and time (138.6 ± 4.3, 139.3 ± 4.3 and 162.8 ± 11.7 mg/g for days 1, 22 and 45, respectively). The protein was higher in the hepatopancreas of DF&SF animals and on day 45 (Table 4.3). Total lipids, saturated, monosaturated and n-6 PUFA, and linoleic acid (LA) were affected by feed and time, and interaction was observed. Total n-6 PUFA, ARA, EPA and DHA were only affected by

time although there was significant interaction between feed and time. Total lipid and fatty acids of the hepatopancreas were significantly lower only on day 22 for DF&NF females (Table 4.3).

Ovary protein was similar between both groups over time and was not affected by the feed and no interaction over time was observed (Table 4.3). There was time effect and interaction with feed on carbohydrate (Table 4.3). Carbohydrate was significantly lower in the DF group than DF&NF treatment on day 22 and it was also significant lower than the later time point. There was no effect of feed and time on total lipid and EPA but interaction between the factors was observed and levels were significantly lower for DF&NF on day 22 (Table 4.3). There was no effect of feed, time and interaction of feed and time on total monosaturated and saturated fatty acids, total n-3 PUFA and DHA (Table 4.3). Total n-6 PUFA (6.1 ± 0.9 and 9.0 ± 0.9 mg/g for DF&NF and DF, respectively) and LA (4.8 ± 0.7 and 7.5 ± 0.8 mg/g for DF&NF and DF, respectively) were only affected by feed. Total n-6 PUFA and LA were significantly higher in DF group. ARA was not affected by feed and time interaction, however there was time effect (1.1 ± 0.1, 0.5 ± 0.1 and 0.7 ± 0.1 mg/g for days 1, 22 and 45, respectively) (Table 4.3). ARA was significantly lower on day 45.

To confirm the extent to which the hepatopancreas lipid content affected the ovary lipid levels, there was a need to run regression analysis. The coefficient of determination on the relationship between total lipid in the hepatopancreas and ovary was lower (r^2= 0.49) which indicate that there are 51% of other factors contributing to ovary lipid content. However, there was significant relationship between the two factors (r= 0.7).

Table 4. 3: Biochemical contents of hepatopancreas (HP) and ovaries (OV) from female fed dry feed and fresh-frozen natural feed (squid and polychaete) (DF&NF) and only dry feed (DF) during pre-maturation. (Mean ± SE) (n = 1).

Time (Days)	**1**		**22**		**45**		**Signficance**		
Parameters (mg/g)	**HP DF&NF**	**HP DF**	**HP DF&NF**	**HP DF**	**HP DF&NF**	**HP DF**	**F**	**T**	**FxT**
Proteins	141.9 ± 6.0	135.2 ± 6.7	144.4 ± 2.2	134.3 ± 7.9	182.4 ± 7.1	143.3 ± 15.1	*	*	ns
Carbohydrates	60.3 ± 3.5	52.4 ± 10.4	65.2 ± 5.5	73.7 ± 6.5	79.2 ± 8.6	67.4 ± 14.7	ns	ns	ns
Total lipids	286.1 ± 29.2^{a}	$276.3 \pm 21.6.4^{a}$	24.9 ± 0.2^{b}	240.7 ± 25.3^{a}	145.8 ± 34.4^{a}	171.2 ± 59.5^{a}	*	*	*
Total saturated	56.3 ± 6.0^{a}	52.0 ± 5.9^{a}	2.9 ± 0.1^{c}	41.1 ± 4.1^{a}	26.9 ± 6.9^{b}	29.4 ± 10.7^{b}	*	*	*
Total monounsaturated	80.7 ± 9.3^{a}	80.4 ± 7.0^{a}	2.1 ± 0.2^{c}	64.1 ± 6.7^{ab}	31.1 ± 9.0^{b}	43.3 ± 14.8^{b}	*	*	*
Total n-6 PUFA	64.7 ± 8.8^{a}	67.9 ± 4.8^{a}	2.0 ± 0.2^{c}	59.4 ± 6.4^{a}	23.6 ± 7.7^{b}	39.3 ± 14.0^{b}	*	*	*
Total n-3 PUFA	34.6 ± 2.1^{a}	27.7 ± 2.1^{a}	3.0 ± 0.0^{b}	23.8 ± 2.4^{a}	24.0 ± 6.8^{a}	16.7 ± 5.9^{a}	ns	*	*
18:2n-6	56.0 ± 8.1^{a}	59.5 ± 4.4^{a}	1.5 ± 0.2^{c}	53.8 ± 6.0^{ab}	20.7 ± 7.0^{b}	34.8 ± 12.6^{b}	*	*	*
20:4n-6	1.2 ± 0.1^{a}	1.0 ± 0.1^{ab}	0.3 ± 0.0^{c}	0.7 ± 0.1^{ab}	0.8 ± 0.1^{ab}	0.7 ± 0.2^{ab}	ns	*	*
20:5n-3	7.6 ± 0.4^{a}	5.4 ± 0.5^{a}	1.2 ± 1.0^{c}	5.2 ± 0.5^{ab}	5.9 ± 1.5^{a}	4.1 ± 1.3^{ab}	ns	*	*
22:6n-3	15.6 ± 1.2^{a}	12.3 ± 0.8^{a}	3.0 ± 0.0^{c}	9.9 ± 0.8^{ab}	13.2 ± 3.7^{a}	7.2 ± 2.8^{ab}	ns	*	*
Parameters (mg/g)	**OV DF&NF**	**OV DF**	**OV DF&NF**	**OV DF**	**OV DF&NF**	**OV DF**	**F**	**T**	**FxT**
Proteins	221.5 ± 10.2	219.1 ± 2.3	236.4 ± 15.3	192.1 ± 31.5	217.4 ± 3.9	213.3 ± 2.4	ns	ns	ns
Carbohydrates	21.5 ± 2.6^{a}	29.6 ± 2.9^{ab}	40.2 ± 3.7^{a}	21.2 ± 2.0^{b}	32.7 ± 2.0^{ab}	39.9 ± 3.7^{a}	ns	*	*
Total lipids	64.3 ± 6.8^{a}	56.7 ± 9.5^{a}	28.4 ± 2.9^{b}	64.6 ± 5.9^{a}	47.3 ± 12.6^{ab}	42.3 ± 3.7^{ab}	ns	ns	*
Total saturated	11.0 ± 1.5	10.2 ± 2.5	4.5 ± 0.5	11.2 ± 1.0	8.5 ± 2.5	7.5 ± 0.5	ns	ns	ns
Total monounsaturated	10.2 ± 1.5	10.5 ± 2.6	3.7 ± 0.5	11 ± 1.2	7.0 ± 2.2	7.1 ± 0.9	ns	ns	ns
Total n-6 PUFA	8.6 ± 1.4	9.1 ± 2.3	4.0 ± 0.5	10.9 ± 1.2	5.6 ± 1.6	7.0 ± 0.6	*	ns	ns
Total n-3 PUFA	8.6 ± 1.0	7.2 ± 1.8	4.4 ± 0.8	8.6 ± 0.7	7.9 ± 2.1	5.9 ± 0.6	ns	ns	ns
18:2n-6	6.8 ± 1.2	7.3 ± 2.0	2.9 ± 0.4	9.4 ± 1.1	4.6 ± 1.5	5.8 ± 0.5	*	ns	ns
20:4n-6	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.2	0.5 ± 0.0	0.4 ± 0.0	ns	*	ns
20:5n-3	3.4 ± 0.3^{a}	2.5 ± 0.5^{a}	1.9 ± 0.1^{b}	3.4 ± 0.3^{a}	2.9 ± 0.7^{ab}	2.5 ± 0.2^{ab}	ns	ns	*
22:6n-3	4.0 ± 0.6	3.4 ± 0.9	2.1 ± 0.7	3.9 ± 0.3	4.0 ± 1.2	2.6 ± 0.3	ns	ns	ns

Rows with different superscript letters are significantly different ($p < 0.05$, Tuckey test).F = Feed; T = Time; ns – not significant.

4.3.2. Reproductive performance, broodstock mortality and biochemical profiles

Reproductive performance results and broodstock mortality data are presented in Table 4.4. Female mortality and spawning events (percentage of mated female that released eggs in spawning tanks) were similar and not affected by feed or ablation and there was no interaction between the factors. Hatching rate was affected by feed and it was higher in DF&NF group (54.3 ± 0.0 and 50.1 ± 0.0 % for DF&NF and DF, respectively). There was no interaction between feed and ablation, however ablation had a significant effect on mating success (4.8 ± 0.0 and 7.0 ± 0.0% for NAF and AF, respectively), number of eggs per spawned female per day (164015 ± 4650 and 143473 ± 2305 for NAF and AF, respectively), number of nauplii per spawned female per day (85379 ± 1683 and 73208 ± 1494 for NAF and AF, respectively), number of eggs per tank per day (888181 ± 41269 and 1153794 ± 32068 for NAF and AF, respectively), and number of nauplii per tank per day (479789 ± 22584 and 592596 ± 16905 for NAF and AF, respectively). Mating success

and tank production parameters of AF were significantly higher than NAF animals, although the latter group had significantly higher fecundity (number of eggs or nauplii per spawned female per day). There was interaction between feed and ablation on fertilization and both factors had a significant effect on this parameter. NAF from DF&NF group had a significantly higher fertilization rate than all other subgroups.

Hepatosomatic and gonadosomatic indexes among the treatments were similar over time (Day 26th and 59th post start of reproduction) (Figure 4.3). There was significant interaction effect of feed, ablation and time on crude protein, total lipid, total saturated and monounsaturated fatty acids, LA, ARA and EPA contents of the hepatopancreas (Table 4.5). Crude protein was significantly lower only on day 26 for NAF from DF&NF. Total lipid, total saturated and monounsaturated fatty acids, LA, ARA and EPA were significantly higher in the hepatopancreas of NAF from DF&NF group at the end (59th day post start of reproduction). There was no interaction effect of feed, ablation and time on total n-6 and n-3 PUFAs, and DHA but there was significant effect of ablation on total n-6 PUFA (2.6 ± 0.6 and 1.5 ± 0.2 mg/g for NAF and AF, respectively), total n-3 PUFA (12.8 ± 1.6 and 8.5 ± 0.9 mg/g for NAF and AF, respectively) and DHA (7.8 ± 1.0 and 5.0 ± 0.5 mg/g for NAF and AF, respectively). Furthermore, there was also a significant effect of time on total n-6 PUFA (1.4 ± 0.2 and 2.7 ± 0.6 mg/g for days 26 and 59, respectively), total n-3 PUFA (8.1 ± 0.8 and 13.3 ± 1.5 mg/g for days 26 and 59, respectively) and DHA (5.0 ± 0.6 and 7.8 ± 0.9 mg/g for days 26 and 59, respectively). These parameters were significantly higher in NAF than AF and on day 59 post reproduction period.

In ovaries, no interaction of the three variables was observed on all biochemical parameters and their biochemical contents (protein, total lipid, total saturated and monounsaturated fatty acids, total n-6 and n-3 PUFA, LA, ARA, EPA and DHA) (Table 4.5). However, there was a significant effect of ablation on LA (1.2 ± 0.1 and 0.8 ± 0.1 mg/g for NAF and AF, respectively) and DHA (8.8 ± 0.7 and 7.5 ± 0.3 mg/g for NAF and AF, respectively). There was also a significant effect of time on ARA (0.68 ± 0.02 and 0.73 ± 0.02 mg/g for days 26 and 59, respectively) and DHA (9.0 ± 0.7 and 7.4 ± 0.2 mg/g for days 26 and 59, respectively). The feed had a significant effect only on DHA (7.5 ± 0.3 and 8.8 ± 0.7 mg/g for DF&NF and DF, respectively). LA and DHA were significantly

higher in NAF than AF. ARA was significantly lower on day 26 whilst DHA on day 59. DHA was significantly higher in DF than DF&NF group.

There was an interaction effect of feed, ablation and time on all biochemical parameters of eggs (Table 4.6). Total lipid, saturated and monounsaturated fatty acids were significantly lower at beginning (Days 7&14 post reproduction) than at the middle (Days 35&42 post reproduction) and end (Day 59 post reproduction). Total n-6 PUFA of eggs was significantly lower at the end of reproduction for all treatments, besides the DF group was significantly higher at beginning (only NAF) and middle of reproduction (NAF and AF). Egg total n-3 PUFA content increased significantly over time. LA of eggs followed a similar pattern to n-6 PUFA content. ARA was significantly lower only at beginning in all treatments. EPA was also significantly lower at the beginning in all treatments, however at the middle and end of reproduction, only eggs from NAF of both groups exhibited significantly higher EPA than AF. Egg DHA increased over time, although the egg content of AF was significantly lower than NAF during the middle period of reproduction.

Interactions between feed, ablation and time were demonstrated on all biochemical parameters of nauplii (Table 4.6) was not observed for total n-6 PUFA and LA. However, there was effect of feed, ablation and time on total n-6 PUFA (1.4 ± 0.0 and 1.7 ± 0.1 mg/g for DF&NF and DF, respectively), (1.6 ± 0.1 and 1.5 ± 0.1 mg/g for NAF and AF, respectively) and (1.5 ± 0.1, 1.8 ± 0.1 and 1.2 ± 0.0 mg/g for days 7&14, 35&42 and 59, respectively), respectively. Effects of feed, ablation and time were also observed on LA (1.0 ± 0.1 and 1.4 ± 0.2 mg/g for DF&NF and DF, respectively), (1.3 ± 0.2 and 1.1 ± 0.1 mg/g for NAF and AF, respectively) and (1.7 ± 0.2, 1.2 ± 0.1 and 0.6 ± 0.0 mg/g for days 7&14, 35&42 and 59, respectively), respectively. Nauplii from the DF group had a higher content of n-6 PUFA and LA, than from DF&NF. NAF produced nauplii with a higher level of n-6 PUFA and LA than AF. Furthermore, the level of the two nutrients decreased significantly over time.

Nauplii lipid was significantly higher at the end of reproduction in all treatments except for AF from DF&NF group. Total saturated and monosaturated fatty acids were not different over time, although nauplii from NAF and AF of DF&NF and DF, respectively, were significantly higher at the end. The nauplii total n-3 PUFA increased over time and with significant observation at the end of reproduction period in all treatments. ARA, EPA

and DHA were significantly higher only at the end of reproduction, except for ARA of nauplii from AF and NAF of DF&NF and DF, respectively, which were similar to previous time points. On the other hand EPA and DHA had similar tendency to ARA but it was observed only with nauplii of AF from the DF&NF group.

4.3.3 Larviculture

There was no significant interaction between feed and ablation on growth parameters, survival to salinity stress test, final survival and biochemical parameters (Table 4.7). However, there was a significant effect of ablation on survival to salinity stress test, (89.2 ± 1.8 and 81.4 ± 2.5 % for NAF and AF, respectively), and of feed only on ARA levels (0.42 ± 0.0 and 0.36 ± 0.0 mg/g for DF&NF and DF, respectively). No significant difference was observed in larval stage indexes at protozoea I (ZI), mysis I (MI), postlarvae I (PLI) and 10 (PL10), final survival and weight, and biochemical parameters (Total lipids and fatty acids, except ARA). Postlarvae from NAF had significantly higher survival to salinity stress test compared to postlarvae from AF.

Table 4. 4: Reproductive performance and mortality of non-ablated (NAF) and ablated (AF) female from group fed dry feed and fresh-frozen natural feed (squid and polychaete) (DF&NF), and only dry feed (DF) during pre-maturation conditioning (Mean ± SE) (n = 3).

	DF&NF		DF		Significance		
Parameters	**NAF**	**AF**	**NAF**	**AF**	**F**	**A**	**FxA**
Mating success per day (%)	5.8 ± 0.5	6.9 ± 0.6	3.8 ± 0.1	7.0 ± 0.7	ns	*	ns
Spawning event per day (%)	92.1 ± 1.9	90.0 ± 0.9	92.3 ± 0.7	91.8 ± 1.9	ns	ns	ns
Hatching rate per day (%)	55.5 ± 1.0	53.1 ± 1.4	50.6 ± 1.4	51.3 ± 1.0	*	ns	ns
Fertilization rate (%)	78.4 ± 0.5[a]	70.5 ± 1.4[b]	69.7 ± 2.1[b]	68.6 ± 0.5[b]	*	*	*
Number of eggs/spawned female/day	158090 ± 8212	140364 ± 2351	169938 ± 2341	146582 ± 3372	ns	*	ns
Number of nauplii/spawned female/day	85708.1 ± 1984.1	71942 ± 2261	85049 ± 3180	74474 ± 2109	ns	*	ns
Number of eggs/tank/day	924598 ± 70823	1142248 ± 66564	851764 ± 46620	1165342 ± 24034	ns	*	ns
Number of nauplii/tank/day	506501 ± 34489	590934 ± 35189	453077 ± 25441	594259 ± 13703	ns	*	ns
Mortality of female per day (%)	5.0 ± 1.2	3.8 ± 0.3	3.5 ± 0.1	4.9 ± 0.9	ns	ns	ns

Rows with different superscript letters are significantly different (p <0.05, Turkey test). F = Feed; A = Ablation; ns – not significant.

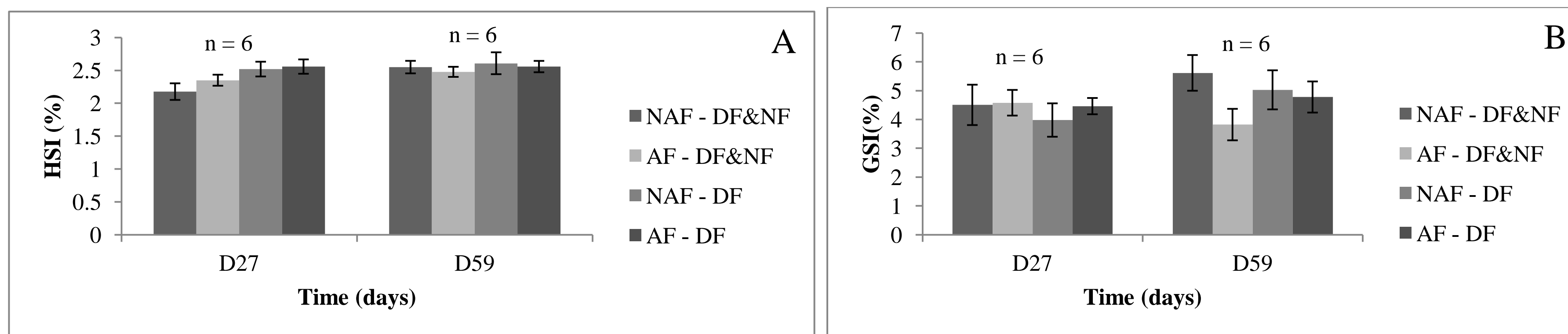

Figure 4. 3: Hepatosomatic index (HSI) (A) and gonadosomatic index (GSI) (B) of non-ablated (NAF) and ablated (AF) female from group fed dry feed and fresh-frozes natural feed (squid and polychaete) (DF&NF), and only dry feed (DF) during pre-maturation. There was no significant difference (p > 0.05, Tuckey test).

Table 4. 5: Biochemical contents of hepatopancreas (HP) and ovaries (OV) of non-ablated (NAF) and ablated (AF) group from female fed dry feed and fresh-frozen natural feed (squid and polychaete) (DF&NF,) and only dry feed (DF) during pre-maturation (Mean ± SE) (n = 3).

	Day 26**				Day 59**									
	DF&NF		DF		DF&NF		DF		Significance					
Parameters (mg/g)	**HP NAF**	**HP AF**	**HP NAF**	**HP AF**	**HP NAF**	**HP AF**	**HP NAF**	**HP AF**	**F**	**A**	**T**	**F x A**	**F x T**	**A x T**
Proteins	182.3 ± 3.1^{ab}	164.2 ± 4.6^{ab}	160.4 ± 5.5^{b}	193.4 ± 9.8^{a}	169.7 ± 6.4^{ab}	178.9 ± 1.6^{ab}	176.3 ± 6.4^{ab}	192.5 ± 6.8^{ab}	ns	*	ns	*	ns	ns
Total lipids	49.6 ± 4.8^{b}	43.8 ± 12.7^{b}	41.9 ± 6.6^{b}	45.5 ± 10.2^{b}	163.7 ± 26.8^{a}	46.0 ± 11.5^{b}	51.5 ± 10.0^{b}	65.4 ± 10.2^{b}	*	*	*	*	*	*
Total saturated	8.1 ± 0.9^{b}	4.0 ± 0.3^{c}	6.7 ± 1.4^{b}	7.1 ± 2.4^{b}	23.2 ± 2.5^{a}	5.1 ± 0.2^{c}	9.7 ± 2.3^{b}	11.8 ± 2.8^{b}	*	*	*	*	*	ns
Total monounsaturated	6.5 ± 0.6^{bc}	2.7 ± 0.4^{c}	5.8 ± 1.3^{bc}	6.2 ± 2.1^{b}	23.2 ± 1.9^{a}	4.6 ± 0.4^{bc}	8.0 ± 2.0^{b}	10.9 ± 2.6^{b}	*	*	*	*	*	*
Total n-6 PUFA	1.6 ± 0.1^{b}	0.8 ± 0.0^{b}	1.4 ± 0.2^{b}	1.7 ± 0.6^{b}	4.8 ± 0.4^{a}	1.7 ± 0.4^{b}	1.8 ± 0.3^{b}	2.7 ± 0.1^{b}	ns	*	*	*	*	ns
Total n-3 PUFA	11.2 ± 1.8^{b}	6.1 ± 0.4^{c}	8.4 ± 1.6^{bc}	8.7 ± 2.8^{bc}	22.0 ± 2.8^{a}	5.6 ± 0.3^{c}	11.1 ± 2.4^{b}	13.4 ± 2.6^{b}	ns	*	*	*	ns	ns
18:2n-6	0.4 ± 0.1^{b}	0.3 ± 0.1^{b}	0.4 ± 0.1^{b}	0.6 ± 0.3^{b}	2.4 ± 0.5^{a}	0.5 ± 0.1^{b}	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}	*	*	*	*	*	*
20:4n-6	0.7 ± 0.0^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	1.7 ± 0.3^{a}	0.7 ± 0.1^{b}	0.8 ± 0.1^{b}	0.8 ± 0.1^{b}	ns	*	*	*	ns	ns
20:5n-3	3.6 ± 0.2^{bc}	2.1 ± 0.1^{c}	2.5 ± 0.4^{c}	2.8 ± 0.7^{c}	8.9 ± 2.1^{a}	3.5 ± 0.6^{bc}	3.5 ± 0.5^{bc}	4.6 ± 0.7^{b}	ns	*	*	*	ns	ns
22:6n-3	7.0 ± 1.7^{ab}	3.7 ± 0.2^{c}	5.3 ± 1.1^{b}	5.3 ± 1.9^{b}	12.8 ± 1.2^{a}	3.3 ± 0.2^{c}	6.8 ± 1.7^{ab}	6.1 ± 1.6^{ab}	ns	*	*	ns	ns	*
Parameters (mg/g)	**OV NAF**	**OV AF**	**OV NAF**	**OV AF**	**OV NAF**	**OV AF**	**OV NAF**	**OV AF**	**F**	**A**	**T**	**F x A**	**F x T**	**A x T**
Proteins	233.4 ± 1.6	239.6 ± 8.4	216.1 ± 4.2	224.6 ± 3.7	213.1 ± 8.7	219.5 ± 2.0	223.2 ± 6.5	226.8 ± 5.9	ns	ns	ns	ns	*	ns
Total lipids	68.2 ± 4.3	67.1 ± 1.5	66.9 ± 0.7	68.4 ± 1.9	65.1 ± 2.6	59.5 ± 5.6	66.0 ± 1.1	63.6 ± 1.9	ns	ns	ns	ns	ns	ns
Total saturated	12.6 ± 0.9	13.5 ± 0.6	12.5 ± 0.2	13.1 ± 0.5	12.8 ± 0.8	11.7 ± 1.6	13.1 ± 0.5	13.0 ± 0.3	ns	ns	ns	ns	ns	ns
Total monounsaturated	11.1 ± 0.9	12.5 ± 0.3	11.1 ± 0.2	11.3 ± 0.5	12.0 ± 0.9	11.3 ± 1.4	12.0 ± 0.7	12.2 ± 0.3	ns	ns	ns	ns	ns	ns
Total n-6 PUFA	2.5 ± 0.1	1.7 ± 0.1	2.5 ± 0.1	2.3 ± 0.2	2.4 ± 0.0	1.9 ± 0.2	2.9 ± 0.6	1.8 ± 0.1	ns	ns	ns	ns	ns	ns
Total n-3 PUFA	13.8 ± 1.0	13.4 ± 0.8	14.4 ± 0.2	13.4 ± 0.3	13.6 ± 0.5	12.3 ± 1.5	13.7 ± 0.3	12.6 ± 0.5	ns	ns	ns	ns	ns	ns
18:2n-6	1.1 ± 0.2	0.7 ± 0.1	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.0	0.8 ± 0.1	1.5 ± 0.5	0.7 ± 0.1	ns	*	ns	*	ns	ns
20:4n-6	0.8 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	ns	ns	*	ns	ns	ns
20:5n-3	4.9 ± 0.4	3.9 ± 0.2	4.7 ± 0.2	4.2 ± 0.1	4.7 ± 0.1	4.6 ± 0.5	4.8 ± 0.3	4.4 ± 0.1	ns	ns	ns	ns	ns	ns
22:6n-3	8.2 ± 0.6	8.8 ± 0.6	8.9 ± 0.1	8.4 ± 0.3	8.0 ± 0.4	7.0 ± 0.9	8.0 ± 0.2	7.4 ± 0.3	*	*	*	ns	ns	ns

** post start of reproduction. F = Feed; A = Ablation; T = Time; ns – not significant. Rows with different superscript letters are significantly different ($p < 0.05$, Tuckey test).

Table 4. 6: Total lipids and fatty acids contents of eggs and nauplii of non-ablated (NAF) and ablated (AF) females from group fed dry feed and fresh-frozen natural feed (squid and polychaete) (DF&NF) and only dry feed (DF) during pre-maturation (Mean ± SE) (n = 3). Eggs and nauplii on days 7&14 and 35&42 were pooled (i.e. from days 7 and 14 or 35 and 42).

	Days 7&14**				**Days 35&42****				**Day 59****										
	DF&NF		**DF**		**DF&NF**		**DF**		**DF&NF**		**DF**		**Significance**						
Parameters (mg/g)	**Eggs NAF**	**Eggs AF**	**Eggs NAF**	**Eggs AF**	**Eggs NAF**	**Eggs AF**	**Eggs NAF**	**Eggs AF**	**Eggs NAF**	**Eggs AF**	**Eggs NAF**	**Eggs AF**	**F**	**A**	**T**	**F x A**	**F x T**	**A x T**	**F x A x T**
Total lipids	41.4 ± 0.8^{d}	43.9 ± 1.0^{cd}	45.3 ± 1.9^{cd}	43.8 ± 0.5^{cd}	50.4 ± 1.0^{ab}	50.9 ± 0.7^{ab}	51.3 ± 1.4^{ab}	51.1 ± 1.3^{ab}	47.8 ± 0.1^{bc}	52.1 ± 0.7^{ab}	53.5 ± 1.5^{ab}	48.5 ± 0.8^{abc}	ns	ns	*	*	ns	ns	*
Total saturated	7.7 ± 0.1^{c}	7.8 ± 0.1^{c}	7.9 ± 0.2^{c}	8.1 ± 0.0^{c}	8.7 ± 0.1^{b}	8.5 ± 0.3^{b}	9.0 ± 0.2^{b}	9.1 ± 0.2^{b}	9.6 ± 0.2^{ab}	10.4 ± 0.2^{a}	9.8 ± 0.1^{ab}	9.3 ± 0.1^{ab}	ns	ns	*	*	ns	ns	*
Total monounsaturated	7.2 ± 0.1^{d}	7.4 ± 0.1^{d}	7.4 ± 0.1^{d}	7.9 ± 0.1^{dc}	8.0 ± 0.1^{c}	8.2 ± 0.2^{c}	8.1 ± 0.2^{c}	8.4 ± 0.3^{bc}	8.8 ± 0.2^{b}	9.8 ± 0.2^{a}	8.9 ± 0.1^{b}	8.8 ± 0.1^{b}	ns	ns	*	*	ns	ns	*
Total n-6 PUFA	2.4 ± 0.0^{b}	1.7 ± 0.0^{c}	3.3 ± 0.3^{a}	2.5 ± 0.3^{b}	2.0 ± 0.0^{b}	2.4 ± 0.1^{b}	3.2 ± 0.1^{a}	3.2 ± 0.2^{a}	1.8 ± 0.0^{c}	1.6 ± 0.0^{c}	1.9 ± 0.0^{c}	1.4 ± 0.0^{d}	ns	*	*	*	ns	*	*
Total n-3 PUFA	7.6 ± 0.1^{d}	7.3 ± 0.0^{d}	7.6 ± 0.2^{d}	7.6 ± 0.2^{d}	9.0 ± 0.2^{b}	8.1 ± 0.2^{c}	9.1 ± 0.2^{b}	8.4 ± 0.2^{c}	9.9 ± 0.2^{b}	9.7 ± 0.2^{b}	10.3 ± 0.1^{ab}	9.0 ± 0.1^{b}	*	*	*	*	*	*	*
18:2n-6	1.6 ± 0.0^{b}	1.0 ± 0.0^{c}	2.5 ± 0.3^{a}	1.6 ± 0.3^{b}	1.2 ± 0.0^{b}	1.6 ± 0.0^{b}	2.2 ± 0.0^{a}	2.2 ± 0.2^{a}	0.9 ± 0.0^{c}	0.8 ± 0.0^{c}	1.0 ± 0.0^{c}	0.6 ± 0.0^{d}	*	*	*	*	*	*	*
20:4n-6	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.4 ± 0.0^{b}	*	*	*	*	*	*	*
20:5n-3	2.6 ± 0.0^{d}	2.5 ± 0.0^{d}	2.6 ± 0.1^{d}	2.3 ± 0.1^{e}	3.1 ± 0.1^{b}	2.7 ± 0.1^{c}	3.2 ± 0.1^{b}	2.8 ± 0.1^{c}	3.4 ± 0.1^{ab}	3.2 ± 0.1^{b}	3.4 ± 0.0^{a}	2.9 ± 0.0^{b}	*	*	*	*	*	*	*
22:6n-3	4.5 ± 0.1^{e}	4.4 ± 0.0^{e}	4.3 ± 0.2^{e}	4.6 ± 0.0^{e}	5.4 ± 0.1^{b}	4.9 ± 0.1^{d}	5.4 ± 0.1^{b}	5.1 ± 0.1^{d}	5.9 ± 0.1^{a}	5.9 ± 0.1^{b}	6.2 ± 0.1^{a}	5.5 ± 0.1^{c}	ns	*	*	*	*	*	*
Parameters (mg/g)	**Nauplii NAF**	**Nauplii AF**	**Nauplii NAF**	**Nauplii AF**	**Nauplii NAF**	**Nauplii AF**	**Nauplii NAF**	**Nauplii AF**	**Nauplii NAF**	**Nauplii AF**	**Nauplii NAF**	**Nauplii AF**	**F**	**A**	**T**	**F x A**	**F x T**	**A x T**	**F x A x T**
Total lipids	31.9 ± 0.5^{cd}	31.7 ± 1.0^{cd}	32.1 ± 0.5^{cd}	30.4 ± 1.1^{d}	29.2 ± 0.8^{d}	29.9 ± 1.0^{d}	34.6 ± 1.1^{bcd}	34.5 ± 1.0^{bcd}	40.0 ± 1.0^{ab}	33.8 ± 1.8^{c}	36.6 ± 1.5a^{bc}	41.5 ± 1.8^{a}	*	ns	*	*	*	ns	*
Total saturated	5.6 ± 0.2^{b}	5.7 ± 0.2^{b}	5.4 ± 0.2^{b}	5.7 ± 0.1^{b}	5.8 ± 0.2^{b}	5.6 ± 0.1^{b}	6.6 ± 0.3^{b}	6.2 ± 0.2^{b}	6.9 ± 0.3^{a}	5.9 ± 0.4^{b}	5.8 ± 0.2^{b}	7.6 ± 0.1^{a}	*	ns	*	ns	*	ns	*
Total monounsaturated	5.2 ± 0.1^{bc}	5.3 ± 0.1^{bc}	4.9 ± 0.1^{c}	5.4 ± 0.1^{bc}	5.3 ± 0.2^{bc}	5.2 ± 0.1^{bc}	5.8 ± 0.2^{bc}	5.9 ± 0.2^{bc}	6.2 ± 0.3^{ab}	5.4 ± 0.3^{bc}	5.1 ± 0.1^{bc}	7.0 ± 0.1^{a}	*	*	*	*	*	ns	*
Total n-6 PUFA	1.3 ± 0.3	1.1 ± 0.3	1.7 ± 0.1	1.5 ± 0.0	1.6 ± 0.0	1.6 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	*	*	*	ns	*	*	ns
Total n-3 PUFA	5.6 ± 0.2^{bc}	5.2 ± 0.2^{c}	5.1 ± 0.2^{c}	4.8 ± 0.1^{c}	5.5 ± 0.2^{bc}	5.0 ± 0.1^{c}	6.3 ± 0.2^{b}	5.3 ± 0.2^{bc}	6.8 ± 0.3^{ab}	5.4 ± 0.4^{bc}	6.0 ± 0.2^{b}	7.1 ± 0.1^{a}	ns	*	*	ns	*	ns	*
18:2n-6	0.7 ± 0.3^{b}	0.6 ± 0.0	1.1 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	*	*	*	ns	*	*	ns
20:4n-6	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{a}	ns	*	*	ns	*	ns	*
20:5n-3	1.8 ± 0.1^{b}	1.8 ± 0.1^{b}	1.8 ± 0.1^{b}	1.6 ± 0.1^{c}	1.9 ± 0.1^{b}	1.7 ± 0.1^{b}	2.2 ± 0.1^{ab}	1.8 ± 0.1^{b}	2.4 ± 0.1^{a}	1.9 ± 0.1^{b}	2.1 ± 0.1^{a}	2.4 ± 0.1^{a}	ns	*	*	ns	*	ns	*
22:6n-3	3.3 ± 0.2^{bc}	3.1 ± 0.1^{c}	3.0 ± 0.1^{c}	3.0 ± 0.0^{c}	3.3 ± 0.1^{c}	2.9 ± 0.1^{c}	3.7 ± 0.1^{bc}	3.1 ± 0.1^{c}	4.0 ± 0.2^{a}	3.2 ± 0.2^{bc}	3.5 ± 0.1^{ab}	4.3 ± 0.1^{a}	*	*	*	ns	*	ns	*

** post start of reproduction. F = Feed; A = Ablation; T = Time; ns – not significant. Rows with different superscript letters are significantly different ($p < 0.05$, Tuckey test).

Table 4. 7: Growth performance, final survival, survival to salinity stress test (SST,) total lipids and fatty acids contents of *L. vannamei* PLs (n = 4) originated from non-ablated (NAF) and ablated (AF) female which were previously fed dry feed and natural feed (squid and polychaete) (DF&NF),and only dry feed (DF) during pre-maturation (Mean ± SE) (n = 4).

	DF&NF		DF		Significance		
Parameters	**NAF**	**AF**	**NAF**	**AF**	**F**	**A**	**FxA**
LSI at Z1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	ns	ns	ns
LSI at M1	3.9 ± 0.0	3.7 ± 0.2	3.7 ± 0.1	3.9 ± 0.1	ns	ns	ns
LSI at PL1	6.7 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.7 ± 0.09	ns	ns	ns
LSI at PL10	15.4 ± 0.2	15.3 ± 0.2	15.6 ± 0.1	15.6 ± 0.1	ns	ns	ns
Survival to SST PL10-11 (%)	88.5 ± 2.9	82.7 ± 5.1	90.0 ± 2.5	80.0 ± 1.2	ns	*	ns
Final weight (mg)	2.2 ± 0.0	2.2 ± 0.3	2.4 ± 0.3	2.2 ± 0.0	ns	ns	ns
Final Survival (%)	37.1 ± 3.9	34.8 ± 1.6	41.0 ± 3.7	30.4 ± 2.2	ns	ns	ns
Parameters (mg/g)	**NAF**	**AF**	**NAF**	**AF**	**F**	**A**	**FxA**
Total lipids	22.9 ± 1.4	20.0 ± 0.4	19.3 ± 0.8	19.7 ± 0.9	ns	ns	ns
Total saturated	2.4 ± 0.1	2.3 ± 0.0	1.8 ± 0.4	2.4 ± 0.1	ns	ns	ns
Total monounsaturated	2.0 ± 0.1	1.9 ± 0.0	1.5 ± 0.3	2.2 ± 0.1	ns	ns	ns
Total n-6 PUFA	1.5 ± 0.3	1.5 ± 0.0	1.2 ± 0.2	1.7 ± 0.1	ns	ns	ns
Total n-3 PUFA	1.9 ± 0.2	1.9 ± 0.0	1.4 ± 0.3	1.9 ± 0.1	ns	ns	ns
18:2n-6	1.0 ± 0.0	1.1 ± 0.0	0.8 ± 0.2	1.2 ± 0.1	ns	ns	ns
20:4n-6	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	*	ns	ns
20:5n-3	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.0	ns	ns	ns
22:6n-3	0.9 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	ns	ns	ns

LSI = Larval stage index. F = Feed; A = Ablation; ns – not significant. Rows with different superscript letters are significantly different ($p < 0.05$, Tuckey test).

4.4. Discussion

The main focus of this study was to evaluate the potential of natural feed (squid and polychaete) in pre-maturation on performance of non-ablated female and their offspring quality. This feeding protocol did not improve non-ablated production parameters (i.e. eggs and nauplii per female or per tank) but improved fertility. The quality of the offspring was not affected by natural feed.

Maturation and gonadosomatic indices have been used to indicate ovary development and to identify the advanced maturation stage of *L. vannamei* (Chamberlain and Lawrence, 1981; Harrison, 1990; Palacios et al., 1999; Alfaro et al., 2004). Although there was limitation in replication of the experimental units, these indices showed that DF&NF females had apparently slower ovarian development than the DF. Increases in GSI and ovarian development during sexual maturation also indicated nutrient accumulation (Harrison, 1990; Wouters et al., 2001a). In penaeid shrimp, nutritional factors play a critical role in the stimulation of sexual maturation (Chamberlain and Lawrence, 1981; Harrison, 1990; Naessens et al., 1997). Slower increases in maturation index and GSI demonstrated delayed nutrient accumulation in DF&NF females. This response is directly related to the nature and amount of supplied feed during pre-maturation. DF&NF females were underfed in first 4 weeks due to unintentional mistake when determining right amount of natural feed (Squid and polychaetes) based on wet weight approach.

However, the variation in ovarian total lipid over the pre-maturation period suggests that lipid was the main nutrient contributing to the rapid ovary development as crude protein and carbohydrate levels were similar to those found in ovaries from DF&NF females, although the latter nutrient was lower in DF on day 22. Lipids are a necessary source of energy for shrimp broodstock, eggs and pre-feeding larva (Harrison, 1990; Wouters et al., 2001b; Glencross, 2009). Therefore, DF females advanced maturation earlier because they initially received more food (due to a higher DM) and lipid. Besides, both treatments had started with a feeding rate of 12% bodyweight per day; 8% (as fed) bodyweight of natural feed and 4% of dry feed (following commercial practice) it was realised that the DF&NF females were receiving a ration of lower DM content compared to the DF treatment that were receiving12% of

the dry feed. Therefore, these levels were clearly sub-optimal for supporting maturation advancement of DF&NF females as it was demonstrated by maturation and gonadosomatic indices on day 22. However, they responded rapidly to the change in feeding rate from day 29 (middle of pre-maturation period) onwards, reaching a similar level of maturity to the controls by days 45 to 60 as was demonstrated by the maturation and gonadosomatic indices.

The hepatopancreas has been suggested as the main lipid storage and processing for the postembryonic stage of shrimp (Harrison, 1990). In addition, it is considered as a major source of lipid accumulation in the maturing ovary of *Penaeus japonicus* (Teshima and Kanazawa, 1983; Teshima et al., 1988; Teshima et al., 1989) and *P. setiferus* (Castille and Lawrence, 1989). In the present study, although the total ovarian lipid had a relationship with levels in the hepatopancreas; almost half of the response for ovarian lipid accumulation in *L. vannamei* in this study was not directly related to hepatopancreas. Wouters et al. (2001b) found that lipid accumulation in the ovaries of *L. vannamei* happened prior to the decrease of lipid in the hepatopancreas. The amount of lipids accumulated in the ovaries of *Chorismus antarcticus* (Clarke, 1982) and *Penaues aztecus* (Castille and Lawrence, 1989) exceeded the amount stored in the hepatopancreas. These findings imply that the source of ovary lipid accumulation is species specific. The rise or maintenance of ovarian lipid quantities in ovaries may express the increase in lipid synthesis, dietary lipid intake, and/or a transfer of body lipid reserves to the developing ovaries (Teshima, et al. 1988). Cavalli (2000) and Wouters et al. (2001b) have suggested that maturing *M. rosenbergii* and *L. vannamei* are probably dependent on dietary lipids for normal lipid accumulation in the ovaries.

At the middle of pre-maturation (Day 22), total lipid in both the hepatopancreas and ovaries of DF&NF female was lower and consistent with a lower maturation index. Shrimp are known to be able to use nutrients reserves in their organs, i.e. hepatopancreas/ovaries, if there are not enough dietary nutrients for biological process (Harrison, 1990; Rosas et al., 2001; Rosas et al., 2002). Therefore DF&NF female might have used the lipid reserve to compensate for sub-optimal levels of dietary lipid. Squid and polychaete have lower dry weight than commercial dry feed which affects food consumption rate (Chamberlain and Lawrence, 1981a). At the beginning, animals

in the DF&NF treatment and DF were offered diet of 12% biomass, however it appears that the percentage of polychaete and squid offered at that period was not enough to fulfil dietary requirements due to their lower DM. Therefore this might have switched lipid metabolism in DF&NF once the animals began to receive experimental diets. Previously, those females that had been fed only a commercial dry diet (Camarón Barrancones Levante Reprodutores NT: 40% Protein and 8% Crude Lipid, Cargill, Honduras) in broodstock ponds at the farm and the animals had already started accumulating lipids in their ovaries, according to our baseline results (Day 1 of pre-maturation). Dry feed had only one more essential fatty acid (Linoleic acid) than squid and polychaete; however the DF females had comparable levels of DHA in hepatopancreas (except on day 22 was higher) and ovaries to DF&NF group. It seems that some of the hepatopancreas and ovarian DHA of DF female was derived from other sources such as lipid synthesis or transfer of body lipid reserves as suggested by Teshima, et al. (1988). In addition, shrimp are considered to have a very limited ability to synthesize lipids, such as highly unsaturated fatty acids (HUFA) *de novo* (Harrison, 1990; Wouters et al., 2001ab). This might explain the DHA results in DF. However, it also can be explained by higher feeding rate observed in DF females.

The feed also affected the lower concentration of protein observed in the hepatopancreas of DF animals as dry feed had lower protein content than squid and polychaete. However, the hepatopancreas protein increased over time which confirms the nutrient accumulation role of this organ over the maturation period for reproduction (Harrison, 1990; Wouters et al., 2001b). Protein concentration in the ovaries did not increase simultaneously with ovarian mass of *L. vannamei* in our study, in contrast to results reported by Jeckel et al. (1989) who observed a concomitant increase in ovary mass and protein concentrations in shrimp *Pleoticus muelleri*. The difference in ovarian carbohydrate on day 22 might be associated with a technical error during carbohydrate analyses of ovarian samples due to the very low amount of sample required for the procedure.

The role of different natural feeds items and feeding practices in maturation and reproduction is not fully understood (Wouters et al. 2001a; Hoa et al.2009) and their performance and biochemical aspects remain to be clarified (Wouters et al. 2001a).

Similarly, very little is known about pre-maturation conditions to stimulate ovarian development. Squid and marine worms have been used as crucial dietary components due to their positive effects on penaeid shrimp reproduction and their high quality nutritional composition. Squid and polychaetes had higher levels of polyunsaturated fatty acids, mainly ARA, EPA and DHA which support what have been reported by Wouters et al. 2001a, Hoa et al., 2009 and Emerenciano et al., 2013.

Reproductive performance of shrimp females relies on high quality oocyctes and male sperm (Ceballos-Vásquez, et al., 2004). The mean values of spermatophore weight and sperm count observed in the current work are within the range reported for *L. vannamei* in previous work (Leung-Trujillo and Lawrence, 1985; Heitzmann, et al., 1993; Perez-Velazquez et al., 2001; Wouters et al., 2002; Ceballo-Velazquez et al., 2003; Perez-Velazquez et al., 2003; Ceballo-Velazquez et al., 2004; Braga et al., 2015). Furthermore the percentage of dead sperm were also similar to that observed for *L. vannamei* in other research (Leung-Trujillo and Lawrence, 1985; Ceballo-Velazquez et al., 2003; Ceballo-Velazquez et al., 2004), except in Braga et al. (2015) who observed very lower values (0 – 5.84%). Ceballo-Velazquez et al. (2003) has suggested that sperm count may depend on culture conditions. This might explain the lower percentage of dead sperm obtained in a previous study on *L. vannamei* because of the biofloc system used to hold them (Braga et al., 2015) whilst the current study was based on a clear water system. Similar values of total sperm count have been observed in other species such as *P. monodon* (Leelatanawit et al., 2014) and *P. setiferus* (Leung-Trujillo and Lawrence, 1985).

Nutrition can affect spermatophore and sperm quality of male shrimp in captivity (Leung-Trunjillo and Lawrence, 1987; Harrison, 1990; Perez-Velazquez et al., 2003; Ceballos-Vázques et al., 2004; Braga et al., 2010; Braga et al., 2015). Spermatophore weight in the current study was not affected by feed. Contrary to the current study, spermatophore weight of *P. monodon* male fed only polychaete was found to be significantly higher than those of commercial pellet (dry feed) (Leelatanawit et al., 2014). Memon et al., (2012) also found a significant increase in spermatophore mass of *P. merguiensis* fed polychaetes, squid, or cockles. This difference might be associated to feeding rate as the male from the treatment given

squid and polychaete were underfed. In addition, squid and polychaete may have not increased total sperm count due to underfeeding observed in pre-maturation period. Although the effect of natural feed on increasing sperm count has been reported using squid for *P. merguiensis* (Memon et al., 2012) and polychaete for *P. monodon* (Leelatanawit et al., 2014), in this study the sperm count increased over time which suggest that it is important to monitor sperm quality during pre-maturation as a measure to improve male selection to maturation tanks.

Although there was underfeeding at beginning of pre-maturation in DF&NF, an assessment of long term impact of broodstock from DF&NF and DF group in pre-maturation was conducted by evaluating their reproductive performance and offspring quality. The assessment was done because by the end of pre-maturation, both females from DF&NF and DF had similar maturation development. Feeding fresh-frozen squid and polychaete during pre-maturation did not have effect on spawning frequency of NAF. This result suggests that mated *L. vannamei* (with attached spermatophore) from DF&NF group in pre-maturation will spawn (release the eggs in spawning tanks) in the similar way as DF, regardless of being NAF or AF. Mating success has been reported to be higher in AF than NAF *L. vannamei* in Chapter 3 and by Chamberlain and Lawrence (1981b), and Palacios et al. (1999b)) due to the effect of unilateral eyestalk ablation. Similar results were found in this study where natural feed did not influence mating success while ablation significantly increased mating success. Xu et al. (2017) observed that ARA supplementation in the diets for early maturation stages enhanced the final reproductive performance of *L. vannamei*. Female blue shrimp (*L. stylirostris)* had better overall spawning performance if they were sourced from a biofloc based system (Emerenciano, et al. 2011). Cardona et al. (2016) also observed that mating success of *L. stylirostris* broodstock previously raised in biofloc systems was dramatically improved compared to those reared extensively in an earthen pond. Emerenciano et al. (2013) observed that mating success of *L. vannamei* previously reared using biofloc technology and fed dry feed, mussel and squid was improved compared to brooders not receiving such feeds. Improvements observed by Emerenciano, et al. (2011) and Cardona et al. (2016) appear related to the complementary dietary lipid or unknown bioactive compounds from biofloc aggregates, whilst in Emerenciano et al. (2013) it was due to the combination of biofloc, mussel and squid. Contrary to the present study,

there was no observed positive influence of natural feed on mating success, except on fertilization and hatching rates. It is most likely that mating success was affected by underfeeding during pre-maturation whilst fertilization and hatching rate did not. The positive effect observed in fertilization and hatching rates can be attributed possibly to other nutritional factors (Naessens et al. 1997; Ibarra et al., 1998) which cannot clearly be explained based on this study data.

Emerenciano et al. (2013) and Cardona et al. (2016) have reported that *L. vannamei* and *L stylirostris* broodstock exhibited higher fecundity if reared in a biofloc system or fed natural feed (mussel and squid) during pre-maturation. In the current study, feeding squid and polychaete in pre-maturation did not improve individual female eggs and nauplii production. However, NAF had better individual production (eggs and nauplii/female) than AF, confirming previous observations from Chapter 3 which showed the similar effect of non-ablation. In addition, natural feeds did not improve tank production parameters of NAF to similar level of AF group. The similar effect of non-ablation on tank production parameters was reported in Chapter 3.

In this study daily broodstock mortality was unaffected by ablation in both feeding treatments. Chamberlain and Lawrence (1981b) and Palacios et al., (1999a) also found similarity in overall mortality rate on their study with *L. vannamei*, 41.7% and 48.3%, and 13% and 17% to NAF and AF after 97 and 90 days respectively. In previous work (Chapter 3), daily mortality of NAF *L. vannamei* females was significantly lower than AF. Similar results have been reported for *P stylirostris* and *P. monodom* (Santiago Jr., 1977). Differences between reports may be associated with maturation and spawning conditions, time at which the study was conducted or the broodstock genetic line/sources. For instance, the maturation facilities used in previous work (Chapter 3) were the same for the current study; however broodstock of a different genetic line and the timing of the experiment (end of annual production cycle which in general the hatchery report higher mortalities) were used.

Boucard et al. (2004) observed depletion of nutritional reserves (i.e. total protein and lipid) in hepatopancreas of spawning shrimp (*Fenneropenaeus indicus*). In contrast, the present study observed NAF from DF&NF group had higher levels of total lipid, total saturated and monounsaturated fatty acids, LA, ARA and EPA in their

hepatopancreas when compared to other females (which did not show nutrient depletion over time) at the end of the experiment. This indicates that females fed natural feed had higher nutrient reserves in their hepatopancreas suggesting that their working life could be extended, with the likelihood of economic benefits as the brodstock account for 20 to 40% of nauplii production cost (see Chapter 6). Total n-6 and n-3 PUFAs, and DHA were higher in hepatopancreas from NAF than AF. LA and DHA were higher in ovaries from NAF than AF. This corroborates the findings from other works who report deterioration of AF due to unilateral eyestalk ablation (Santiago, 1977; Primavera, 1982; Palacios et al., 1999b).

Total lipid, saturated and monosaturated fatty acids, total n-3 PUFA, EPA and DHA of eggs were significantly lower in all treatments only at the beginning of the reproduction period. ARA, EPA and DHA of nauplii were significantly higher only at the end of reproduction, except for ARA of nauplii from AF and NAF of DF&NF and DF, respectively, which were similar to previous time points. On the other hand EPA and DHA demonstrated a similar trend to ARA but it was observed only with nauplii of AF from DF&NF group. Biochemical components (i.e. total lipid, fatty acids) of shrimp eggs can be used as predictive criteria in assessing spawn quality (Racotta et al., 2003). From the current study results, it appears that spawn quality was not good during first period of reproduction in all treatments. Abortive or low-viability spawns are produced immediately after eyestalk ablation (Primavera, 1985), due to stress associated with the procedure (Palacios et al. 1999b). However, in this study such effect of eyestalk ablation was not observed as the results were similar between NAF and AF regardless of the feeding history at this early stage. Arcos et al. (2003) also did not find difference in spawn production variables (i.e. biochemical parameters) obtained 1-3 days after eyestalk ablation; however they were similar to those obtained after several weeks. Shrimp broodstock condition is important for production of good quality offspring and it has both a nutritional (i.e. has high levels of nutrients reserves) (Naessens et al., 1997; Wouters et al., 2001ab; Perez-velazquez and Gonzaez-feliz, 2003; Racotta et al., 2003; Hoa et al., 2009; Emerenciano et al. 2013; Emerenciano et al., 2014; Braga et al. 2015; Cardona et al. 2016) and physiological component (i.e. no hormone imbalance) (Chamberlain and Lawrence, 1981b; Lawrence, 1983; Palacios et al., 1999ab; Racotta et al., 2003). Although lower total n-6 PUFA and LA were observed in eggs for all

treatments, the eggs from DF group had higher contents of the two nutrients at the beginning (only NAF) and middle of reproduction (NAF and AF). In addition, nauplii from DF group exhibited a higher content of total n-6 PUFA and LA. These results corroborate other studies suggesting that shrimp offspring quality can be affected by composition of broodstock feed (Wouters et al. 2001a; Racotta et al., 2003). Emerenciano et al. (2013) observed that females that received mussel and squid in pre-maturation presented higher levels of HUFA in eggs. In this study, the effect was not reflected in the eggs/larvae. This might be related to higher feeding rate observed in DF females during pre-maturation.

Eggs of NAF from both treatments had significantly higher EPA than AF at the middle and end of reproduction. Similarly, DHA of AF was significantly lower than NAF at the middle of reproduction. ARA was significantly lower only at the end of reproduction on AF from DF. Nauplii lipid, EPA and DHA were significantly lower at the end of reproduction in AF from DF&NF group. Furthermore, nauplii from NAF had higher total n-6 PUFA and LA. These results indicate that eyestalk ablation can indeed lower the nutritional reserves of the shrimp offspring as it has been suggested in previous work (Wickins and Lee, 2002; Racotta et al., 2003), except Palacios et al. (1999b) whom did not find significant difference in biochemical composition of eggs and nauplii from NAF and AF of same spawns.

Total n-6 PUFA and LA were the only nutrients which decreased over time in nauplii. Offspring quality (i.e. biochemical content) of the shrimp broodstock can deteriorate over time as a result of the broodstock ablation or time spent in the maturation tanks (Primavera, 1982; Ibarra et al., 1998; Palacios et al., 1999b; Racotta e tal., 2003). The results from total n-6 PUFA and LA suggest that the deterioration on biochemical contents in nauplii does not occur at same time with other fatty acids, mainly the essential one (i.e. EPA, DHA).

It appears that natural feed (squid and polychaete) effect on pre-maturation is not reflected in larval performance. The larviculture results presented little evidence that larval growth and development over time was affected by the broodstock feeding practice, based on larval stage index (LSI), final survival and weight of PLs, total lipids and fatty acids (except ARA). However, larvae from NAF group had higher survival to

salinity stress test. Palacios et al. (1999b) and the study from Chapter 3 also did not find significant difference when comparing larval growth and survival from AF and NAF *L. vannamei* females. Although the study from Chapter 3 found that PLs from NAF were significantly more resilient to a salinity stress test.

Overall, NAF broodstock exhibited lower reproductive performance than AF, although they had higher fecundity. In addition, NAF had better nutrient reserves in hepatopancreas and ovaries when they are previously fed squid and polychaete in pre-maturation. Eggs and nauplii from NAF had an apparent better nutrient than their counterpart; however this was not reflected in postlarvae growth performance and survival, although postlarvae from NAF were more resilient to stress. Underfeeding in female fed fresh-frozen natural feed during pre-maturation might have affected NAF reproductive performance, mainly the mating success.

Chapter 5: Disease resistance of postlarvae and juveniles from non-ablated Pacific whiteleg shrimp, *Litopenaeus vannamei*, broodstock post-challenged with pathogenic isolates of *Vibrio parahaemolyticus* (VpAHPND) and white spot disease (WSD)

5.1 Introduction

Growth performance and final survival of offspring produced from NAF broodstock have been demonstrated to be similar to those from AF broodstock in larviculture, nursery and grow-out (Chapter 3). Salinity stress tests, however, suggest that NAF can produce more resilient animals (Chapter 3 and 4).

The global shrimp farming industry has been affected by regular outbreaks of disease causing catastrophic crop failures with severe financial losses (Cock et al., 2009; Tran et al., 2013; Shinn et al., 2018b). Acute hepatopancreatic necrosis disease (AHPND), or Early Mortality Syndrome (EMS) as it is more commonly known, the microsporidian *Enterocytozoon hepatopenaei* (EHP) and white spot virus disease (WSD) are the top bacterial, parasitic and viral diseases impacting whiteleg shrimp production (Phuoc et al., 2009; Lightner et al., 2012; Sajali et al., 2019). AHPND is caused by pathogenic isolates of *Vibrio parahaemolyticus* (*Vp*), and a number of other *Vibrio* spp., that carry a plasmid encoding two *Pir*-like toxins which cause progressive degeneration of the shrimp hepatopancreas (Sajali et al., 2019). Infection often results in acute episodes of mortality in *L. vannamei* postlarvae (PL) within the first 20-35 days after stocking in nursery or grow-out ponds (Lightner and Redman, 2012; Tran et al., 2013; De Schryver et al., 2014), usually resulting in the complete loss of stock (De Schryver et al., 2014; Sajali et al., 2019). The collective losses attributed to AHPND alone throughout a number of Asian states (i.e. China, Malaysia, Thailand, and Vietnam) and in Mexico across the period of 2009 to 2016 were estimated by Shinn et al. (2018b) to be US$ 23.58 bn.

The Whispovirus commonly referred to as white spot (syndrome) virus (WSSV) responsible for white spot disease (WSD) infects a broad range of crustaceans inhabiting all tropical aquatic environments with temperatures typically ranging from 18 to 30˚C (Lightner et al., 2012; Verma et al., 2017). Infection can similarly result in high rates of mortality which can reach 100% within 3-10 days of infection (Lin et al., 2011; Verma et al., 2017). Since the first report of WSSV infection in Taiwan and the People's Republic of China in 1992 (Chou et al., 1995), the subsequent resultant losses were estimated by Lightner et al. (2012), up to the point of their report, to be in the

order of US$ 8-15 bn. In the same year, Stentiford et al. (2012) estimated that WSD accounts for an annual loss of almost US$1 bn.

The growth performance and final survival of the offspring of NAF shrimp is not different from those of AF shrimp, but previous studies (Chapter 3 and 4) suggest an improvement in their ability to cope with stress measured as survival after salinity stress testing. Salinity stress testing, a common method used by shrimp farmers to check post-larvae quality when sourcing, however, mainly relates to the ability of the PL to withstand environmental stress and does not give any indication of the ability of the shrimp to withstand a disease challenge. The objective of this study was to assess the resilience of postlarvae and juvenile *L. vannamei* produced from AF and NAF broodstock following a disease challenge and test the hypothesis that NAF offspring show higher resistance to disease when challenged with Vp_{AHPND} and WSSV under controlled experimental conditions. Any difference in survival post-challenge would demonstrate if there is any added value for farmers when sourcing PLs from ablated or non-ablated females.

5.2 Materials and methods

5.2.1 Hatchery production of the two shrimp populations

Two postlarvae populations were produced by Syaqua Siam Co. hatchery in Surat Thani Province, Thailand, one from ablated (AF) and the other from non-ablated (NAF) females belonging to the same breeding batch and family. The shrimp lines were from families from genetic breeding program and selected using salinity tolerance as one of the selection criteria. SPF (specific pathogen free) *Litopenaeus vannamei* broodstock with average male and female weights of 38.0 ± 2.0 and 40.0 ± 2.0 g respectively, were used for the production. Four maturation tanks (7×3.5×0.5 m; 2 for males and 2 for females) were stocked with 50 shrimp per tank ($2/m^2$). After one week of acclimatization after stocking, unilateral eyestalk ablation was performed on the females in one tank (Ablated – AF) by cauterization, while in the second tank the females remained intact (non-ablated – NAF).

Water temperature and salinity were maintained at 28.0 ± 0.0 ºC and 30.0 ± 1.0 ppt, respectively. Daily water exchange was applied (50-100%). Photoperiod followed a

natural regime by exposure to ambient sunlight through translucent roof windows in the maturation room. The broodstock were fed six times a day with polychaete worms and squid. Feeding rates were adjusted based on consumption rates and shrimp biomass. One week after ablation, mature females from each treatment were collected and placed in tanks containing males (1 male tank for each treatment group). After 3-4 hours, the mated females were collected from the male tank and placed into separate spawning tanks. Females were removed from the spawning tanks after spawning and returned to their respective maturation tanks. Nauplii were harvested after 16 h using a net (100-micron mesh), dipped in 50 ppm iodine for 60 seconds and then rinsed in running seawater for 5 minutes.

Six plastic tanks (500L) with an initial 300L water volume were stocked with 45,000 stage 5 nauplii at a density of 150/L. Both treatments were set up in triplicate and randomly distributed within a greenhouse. Water temperature, dissolved oxygen, pH, ammonia, nitrite and alkalinity were in 28.5 ± 0.7 ºC, 5.4 ± 0.2 mg/L, 7.8 ± 0.1, 0.1 ± 0.0, < 1 mg/L and 160.2 ± 39.4.6 mg/L $CaCO_3$ respectively. Salinity was adjusted from 30.0 ± 0.0 ppt to 15 ± 0.0 ppt from postlarvae 5. Approximately 30% of the water volume was exchanged daily when the animals reached postlarvae stage. The larval diets consisted of algae (*Thalassiosira* sp.), microparticulate feed (HiPro® from SyAqua Sdn. Bhd.) and live *Artemia*. The type and amount of food was adjusted for each larval stage.

When postlarvae were 15-days old (PL 15), they were shipped to the aquarium and challenge facilities of Fish Vet Group Asia Limited (FVGAL), Benchmark Thailand in Chonburi, Thailand. To avoid bias, a double-blind approach was used throughout the trial and subsequent analysis. The ablation status of the females producing each group of PL (AF or NAF) was not disclosed by SyAqua Siam until the completion of the challenge trials.

5.2.2 Mandatory health checks following the receipt of shrimp

The disease challenge trials were conducted within the aquarium and disease challenge facilities of Fish Vet Group Asia Limited (FVGAL), Benchmark Thailand in

Chonburi, Thailand. A total of 20,000 SPF *P. vannamei* postlarvae 15-day-old (PL_{15}), half of which were derived from NAF and the other half from AF were used in the trial.

Upon receipt at FVGAL, the PL were handled in accordance with local standard operating procedures for the receipt of new stock on site, i.e. the exterior of the transport bags were sprayed with 70% alcohol, then the PL were passed through a 100-micron mesh nylon bag and then surface-disinfected (15-20 sec dip) in a separate vessel containing 0.1 mg/L P.V.-DINE 125® (povidone iodine). The mesh bag and PL were then dipped for 15-20 s in a second vessel containing pre-treated conditioned 15 ppt seawater to rinse the shrimp. The PL were subsequently assigned to three separate 200 L static aerated holding tanks, each stocked in 180 L of pre-treated, dechlorinated 15 ppt seawater; stock was held under quarantine conditions while mandatory disease testing was conducted. For testing, a pooled sample of 150 PL (taken randomly from the holding tanks) per population were screened for seven key shrimp diseases, namely: Vp_{AHPND} by nested PCR; the fungal microsporidian *Enterocytozoon hepatopenaei* (EHP) and WSSV tested for by qPCR using OIE (2019) approved methodologies; for infectious hypodermal and haemotopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), and, yellow head virus (YHV) by iiPCR test kits (GeneReach Biotechnology Corporation, Taichung, Taiwan). Following the confirmation of freedom from all seven diseases, the remaining shrimp were kept in aerated, static tanks (200L) until the first disease challenge.

5.2.3 Maintenance of the two populations of *L. vannamei*

During the holding period, daily 20% water exchanges were performed using 15 ppt water (water pre-treated with 50 mg/L chlorine over a 24+ h period and the residual chlorine driven off by vigorous aeration). The absence of chlorine was confirmed using an orthotolidine-based chlorine test kit (Monitor®; Pet Wonderland Group, Thailand). Water temperature, salinity, dissolved oxygen, pH, alkalinity, unionized ammonia and nitrite were within the following ranges: 27.5 ± 0.1 ºC, 15.0 ± 0.0 ppt, 7.3 ± 0.1 mg/L, 7.5 ± 0.0, 161.5 ± 4.9 mg/L $CaCO_3$, 0.04 ± 0.0 and 0.1 ± 0.0 mg/L respectively. During the culture phase, the shrimp were fed three times daily (08:00 am; 12:00 pm and 04:00 pm) with two types of commercial shrimp feed: for the first 30 days, the animals were fed TNT 400-600 (Charoen Pokphand Co., Bangkok, Thailand) at a rate of 15 – 20% of

total biomass; from day 30 onwards, the shrimp were fed Starbird 5093 S shrimp feed (Charoen Pokphand Co., Bangkok, Thailand) at a rate of 10% body biomass per day.

5.2.4 Survival to salinity stress test

Two days after the receipt of the PL15 at FVGAL and one day before the start of the Vp_{AHPND} challenge, salinity stress tests were conducted on the two populations in quadruplicate (100 PL per replicate with 6.0 mg mean individual weight). Each batch of PL was transferred from 15 ppt seawater into a 1 L beaker with dechlorinated tap water (0 ppt) for 30 mins and then transferred into another 1 L beaker with clear 15 ppt salinity water. After a further 30 mins, the survival (%) of the PL in each replicate was evaluated based on immobility/response after physical stimulation with a pipette.

5.2.5 Vibrio parahaemolyticus preparation

The bacterial inoculum for the challenge was prepared by inoculating isolate FVG0001 (an isolate derived from a Vp_{AHPND} mortality event in *P. vannamei* cultured in Thailand) into tryptone soya broth (TSB) supplemented with 2% NaCl and cultured for 12h at 28°C, shaking at 250 rpm. Thereafter, the bacterial cells were collected by centrifugation at 900×g for 10 mins at 10°C and the resultant bacterial pellet re-suspended in sterile seawater (15 ppt). The number of colony-forming units (CFU mL^{-1}) in the suspension was then determined by measuring the optical density at 600 nm (OD600), where for Vp_{AHPND}, an OD value of 1.0 corresponded to approximately 2.0×10^8 CFU mL^{-1}. The bacterial cell number was then adjusted and verified by viable plate counts following standard methods.

5.2.6 Survival of shrimp postlarvae challenged with Vp_{AHPND}

The Vp_{AHPND} challenge tests followed the methods described in Shinn et al. (2018a) and Sajali et al. (2019).

Pre-challenge: The volume of bacterial suspension required to be added to each vessel for the main challenge was determined by a pre-challenge to assess the pathogen virulence by shrimp mortality using seven concentrations (0.1, 0.45, 0.8, 1.15, 1.5, 1.85, and 2.2 ml of a 2.0×10^8 CFU mL^{-1}, respectively) and selecting the bacterial

concentration required to give ca. 60-70% mortality 96 h post-infection. One day before the pre-challenge, 42 replicate, static, aerated, 20 L tanks, each containing 5 L of 15 ppt clear seawater were set up in a temperature-controlled disease challenge room maintained at 29.0 ± 0.0ºC. A total of 100 PLs per tank were used, with three replicates per dose. The pre-challenge was done for both populations of PL; the average weight of the PL at this stage was 10 mg. The initial volume of water in each tank was 5 L then at 24 h and 48 h post-challenge, an additional 3 L and 2 L of water was added respectively to a final volume of 10 L to maintain water quality. At 72 h post-challenge 50% water was exchanged. Shrimp mortality was assessed every 3 h over the 96 h post-challenge period. Shrimp were fed TNT 400-600 (Charoen Pokphand Co., Bangkok, Thailand) at 20% of the biomass following the same feeding regime as the PL held in the holding tanks.

Main challenge: Based on the pre-challenge results, a challenge dose of 2.0 ml of a 2×10^8 CFU mL^{-1} was selected. This dose resulted in 64% and 33% mortality in populations from AF and NAF respectively at 96 h post-infection. The main challenge was performed under the same conditions as the pre-challenge. For the main challenge, the performance of each population and condition was tested by using five replicate, static, aerated, 20 L tanks, with a total of 100 PL per tank. The groups were Population AF + Vp_{AHPND}; Population NAF + Vp_{AHPND}; Population AF – control with no Vp_{AHPND} added; Population NAF - control with no Vp_{AHPND} added. The PL from both populations had average individual weight of 14 mg at the time of the challenge. A semi-randomized block design was used to allocate the test tanks. The control tanks were isolated from the challenge tanks to prevent cross-contamination. Shrimp mortality was assessed every 3 h over the 96 h post-challenge period.

5.2.7 Challenge trials using white spot syndrome virus

Virus amplification: One week prior to starting the WSSV pre-challenge, 30 shrimp juveniles from population AF were placed in two tanks (10 L; 15 ppt) in a temperature controlled disease challenge room maintained at 26 ± 0.0 ºC. Population AF was selected as it was the weaker performer from the $\mathrm{Vp}_{\mathrm{AHPND}}$ tests to minimise animal use (3Rs). On the first day, the shrimp were fed to satiation with minced tissue from WSSV infected *P. vannamei.* The infected tissue was derived from frozen (-80˚C), WSSV

infected tissue, confirmed free of all other shrimp diseases. After exposure to WSSV infected tissue, the shrimp were fed a normal commercial feed thereafter. The tanks were checked every 3 h for 168 h and any dead or moribund shrimp removed. Moribund shrimp were immediately euthanised in iced water (<4˚C). Euthanised or dead shrimp were then stored at -80 ºC. After 7 days, all the resulting shrimp material was processed – the gills, muscle and pleopods were harvested, and thoroughly macerated to ensure complete mixing of the shrimp tissues. Three random 0.5 g samples were then taken and the titre of WSSV virus determined by qPCR. The macerated tissue was stored in the -80˚C freezer, while the qPCR tests were being conducted and the WSSV pre-tests were set-up.

Determination of the WSSV viral titres in the shrimp tissue for challenge

Quantitative PCR (qPCR) was used to determine the viral titre of the shrimp tissues used for the main WSSV challenge. DNA from macerated *P. vannamei* gill, muscle and pleopod tissue was extracted using a Qiagen DNEasy Blood & Tissue Kit (Qiagen, Hilden, Germany). qPCR was performed using qPCR Green Master Mix LRox (biotechrabbit GmbH, Hennigsdorf, Germany) on a Roche Lightcycler® 96 (Roche Diagnostics GmbH, Mannheim, Germany). The protocol used follows that of Durand and Lightner (2002) approved by OIE (OIE, 2019) for the detection of WSSV using primers WSS1011F (5'-TGG-TCC-CGT-CCT-CAT-CTC-AG-3') and WSS1079R (5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3'). The qPCR conditions used were: an initial denaturation step of 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and then 72°C for 30 sec. A melting curve analysis was performed to estimate the specificity of the method and used to confirm that no secondary products were observed. A negative DNA template control was included in the qPCR assay alongside a serial diluted plasmid DNA standard ($1 \times 10 - 1 \times 10^5$ μL^{-1}; Centex Shrimp, Mahidol University, Thailand) to permit the determination of the WSSV copy number within each sample. From >30g minced tissue resulting from the WSSV amplification step, the WSSV titre was determined from triplicate samples to be $1.81 – 2.37 \times 10^9$ WSSV/0.1 g (av. 2.02×10^9 WSSV/0.1 g).

WSSV pre-challenge: The amount of WSSV infected tissue derived from the WSSV amplification for the main WSSV challenge was determined from a pre-challenge assessing the virulence and mortality of shrimp using three amounts (i.e. 0.1 g, 0.15 g or 0.2 g shrimp^{-1}) of tissue (av. 2.02×10^9 WSSV/0.1 gram). The main aim was to determine the amount which resulted in 60-70% mortality 168 h post-infection. The pre-challenge was performed under the same conditions intended for the main challenge. One day before the pre-challenge 30 static, aerated, 1 L tanks each containing 0.4 L of 15 ppt clear seawater were set up in a temperature-controlled disease challenge room maintained at 26.3 ± 0.0 ºC. Ten single juvenile shrimp (average weight 1.5 ± 0.1 g) replicates were used per assessment dose of tissue. The pre-challenge was performed on shrimp taken from population AF as these shrimp had a significantly higher mortality in the *Vp*AHPND challenge and were regarded at this stage as the "weaker" population. For the infection step, WSSV macerated tissue from the pre-amplification step held at -80˚C was prepared by adding 50 µL of red food grade dye to each 1 g of minced shrimp tissue for 10 minutes before being weighed and allocated to the experimental tanks. The shrimp were not fed for 24 h prior to the start of the experiment. Shrimp were infected by weighing out the relevant amount of tissue and added to each vessel. For the infection step, the relevant amount of infected tissue was placed into the tank and the aeration to the tank switched off (pre-test dose range was 0.1-0.2 g WSSV infected tissue shrimp^{-1}). Shrimp consumption of the entire ration was confirmed by the presence of the red tissue passing into the stomach and intestine of the shrimp and the absence of any remaining free tissue in the experimental tank. The aeration was then switched back on, typically within 15 min. The shrimp were then maintained and monitored regularly. After 24 h, additional 0.4 L water was added to each experimental vessel. At 48, 72, 96, 120 and 144 h post-challenge, 50% of the water in each vessel was replenished. From day 2 of the challenge, the shrimp were maintained on the same feeding regime as the stock held in the main holding tanks. Shrimp mortality was assessed every 3 h over the 168 h post-infection period.

WSSV main challenge: From the pre-challenges, a dose of 0.1 g WSSV-infected tissue (av. 2.02×10^9 WSSV/0.1 gram) was selected as it resulted in 70% mortality of shrimp at 168 h post-infection. The main challenge was performed under the same conditions

as the pre-challenge but using a total of 200, static, aerated, 1 L vessels, each stocked with a single juvenile (i.e. 50 replicates per treatment – 50 × Population AF + WSSV; 50 × Population NAF + WSSV; 50 × Population AF – control not exposed to WSSV; 50 × Population NAF - control not exposed to WSSV). All shrimp used for the experiment were of a similar size and had an average individual weight of 1.4 ± 0.0 g. A semi-randomized block design was used to allocate the test tanks in the challenge room. As with the Vp_{AHPND} challenge, the control treatments were isolated to prevent cross-contamination. The experimental vessels were inspected every 3 h and any dead or moribund shrimp removed. Moribund shrimp were euthanized in pre-iced water where necessary, and then all removed shrimp stored in a -80 ºC freezer. After 168 h post-infection, the gills, pleopods and muscle were harvested from a random sample of shrimp from each population of shrimp and then analysed by qPCR to confirm the presence of WSSV and to determine the titres of WSSV.

5.2.8 Disposal of experimental materials

On completion of each trial, all surviving shrimp were humanely euthanized in pre-iced water (<4˚C), and subsequently incinerated together with other remaining dead shrimp collected during the trials.

5.2.9 Statistical analysis

One-way ANOVA followed by a Tukey test (Zar, 2010) was used to compare survival to salinity stress test in significance level of 0.05. Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. Percentage data were transformed to arcsine square-root prior to analysis. The data are presented as mean ± standard error.

Pairwise Kaplan-Meier survival analyses with subsequent *post-hoc* Mantel-Cox log-rank tests conducted in Excel 2016 were performed to calculate the survival probabilities of each population of shrimp in the Vp_{AHPND} and WSSV trials. All comparisons were conducted at a significance level of 0.05.

5.3 Results

5.3.1 Salinity stress tests and the survival rate of the shrimp post-larvae

No significant survival difference between the two populations was observed after the salinity stress tests. The PL from NAF and AF had 96.5 ± 1.84 and 99.75 ± 0.25 % survivals, respectively.

5.3.2 *Vp*$_{\mathrm{AHPND}}$ challenge

Drop counts confirmed that the growth equated to 2.35E+08 and 2.0E+08 CFU mL for the pre challenge and main challenge, respectively. The PL originating from NAF had significantly better survival (70.4%) than PL from AF (38.8%) at 96 h post-challenge (Figure 5.1). Over the challenge period, a significant difference between the two challenged groups was observed from 9 h post-challenge onwards (Table 5.1). The survival of the control (i.e. un-challenged) shrimp from the NAF and AF 96 h post-challenge was not significantly different between the two populations (100% and 100% for NAF and AF, respectively) (Figure 5.1). The *Vp*$_{\mathrm{AHPND}}$ challenged groups, however, had significantly lower survival than the control groups (Figure 5.1).

5.3.3 Survival of shrimp juveniles following WSSV challenge

The survival of the shrimp from NAF (62%) at 168 h post-infection was higher than that of the shrimp from AF (48%) but the difference was not significantly different. There were, however, significant differences between the two populations at 65 to 75 h post-challenge (Table 5.1; Figure 5.2). No significant difference was observed in the survival of non-challenged animals from both groups 168 h post-challenge (98% and 98% for NAF and AF, respectively). However, the WSSV challenged groups had significantly lower survival than the control groups (Figure 5.2). Terminal disease testing of a random selection of moribund and dead shrimp from each population confirmed death due to WSSV infection (AF (n = 3), av. 1.21×10^9 copies (range $1.09\text{-}1.31 \times 10^9$) WSSV copies / 0.1 g ; NAF (n = 3), av. 1.40×10^9 (range $1.37 - 1.44 \times 10^9$) WSSV copies / 0.1 g).

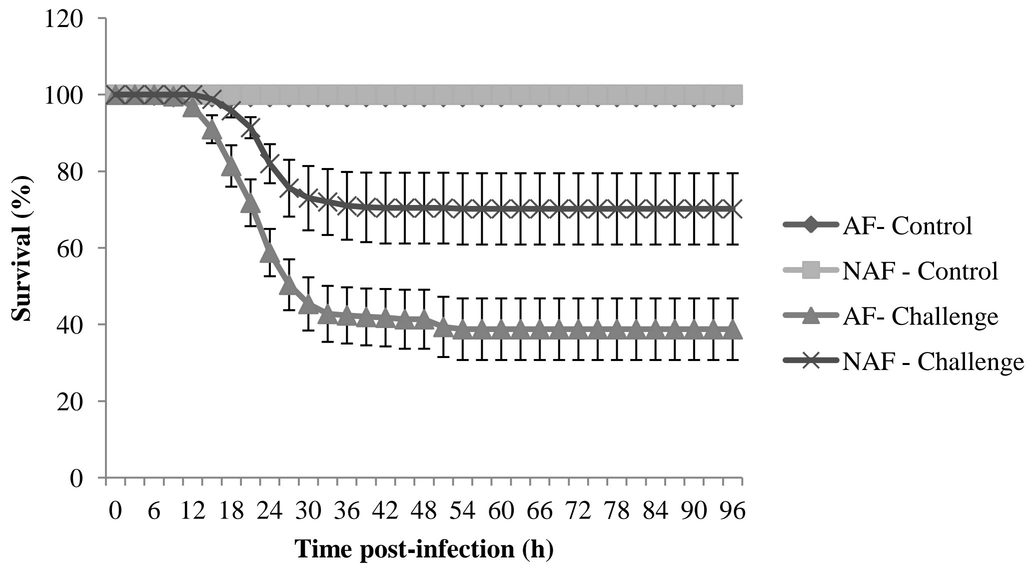

Figure 5. 1: Survival of non-challenged and *Vibrio parahaemolyticus*-challenged *L. vannamei* postlarvae (PL17) originating from non-ablated female (NAF) and ablated female (AF) broodstock. Significant difference between the two challenged groups was observerd from 9h post-challenge ($p < 0.05$, Mantel-Cox test).

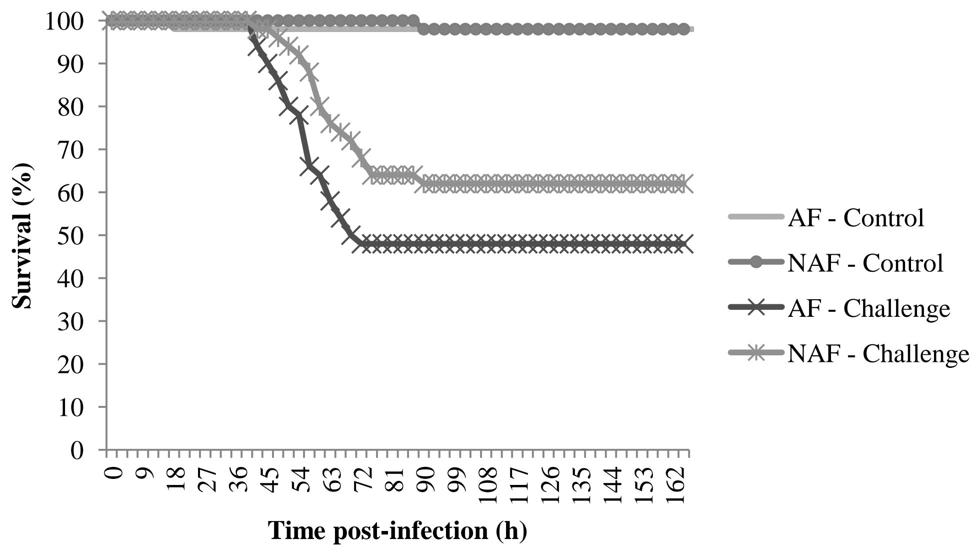

Figure 5. 2: Survival of non-challenged and WSSV-challenged *L. vannamei* juveniles originating from non-ablated female (NAF) and ablated female (AF) broodstock. No significant difference between the challenged groups was observed 162 h post-challenge ($p > 0.05$, Mantel-Cox test).

Table 5. 1: P values observed at each time point when challenged animals from non-ablated (NAF) and ablated (AF) with *Vibrio parahaemolyticus* or WSSV were compared.

	Observed P values between NAF&AF for each challenge			Observed P values between NAF&AF for each challenge	
Time (h)	***Vp* AHPND**	**WSSV**	**Time (h)**	***Vp* AHPND**	**WSSV**
0	NS	NS	84	2.40E-23	0.06
3	NS	NS	87	2.40E-23	0.06
6	0.16	NS	90	2.40E-23	0.09
9	6.15E-05	NS	93	2.40E-23	0.09
12	2.93E-08	NS	96	2.40E-23	0.09
15	1.86E-12	NS	99		0.09
18	3.07E-15	NS	102		0.09
21	7.44E-16	NS	105		0.09
24	5.22E-17	NS	108		0.09
27	3.09E-19	NS	111		0.09
30	7.03E-21	NS	114		0.09
33	2.45E-20	NS	117		0.09
36	2.28E-20	NS	120		0.09
39	2.20E-20	NS	123		0.09
42	8.59E-21	NS	126		0.09
45	8.59E-21	0.32	129		0.09
48	6.95E-23	0.099	132		0.09
51	2.40E-23	0.08	135		0.09
54	2.40E-23	0.04	138		0.09
57	2.40E-23	0.05	141		0.09
60	2.40E-23	0.01	144		0.09
63	2.40E-23	0.06	147		0.09
66	2.40E-23	0.04	150		0.09
69	2.40E-23	0.03	153		0.09
72	2.40E-23	0.02	156		0.09
75	2.40E-23	0.03	159		0.09
78	2.40E-23	0.06	162		0.09
81	2.40E-23	0.06	165		0.09
84	2.40E-23	0.06	168		0.09

AF – Ablated female; NAF – Non-ablated female; NS – Not significant

5.4 Discussion

Although unilateral eyestalk ablation facilitates consistent production schedules and increased nauplii production in commercial shrimp hatcheries, it is not a good welfare practice (Little et al., 2018). Eliminating ablation will require hatcheries to accept that this can be done without significant impact on their production and profitability and that there may be additional benefits in adopting a non-ablation approach. The data from Chapters 3 and 4 have demonstrated that it is possible to use NAF under commercial conditions and attain similar productivity with AF, and that the final survival and growth performance in larviculture, nursery and grow-out of their offspring is similar to that of AF.

Salinity stress tests are used by shrimp hatcheries, farmers and researchers to evaluate PL robustness as it is assumed the test serves as a predictor of shrimp performance during the nursery and grow-out phases (Alvaréz et al., 2004; Racotta et al., 2004; Palacios and Racotta, 2007). The response to this test is reported to be dependent on induced (i.e. experimental conditions) or natural (i.e. genetic) variation (Palacios and Racotta, 2007). The results from Chapters 3 and 4 demonstrated that PLs from NAF were significantly more resilient under salinity stress testing. This may suggest that ablation reduces the tolerance of PL to salinity stress. In the current study, however, no difference was noted in the survival of the PLs from NAF or AF in the salinity stress test. The difference to results observed in previous experiments (Chapters 3 and 4) may be at least partly due to differences in the genetics of the shrimp lines and PLs stage used in both experiments. Genetic variation in the particular shrimp line used in the current trial and the experimental conditions may have accounted for the results observed here. The shrimp used in the present study were derived from a SyAqua Siam shrimp line that was selected partly for tolerance to salinity change. A further reason for the observed differences is the salinity used to raise the PL which may affect their subsequent performance under salinity stress testing. In Chapter 3 and 4 the PL used were supplied in full strength seawater (32 ppt) while the PL used for the current trial were already acclimatized to 15 ppt following practices commonly used in Thailand.

Shrimp broodstock condition is essential for production of good quality offspring (Racotta et al., 2003). The basis of this can be nutritional (Naessens et al., 1997; Wouters et al., 2001a,b; Racotta et al., 2003; Hoa et al., 2009; Braga et al., 2015; Cardona et al., 2016) and/or physiological (Chamberlain and Lawrence, 1981b; Lawrence, 1983; Palacios et al., 1999ab; Racotta et al., 2003). Theoretically, NAF broodstock should demonstrate a better overall condition than AF counterparts as the latter has been reported to have physical trauma or stress, physiological imbalance and activation/reduction of immune related genes due to the effect of eyestalk ablation (Taylor et al., 2004; Sainz-Hernandez et at., 2009; Bae et al., 2013). This will consequently be reflected in differential quality of their offspring (Chapter 3 and 4). Although the PL from NAF had a similar rate of survival under salinity stress testing as the PL from AF broodstock in this experiment, there were different survival rates following experimental challenges with two key shrimp pathogens. Under challenge

with Vp_{AHPND}, the survival of the challenged PL from NAF was significantly higher than the PL from AF at 96 h post-challenge. The trial supports the hypothesis posed in Chapters 3 and 4, that ablation can negatively affect offspring quality in terms of their physiological status. Overall, the rates of survival observed in the PL from NAF (70.4%) and AF group (38.8%) are similar to those reported by Sajali et al. (2019) who observed a near 70% survival of shrimp challenged in 50% biofloc and a survival of 40% in the population of shrimp challenged with Vp_{AHPND} in 15 ppt clear seawater. The bacterial community of species found within biofloc forming, either purposefully or consequentially within systems, has been reported to have probiotic or immune-stimulatory effect on shrimp, thus acting as a biocontrol technique (Avnimelech, 2014; Shinn et al., 2018a; Sajali et al., 2019). In this study the challenge was conducted using clear water, therefore there would be expected that our results from NAF or/and AF would be similar to those reported by Sajali et al. (2019) in clear water as no immune-stimulatory effect would be observed in absence of bioflocs. The current study, however, suggest that PLs from NAF have better immune systems than AF, as they exhibited similar survival to the shrimp challenged in biofloc by Sajali et al. (2019). .

The mechanisms that lead to this improvement could be multifarious and most likely linked to enhancement in the immune status of the offspring from non-ablated broodstock. However, as no measurements of immune response were conducted in this study the mode of action for enhanced robustness remains to be confirmed. In addition, the future studies should also assess antimicrobial effects of fatty acids with Vp_{AHPND} in offspring from NAF and AF as they have been demonstrated to show antagonic activity against a wide range of microorganisms, including bacteria (Desbois, et al., 2008; Desbois et al., 2009; Desbois and Smith, 2010; Desbois and Lawlor, 2013; Desbois, 2013).

When the same two populations of shrimp were challenged with WSSV, there was no statistical difference between the two challenged groups at the conclusion of the experiment (168 h post-challenge). At intermediate times (54 h and 75 h post-challenge) the NAF population survival was significantly higher than that of the AF. The higher survival of juveniles from NAF, although not statistically significant, suggests that there may be some slight disadvantage of ablation on the offspring's

ability to withstand a WSSV challenge but that the current experimental design was inadequate to demonstrate this.

It has long been recognized, however, that ablation can also cause physiological imbalance and compromise the immunological health of broodstock (Palacios et al., 1999ab; Sainz-Hernandez, et al., 2009; Bae et al., 2013; Treerattrakool et al., 2014; Das et al., 2015). Ablation can also lower the nutritional reserves of the offspring (Wickins and Lee, 2002; Racotta et al., 2003). This study and previous studies (Chapters 3 and 4), confirms that ablation has an impact not only on the female broodstock, but that negative effects are carried on through to the offspring. This indicates that any negative impacts from adopting a non-ablation policy can potentially be offset by a higher survival to disease outbreaks and this will provide important benefits to the industry as a whole through reducing losses at the farm level. It will also remove animal welfare concerns associated with the practice of ablation that could affect access to key markets.

The higher survival of the PL from NAF post-challenge with Vp_{AHPND} under laboratory controlled conditions suggest that if farmers stock their nursery tanks/ponds with PLs from NAF, there is increased likelihood of significantly improving the survival of stock compared to PLs from AF when shrimp are exposed to Vp_{AHPND} within the first days of stocking. Similarly, a higher rate of survival of juveniles from NAF parents stocked in grow-out ponds may be observed in first days of WSSV exposure. Vp_{AHPND} infections can result in the complete loss of stock (De Schryver et al., 2014; Sajali et al., 2019), which has been estimated to have resulted in accumulated losses of ca. US$ 23.58 bn in 8 years (2009-2016) across Vietnam, Thailand, Malaysia, China and Mexico (Shinn et al., 2018b). Lightner et al. (2012) also reported losses of US$8 – $15 bn due to WSSV. The higher survival observed in PL and juveniles from NAF might, therefore, reduce the levels of loss and deliver economic benefits to farmers.

In conclusion, these results and those reported in previous chapters support the fact that eyestalk ablation as a management practice can be phased out to move the sector towards higher welfare production standards. A further benefit of this, as these results show, is that there is compelling economic argument of the benefits of non-ablation as results now confirm growth performance and survival under normal

conditions are not compromised and in fact survival in response to typical pathogens (Vp_{AHPND} and WSSV) is likely to be higher in PLs and juveniles from non-ablated animals, Validation at the farm level of the current study's findings is now needed.

Chapter 6: Economics of non-ablated Pacific white shrimp (*Litopenaeus vannamei*) hatchery based system and their contribution to larviculture and grow-out production costs

6.1 Introduction

Maturation facilities using NAF can attain a similar level of productivity (eggs and nauplii per tank/day) as ablated animals if the male:female sex ratio is increased from 1:1 to 1:2 (Chapter 3 and 4). The offspring produced by non-ablated females have similar growth and final survival to those from ablated female from early development stage to marketable size shrimp (Chapters 3 and 4). However, salinity stress tests indicated that offspring from non-ablated female are more robust (2.3 to 3% more survival) (Chapters 3 and 4). This has been confirmed by the higher survival observed for postlarvae (70%) and juveniles (62%) from non-ablated females when challenged with Vp_{AHPND} and WSSV, respectively, as presented in Chapter 5.

Understanding the factors affecting shrimp production costs allow hatchery or farm managers to control and optimize the economics of their operation (Juarez and Marinez-Cordero, 2004). The profitability of a shrimp hatchery or farm depends on three major factors: production cost, volume of product (larvae, postlarvae, juveniles or marketable size shrimp produced) and their respective market price (Naegel, 2010), market price reflecting their perceived value to a buyer. The hatchery production cost is affected by many factors including the condition of the shrimp broodstock and the use of techniques such as ablation that can change mating success and system productivity.

Switching from using unilateral eyestalk ablated broodstock to management of non-ablated shrimp has been associated with lower hatchery productivity (Chapters 3 and 4). Maintaining production levels of larvae, without expanding hatchery facilities, would require an increase in the number of non-ablated females stocked, with all the associated costs with potentially, knock on increased cost of larvae and postlarvae and costs to market shrimp producers. However the higher survival observed on non-ablated offspring under laboratory disease challenge (Vp_{AHPND} and WSSV) might be expected to offset these higher costs by reducing the costs and risk to market shrimp farmers.

Therefore, this study assessed the effect of shifting from using ablated (AF) to non-ablated (NAF) *L. vannamei* female for a commercial scale hatchery on the production cost of larvae (nauplii) and postlarvae (PLs). A key element of this is an analysis of the profitability of using NAF offspring (postlarvae and juveniles) at farm level under conditions of disease outbreak (APHND or WSSV).

6.2 Material and methods

6.2.1 Hatchery production cost

Two different sets of financial data (primary and secondary) were used to determine nauplii production cost and one set (secondary data) for postlarvae production cost. Secondary financial data covering 2012 through 2017 on nauplii and postlarvae production at Larviculture del Pacífico (Larvipac), a commercial hatchery of Seajoy company in Honduras where studies from Chapter 3 and 4 were conducted, were provided by the company general manager. The hatchery has been producing *L. vannamei* nauplii and postlarvae for over 20 years and used AF from the time it was set up in 1996 to 2014; but switched to NAF production in 2015 to date. Data for NAF relate to the period 2015-2017, and for AF the period 2012-2014. Another set of financial data related to nauplii production was collected during one experiment conducted under commercial conditions at Larvipac in 2017 (Chapter 4). In this trial the effect of offering natural feed (squid and polychaete) during pre-maturation on reproductive performance of NAF *L. vannamei* was evaluated. Data were collected to assess the impact of feeding regime during pre-maturation on nauplii production cost.

The following information were extracted from secondary financial data sheets of maturation and spawning area: Number of produced nauplii and used broodstock, direct costs (including feed, broodstock, probiotic and stimulants, fertilizer, packing materials and direct labor costs) and indirect costs (including staff costs; benefits and social positions; fuel, oil and lubricants; work equipment and uniform; staff transportation; repair and maintenance; materials and provisions; chemicals for laboratory; security expenses; transport and product dispatch; depreciation and amortization; public services; external services; travel and representations expenses; marketing and representations expense; rent and facilities; provisions and taxes; corporate transport and others expenses).

Data collected from the Chapter 4 experiment (Figure 6.1) included the number of nauplii produced , direct costs (including feed, broodstock, probiotic and stimulants costs) and indirect costs (including cost of staff; benefits and social positions; fuel, oil and lubricants; work equipment and uniform; material transportation; repair and maintenance; materials and provisions; public services; rent and facilities).

In larviculture secondary data extracted included the number of postlarvae produced; direct costs (including nauplii, feed, probiotic and stimulants, fertilizer, packing materials and direct labor) and indirect costs (calculated similarly to the maturation data).

It is important to highlight that the Seajoy Hatchery has both (1) maturation and spawning (to produce nauplii) and (2) larviculture (to produce postlarvae) components (Figure 6.2). Nauplii production cost is the main cost parameter at maturation and spawning area as all facilities and investment used there are to produce nauplii. On another hand postlarvae production cost is the main cost parameter for larviculture and is directly associated to nauplii production costs. Therefore the production cost parameters determined using whether secondary or experimental data will be shown based on their respective production area (Maturation and spawning or larviculture).

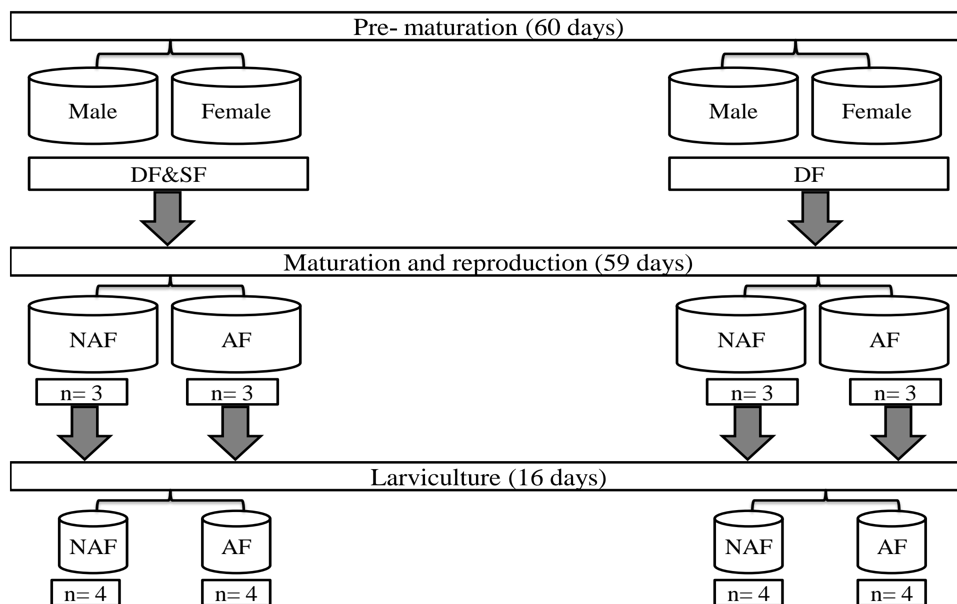

Figure 6. 1: Schematic diagram of experimental design used in Chapter 4. DF&NF - Dry feed and natural feed treatment; DF – Dry feed treatment; NAF – Non-ablated female; AF – Ablated female.

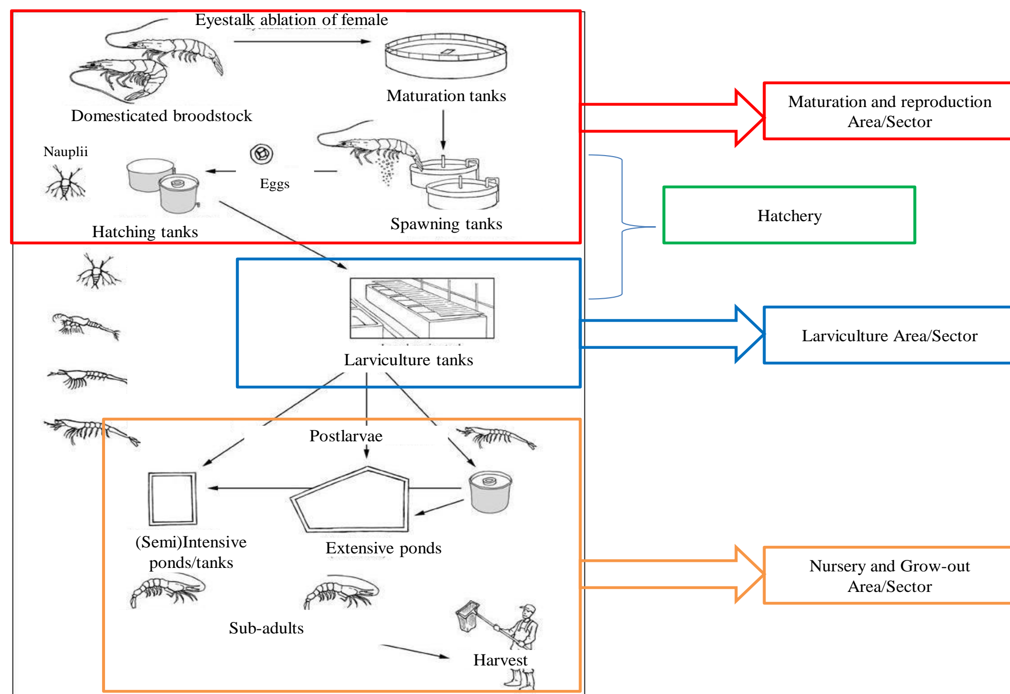

Figure 6. 2: Main production components of *L. vannamei* in captivity. Adapted from FAO, 2009

6.2.2 Nursery and grow-out profitability modeling under disease outbreak

The effect of disease resistance (AHPND or WSSV) on farms profitability under various emergency scenario was modeled using data from nursery and grow-out (Chapter 3 – study I), survival from disease challenges (Chapter 5) and costs (secondary data). When farmers detect mortalities due to disease outbreak and can no longer control the situation, emergency harvest maybe implemented to avoid complete loss of the crop or their investment. Therefore, the models were built based on two different emergency scenarios.

First was considered during nursery period for both farmers who stock directly their postlarvae in grow-out or nursery ponds before moving to other grow-out ponds. AHPND outbreak can occur during any period of a nursery. To model AHPND impact, the following assumptions were made:

- The outbreak occurred at the end of the nursery period (between 20 to 30 days post start of the nursery) where the investment losses are usually higher.
- Farmers harvested surviving animals as a salvage operation to avoid any further losses and stocked them in new and clean grow-ponds to marketable shrimp size.
- Survival post challenge was then calculated as if there is disease outbreak at the end.
- The price of juveniles emergency harvested from nursery, was based on assumed value for to grow-out farmers.

A second scenario was considered during grow-out period. WSSV outbreaks can also occur during any period of a grow-out. To model the WSSV impact, the following assumptions were made:

- The outbreak occurred at the end of the grow-out period when investment losses are usually higher.
- The farmers will harvest the surviving animals as emergency measure to avoid any further losses and sell them as marketable size shrimp.
- Survival post challenge was then calculated as if there is disease outbreak at different time points of grow-out cycle.

6.2.3 Data Analyses

Two approaches were used to analyse the effect of making a small change in the production process (i.e. switch from AF to NAF system). First the secondary data were analyzed using one-way ANOVA followed by a Tukey test (Zar, 2010) in significance level of 0.05. Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. All statistics were run using STATISTICA 7 software. Then a partial budget (Engle, 2010) was used to analyse primary data gathered from Chapter 4.

The impact of disease resistance on profitability was assessed by modeling production cost, revenue, profit and gross margin under normal scenario or disease outbreak for either AHPND or WSSV.

It is important to highlight that the majority of secondary data were not provided with full details of fixed and variables costs. For instance, almost all fixed costs and some variable costs were included in “new” category “indirect cost”, whilst other variable costs were included in “direct cost” category. It was not possible to breakdown the actual cost of each fixed or variable item in the indirect cost category. Therefore, the production cost was determined based on new approach by using indirect and direct costs data.

6.3 Results

6.3.1 Production costs

6.3.1.1 Maturation and spawning, and larviculture

Secondary data indicated that nauplii production cost was significantly higher when the hatchery used NAF rather than AF (Table 6.1). In contrast, no significant difference was observed for postlarvae production cost when the hatchery used nauplii produced using NAF and AF (Table 6.1). Costs related to broodstock and own-produced nauplii contributed to the higher percentage to nauplii and postlarvae production costs when using NAF animals, respectively (Table 6.1). On the other hand indirect costs contributed more when using AF animals, respectively. Feed did not make any significant contribution to nauplii or postlarvae production cost (Table 6.1).

Table 6. 1: Production costs (mean ± SE) of *nauplii* and *postlarvae* (PL) using ablated – AF (n = 3; 2012-2014) and non-ablated – NAF broodstock (n = 3; 2015-2017).

Maturation and spawning	**NAF**	**AF**	**NAF**	**AF**
Costs	**US $/1000 np**		**%**	
Feed	0.08 ± 0.01[a]	0.09 ± 0.01[a]	16.73 ± 1.02[a]	25.56 ± 3.90[a]
Broodstock	0.23 ± 0.01[a]	0.07 ± 0.01[b]	48.66 ± 1.35[a]	19.57 ± 5.18[b]
Indirect cost	0.16 ± 0.01[b]	0.19 ± 0.01[a]	34.02 ± 1.58[b]	54.87 ± 8.78[a]
Nauplii production cost	0.47 ± 0.01[a]	0.35 ± 0.02[b]		
Larviculture	**NAF**	**AF**	**NAF**	**AF**
Costs	**US $/1000 PL**		**%**	
Feed	0.35 ± 0.02[a]	0.45 ± 0.02[a]	14.79 ± 1.43[a]	17.76 ± 0.91[a]
Own nauplii	1.24 ± 0.07[a]	0.67 ± 0.07[b]	51.92 ± 1.95[a]	35.42 ± 2.88[b]
Indirect cost	0.65 ± 0.03[b]	1.06 ± 0.09[a]	27.33 ± 2.48[b]	45.57 ± 3.68[a]
Postlarvae production cost	2.39 ± 0.13[a]	2.04 ± 0.03[a]		

Cost evaluation using data from the Chapter 4 experiment also showed that nauplii production cost was higher when using NAF, regardless of their feeding history in pre-maturation, although the feed, broodstock and indirect costs contributed a similar proportion of the production cost (Table 6.2). However, the nauplii production cost using NAF animals previously supplemented with high quality moist feed was lower than in the NAF group fed only dry feed (Table 6.2).

Table 6. 2: Production cost of *nauplii* from non-ablated (NAF) and ablated (AF) female previously supplemented high quality moist feed (DF&SF) or not (DF).

	DF&SF		**DF**	
Unit/Item	**NAF**	**AF**	**NAF**	**AF**
Nauplii production cost (US $/1000 np)	0.36	0.29	0.41	0.30
% of nauplii production cost				
Feed	32.4	32.4	29.4	29.4
Broodstock	51.3	51.3	53.5	53.5
Indirect costs	16.3	16.3	17.0	17.0

6.3.2 Modeling of disease impact on profitability at nursery and grow-out

Infection with AHPND often results in acute episodes of mortality in *L. vannamei* postlarvae (PL) within the first 20-35 days (nursery period) after stocking in nursery or grow-out ponds. Under normal conditions (no AHPND outbreak) the revenue, profit and gross margin generated using postlarvae from either NAF or AF in semi-intensive nursery are similar (Figure 6.3). However, if there is outbreak of AHPND the revenue and profit are higher in nursery ponds with postlarvae derived from NAF than AF (more US$ 207 and 333/ha of revenue and profit, respectively) (Figure 6.3). In addition, gross margins are also higher in ponds with postlarvae from NAF (more 111 %/ha of gross margin) (Figure 6.3).

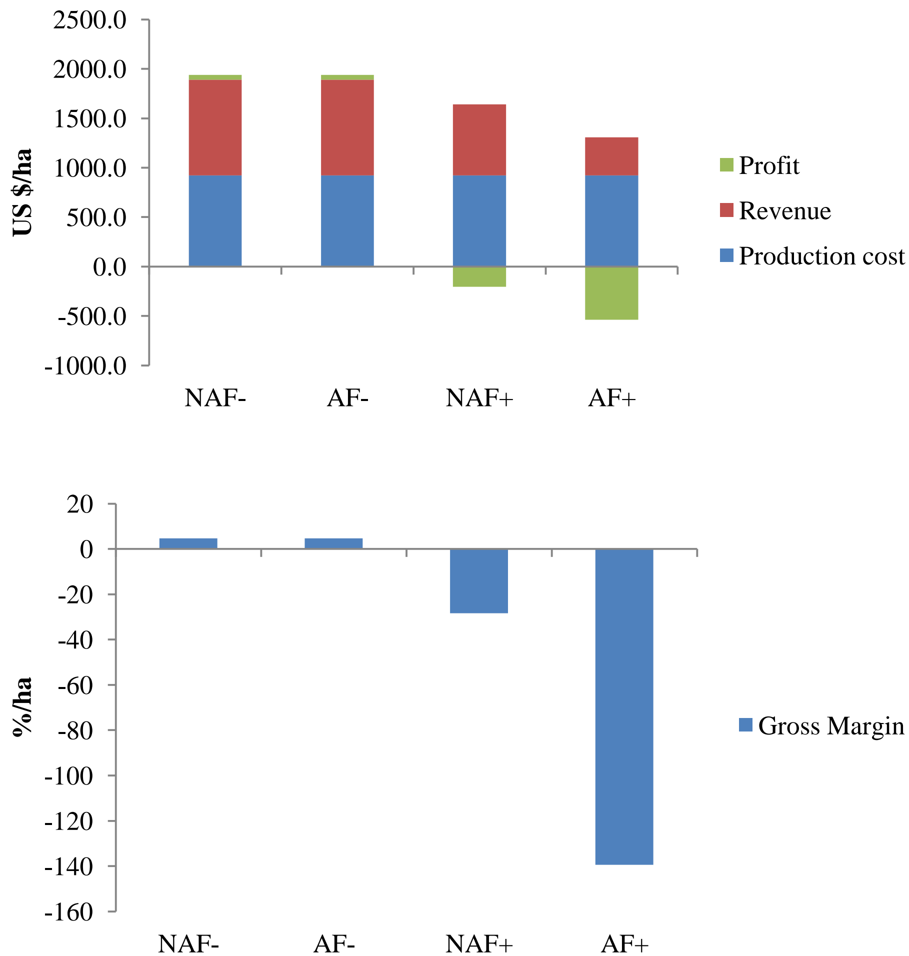

Figure 6. 3: Nursery profitability using postlarvae from NAF and AF without (-) and with AHPND (+).

If , following emergency harvest, surviving shrimp were stocked in new and clean grow-out ponds free of disease and no outbreak was observed until the harvest of marketable shrimp size , both production costs and revenue would be higher in grow-out ponds with animals derived from NAF than AF (more US$ 3750, 5057 and 1307/ha of production cost, revenue and profit, respectively) (Figure 6.4).

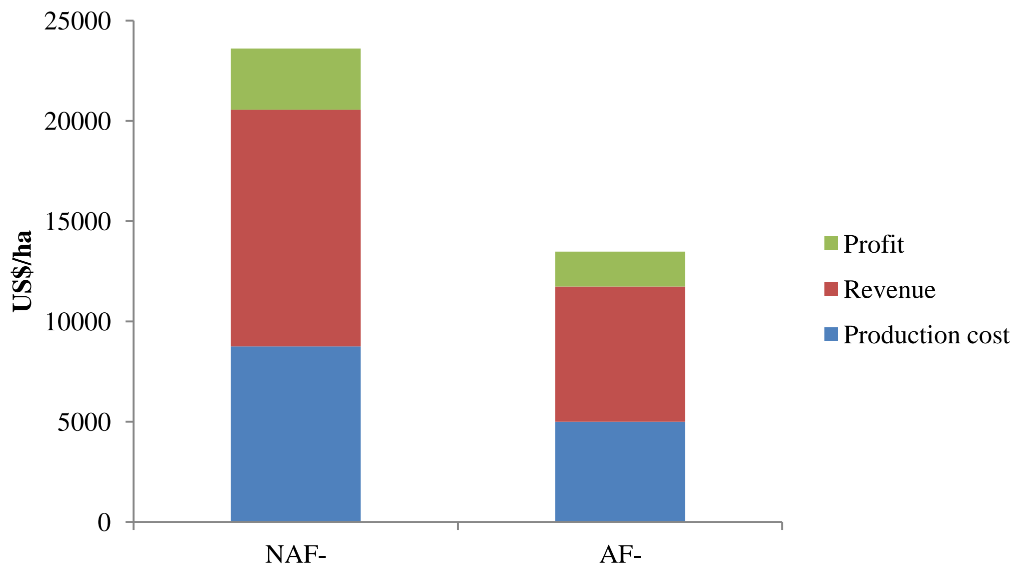

Figure 6. 4: Grow-out profitability using surviving animals during AHPND outbreak in nursery ponds. NAF- and AF- represent absence of disease.

The revenue, profit and gross margin are similar under absence of WSSV outbreak in grow-out semi-intensive ponds stocked with juveniles from either NAF or AF (Figure 6.5). After a WSSV outbreak the revenue and profit are higher in grow-out ponds with shrimp derived from non-ablated than ablated broodstock (more US$ 1226 and 1111/ha of revenue and profit, respectively) (Figure 6.5). In addition, gross margin is also higher in ponds with postlarvae from NAF (more 35%/ha of gross margin)

(Figure 6.5). When looking at the whole production cycle, revenue, profit and gross margin are higher in ponds stocked with juveniles derived from NAF than AF (Figure 6.6).

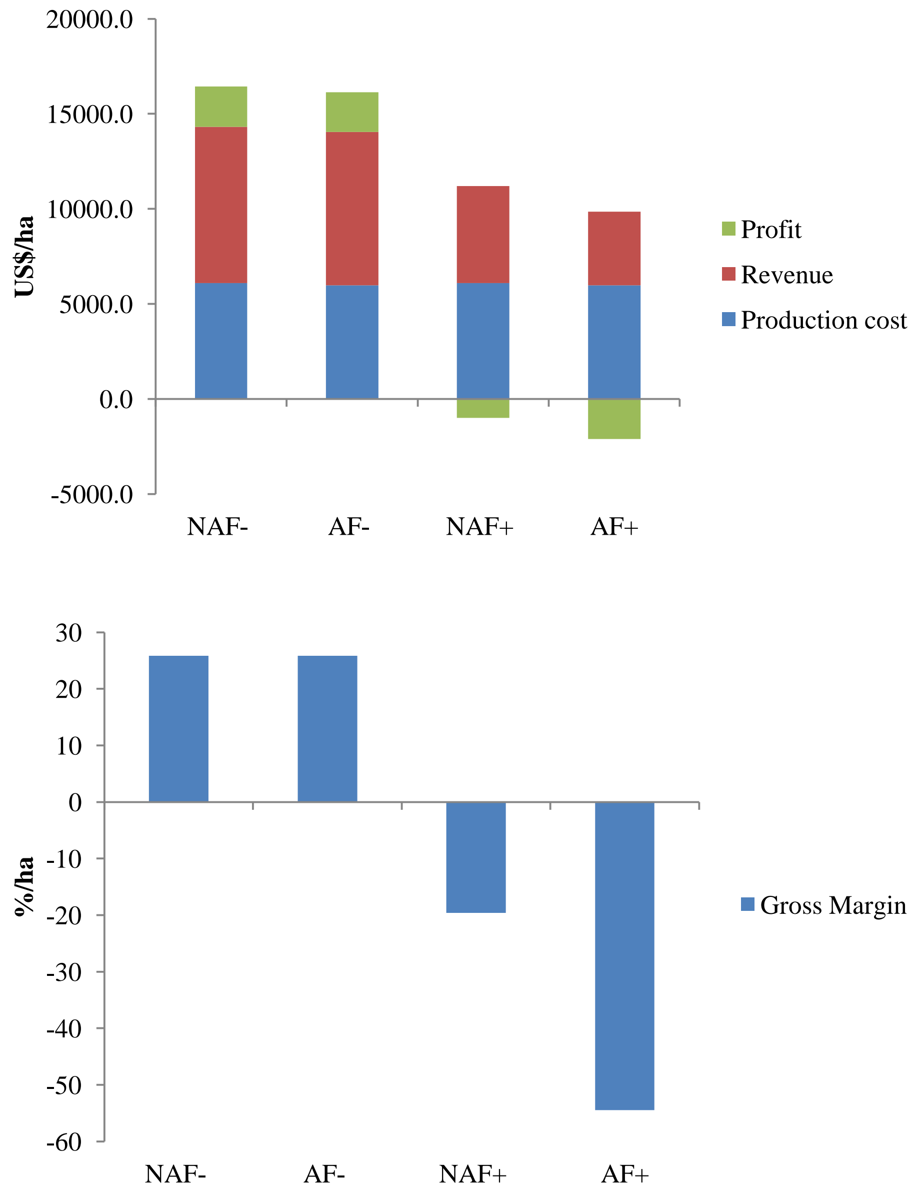

Figure 6. 5: Grow-out profitability using juveniles from NAF and AF without (-) and with WSSV (+).

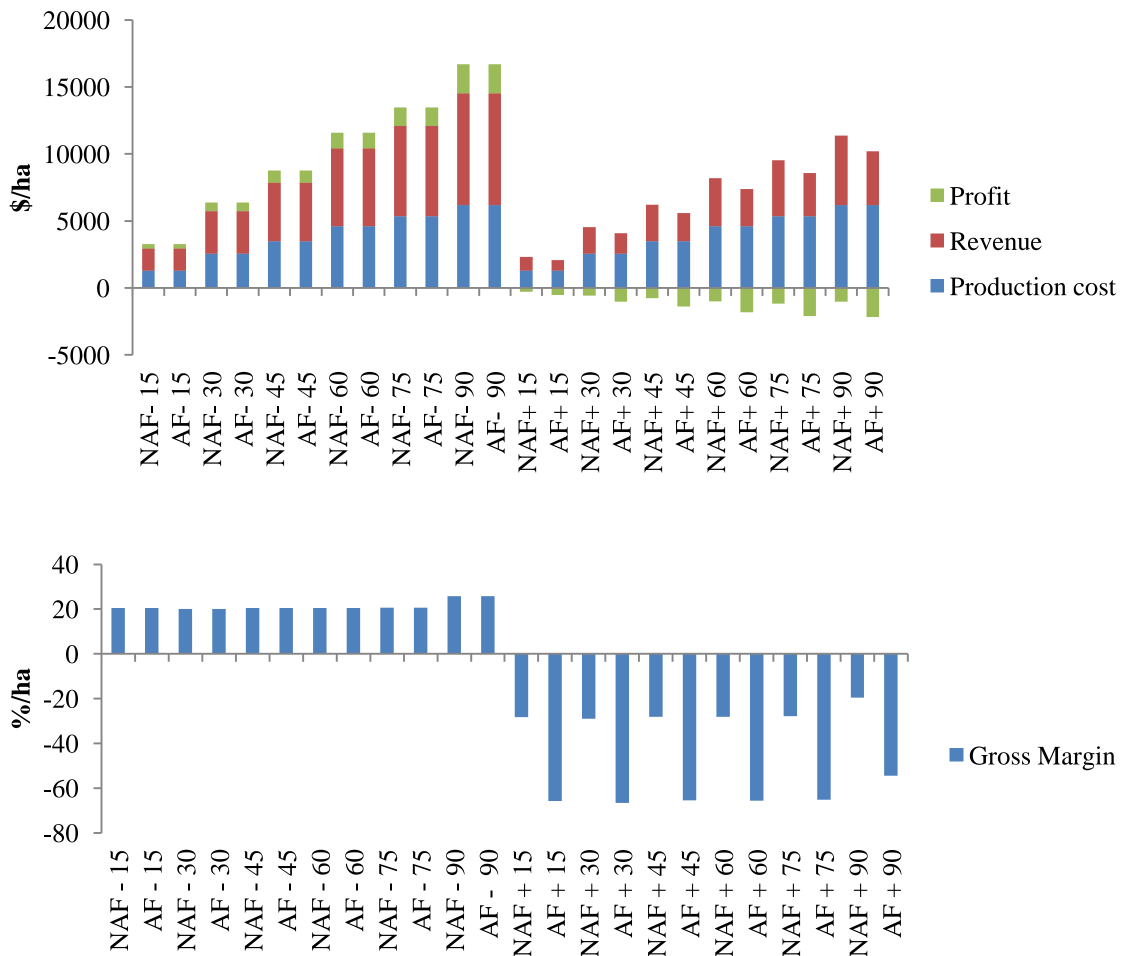

Figure 6. 6: Grow-out profitability using juveniles from NAF and AF without (-) and with WSSV (+) over the production cycle.

6.4 Discussion

The production cost of PLs depends in several factors i.e. nauplii, broodstock, feed, size of operation and maturation syste.. The number of nauplii produced by a hatchery has a direct effect on total production cost (Naegel, 2010). Therefore any change on nauplii production process, such as changing broodstock strain, increasing broodstock number, or operation system, will be immediately expressed in production cost. Secondary data suggest that switching from AF to NAF hatchery system will increase nauplii production cost by 34%, a result that was supported by the primary data presented in Chapter 4. There was also increase in production cost with NAF (by 19% to the group fed squid and polychaete, and 29% to broodstock fed only dry diet) but it is

important to highlight that the rise by 19% is underestimated value as there was underfeeding in the female fed squid and polychaete during pre-maturation. Increased broodstock cost related directly to the requirement for a greater number of breeding animals and the additional feed required. The results can be explained by two factors. First, when the company switched to NAF system there was an increase in the number of broodstock required with concomitant increases in costs, this increased nauplii production costs for NAF by 34% compared to AF (i.e. US $ 0.47 of 0.35 /1000 nauplii). The total cost of broodstock has been reported to be one of the main factors in the financial performance of shrimp hatcheries (Shang et al., 1998; Wickins and Lee, 2002), contributing 26.3%, 22.9% and 22% to the production cost in small, medium and large scale hatcheries, respectively (Shang et al., 1998). Although, contribution of indirect costs dropped by 38% with NAF system they did not affect the nauplii production cost as broodstock. Second the amount of produced nauplii account to the nauplii production cost. The number of nauplii per tank produced by NAF previously fed squid and polychaete or not, was significantly lower to AF (Chapter 4), therefore their nauplii production cost was lower here. Furthermore, the direct effect of nauplii numbers is clear between the two NAF groups. Broodstock fed only dry feed had higher production cost (12% more) because it had lower production of nauplii per tank, although not significantly different (Chapter 4).

Besides survival, postlarvae production cost is also a function of nauplii cost (Naegel, 2010).. Although nauplii cost contribution to production cost of postlarvae from NAF was higher than AF, it did not affect the postlarvae production cost. Indirect costs were significantly lower but they did not have any impact on the production cost of the animals from NAF.

The global shrimp farming industry has been affected by regular outbreaks of disease causing catastrophic crop failures with severe financial losses (Cock et al., 2009; Tran et al., 2013; Shinn et al., 2018b). For instance the collective losses attributed to AHPND alone throughout a number of Asian states (i.e. China, Malaysia, Thailand, and Vietnam) and in Mexico across the period of 2009 to 2016 were estimated by Shinn et al. (2018b) to be US$ 23.58 bn. In addition, resultant annual loss from WSSV was estimated by Stentiford et al. (2012), up to the point of their report, to be almost US$1

bn. However, the current study has demonstrated that if the farmers switch to source animals produced on NAF system, they would reduce the level of financial losses caused by AHPND and WSSV as better revenue, profit and gross margin were observed with NAF offspring.

Overall, moving to high welfare practice by stopping unilateral eyestalk ablation in commercial shrimp hatcheries will only increase nauplii production cost. In addition, the move to source animals from NAF will reduce loss after disease challenges and hold a promise towards reducing risk, and along with other measures, improving profits even in the face of pathogen challenges.

In conclusion, NAF broodstock increase nauplii production costs but this does not affect the postlarvae production cost. Farmers will reduce financial losses due to AHPND and WSSV if they source shrimp produced on NAF system. Furthermore, there is still room to reduce nauplii production cost if the number of the broodstock can be reduced by using more productive strains.

Chapter 7: General Discussion and final conclusion

7.1 Reproductive performance and mortality of non-ablated females

Reproductive parameters such as spawning event, hatching rate and fertilization are not affected by non-ablation. The lack of difference between hatching rate of eggs from NAF and AF *L. vannamei* (Chapter 3 and 4) are corroborated by earlier results reported by Chamberlain and Lawrence (1981) and Palacios et al. (1999b) with the same species, and in *Penaeus stylirostris* (Gendrop-Funes and Valenzuela-Espinoza, 1995), and *Penaeus canaliculatus* (Choy, 1987). Natural feed (squid and polychaete) during pre-maturation improved egg hatching rates for NAF, also fertilization rates (Chapter 4). These results confirm the role of fresh-frozen natural feeds to improve spawn quality (fertility and hatching rate) as previously reported by Naessens et al. (1997) and Ibarra et al. (1998).

Mating success has been reported to be higher in AF than NAF *L. vannamei* (Chamberlain and Lawrence, 1981b; Palacios et al. 1999b) due to the effect of unilateral eyestalk ablation (Chapter 3 and 4). This parameter is directly associated with female productivity in terms of number of eggs and nauplii per tank per day. Therefore the productivity was significantly higher in AF (Chapters 3 and 4). Emerenciano et al. (2013) observed that mating success of *L. vannamei* (AF) previously reared in biofloc systems and fed with mussel and squid was improved compared to breeders not receiving such feeds. This is contrary to present study (Chapter 4) where fresh-frozen natural feeds (squid and polychaete) did not improve NAF mating success as their effect was outweighed by ablation. Furthermore, the effect of fresh-frozen feeds on mating success was not reflected, most likely due to underfeeding which occurred in first weeks of pre-maturation when there the amount of those feeds for the shrimp were miscalculated.

The fecundity (number of eggs and nauplii per spawned female per day) from NAF was significantly higher than AF (Chapter 3 and 4). This supports the outcomes observed by Choy (1987) and Emmerson (1980) with *P. canaliculatus* and *P. indicus*, and it sustains the idea that unilateral eyestalk ablation negatively affects other aspects of shrimp female reproductive physiology. Although, no difference in female mortality rate was observed in Chapter 4, the results in Chapter 3 have demonstrated that mortality rates of AF females were almost double that of NAF confirming the trauma

and stress associated with the technique (Bae et al., 2013; Choy, 1987; Taylor et al., 2004). In addition, the differences between mortalities observed in Chapter 3 and 4 may be associated with maturation and spawning conditions, time at which the study was conducted or differences in the broodstock genetic line/sources. For instance, the maturation facilities used in both chapters were the same; however broodstock of a different genetic line and the timing of the experiment (end of annual production cycle which in general the hatchery report higher mortalities) were used.

Unilateral eyestalk ablation is also reported to cause deterioration in female shrimp in either their condition (i.e. nutrition reserves) or offspring quality (Boucard et al., 2004, Santiago, 1977; Primavera, 1982; Palacios et al., 1999b). This has been confirmed in the current study (Chapter 4) which observed higher content of total n-6 and n-3 PUFAs, and DHA in hepatopancreas from NAF than AF. Furthermore, LA and DHA were higher in ovaries from NAF than AF. The positive role of fresh-frozen natural feeds (squid and polychaete) in improving NAF condition were also demonstrated here (Chapter 4) as their hepatopancreas had higher levels of total lipid, total saturated and monounsaturated fatty acids, LA, ARA and EPA when compared to other AF females.

In general, commercial shrimp maturation facilities have relied on unilateral eyestalk ablation to rapidly induce female maturation and meet production demand. Any new approach would have to meet the commercial realities of maturation facilities, but also ensure improved welfare outcomes for the animals being managed. Among the shrimp female reproductive performance parameters (including mating success, spawning frequency, hatching and fertilization rates, and fecundity), the mating success is the most important parameter when evaluating the potential of a new practice as it is directly correlated to the level of egg and nauplii production over time in a maturation facility of a given size and capacity. This is confirmed by the fact that although NAF had higher fecundity (egg and nauplii per spawned female) than AF (Chapter 3), it was not enough to increase overall maturation facility output of egg and nauplii. Other, older studies also found lower mating success using NAF with *L. vannamei* (Chamberlain and Lawrence, 1981b; Palacios et al. 1999b), a finding that was confirmed with the current study (Chapter 3) where mating success for NAF was

around half of AF despite modern production practices (i.e improved shrimp strains, feed quality and maturation system). Moreover, the use of fresh-frozen natural feed (squid and polychaete) in pre-maturation did not improve NAF mating success (Chapter 4). However, improved longevity of NAF together with higher fecundity might compensate for the higher short term productivity of AF. Such tradeoffs in reproductive performance are well known in the terrestrial livestock breeding literature (e.g. De Vries, 2017). Furthermore comparable level of production can be realised by an intensive commercial maturation facility based on NAF system if the managers consider other factors such as the sex ratio used in their production tanks. Normally, hatcheries based on AF system use 1:1 male to female ratio and if they switch to NAF system would require increasing sex ratio to 1:2 male to female in order to get same productivity observed in 1:1 sex ratio. The future focus would still be to find ways of improving NAF mating success. Although fresh-frozen feed did not improve this aspect of performance under the given experimental set up and feeding regimes, it may be a suitable focus for a selective breeding program as little attention has been given to this approach for improving productive performance of NAF.

7.2 Offspring quality

Shrimp broodstock condition (nutrition and/or physiology status) is crucial for production of good quality offspring (Chamberlain and Lawrence, 1981b; Naessens et al., 1997; Wouters et al., 2001ab; Racotta et al., 2003;). NAF broodstock demonstrated apparently better condition (higher total n-6 and n-3 PUFAs, and DHA in hepatopancreas) than AF counterparts in this study (Chapter 4). Theoretically this would consequently be reflected in differential quality of their offspring (Racota et al., 2003). However, the significant effect of non-ablation in this study was not shown in egg diameter, nauplii and postlarvae lengths, larval development and growth measured by larval stage index (LSI), postlarvae final survival and weight in larviculture, growth performance and survival of juveniles and sub-adults in nursery and growth-out, respectively (Chapter 3 and 4).

At some points over the breeding period it was observed that eggs and nauplii produced from NAF had statistically significantly more EPA and DHA. In addition, more ARA and total n-6 PUFA and LA were observed in eggs and nauplii from NAF, respectively (Chapter 4). Other significant impact of non-ablation was observed in higher survival of the postlarvae to salinity stress test (Chapter 3 and 4) and challenge with Vp_{AHPND} (Chapter 5).

Overall, non-ablation condition can improve some nutrient reserves in eggs and nauplii (Chapter 4) which could be reflected in their offspring performance in further development stages (Racotta et al., 2003). However, the current study demonstrated that this advantage is not shown in growth performance and survival of NAF offspring under normal farming conditions (Chapters 3 and 4) perhaps because modern farming practices based on high quality feed can compensate for any nutrient deficiency observed in eggs and nauplii from AF. This is confirmed by the similarity in biochemical contents observed in postlarvae from NAF and AF at the end of larviculture in Chapter 4.

On the other hand, a highly positive and potentially commercially important effect of non-ablation has been observed in the higher survival of their offspring after challenge with environmental stress such as salinity stress test, or pathogen such as bacterial diseases (Chapters 3, 4 and 5). The influence of nutrients on this differential quality of NAF offspring cannot be fully discarded as more parameters apart from total lipids and fatty acids need to be evaluated. However, when the female are ablated there is activation/reduction of immunity related genes (Taylor et al., 2004; Sainz-Hernandez et at., 2009; Bae et al., 2013). Although, these parameters were not measured in the current study, they might explain the differential outcome observed here as theoretically the offspring from AF would have lower expression of immunity related genes. The demonstrable greater robustness of NAF derived animals is a real benefit and would command a commercial premium that would justify adopting this approach over and above the welfare benefits. In addition, it sustains the need to continue on finding ways to improve mating success of NAF in order to also support non-premium shrimp producers to adopt this practice.

7.3 Economic impact of using non-ablated hatchery system

Introducing a non-ablated hatchery based system in commercial hatcheries can improve some shrimp broodstock condition (i.e. nutrient reserve) and offspring quality (higher in some nutrient reserves and robust animals). This move to high welfare practice will be associated with an increase in nauplii production cost (Chapter 6).

The global shrimp farming industry has been affected by regular outbreaks of disease causing catastrophic crop failures with severe financial losses (Cock et al., 2009; Tran et al., 2013; Shinn et al., 2018b). Although the observed laboratory higher survival of animals from NAF to typical diseases in this study (Chapter 5) have not yet been validated at the farm levels, the modeling data (Chapter 6) has shown that if the farmers switch to source animals produced on NAF system, they would reduce the level of financial losses caused by AHPND and WSSV as better revenue, profit and gross margin are observed with NAF offspring.

In summary, adopting high welfare practice by using NAF animals in the commercial shrimp hatcheries might result in some increase in production cost. However, it appears that this increase would not significantly affect the production cost at the larviculture level. Although the cost is not reflected at larviculture, there is still a need to improve the mating success of NAF in order to improve eggs and nauplii productivity and hence reduce the production costs at the maturation and spawning level. On another hand, the possible improvement in revenue, profit and gross margin under disease outbreak when using animals from NAF reinforce the need for the farmers to source shrimp produced in a non-ablated hatchery based system.

7.4 General conclusions

The general goal of this thesis was to provide a body of evidence on potential of using non-ablated female in commercial shrimp hatcheries to support on-going dialogue around the continued use of unilateral eyestalk ablation in modern *L. vannamei* breeding practices.

Commercial hatcheries can use NAF production based system as long as some maturation facilities management practices are changed, i.e. on sex ratio (increased from 1:1 to 1:2), in order to have similar productivity. Normally, maturation facilities are based on AF system use 1:1 male to female ratio and if they switch to NAF system would require increasing sex ratio to 1:2 male to female in order to get same productivity observed in 1:1 sex ratio. In addition, selective breeding program should also be taken into account in order to select successful strains with non-ablation. Although there was underfeeding in the first weeks of pre-maturation the maturation facilities can still consider the use of fresh-frozen feeds (squid and polychaete) in pre-maturation tanks given some advantages on improving the NAF broodstock condition and improving fertilization and hatching rate. Maturation facilities managers and farmers should be confident that offspring derived from NAF perform comparably in all culture stages. Moreover, there is evidence of greater resilience to stress and typical diseases in NAF produced PLs and juvenile. Furthermore, the increase in production cost in a NAF based system is not reflected at larviculture level and the models indicate that there is reduction in financial losses when there is disease outbreak at farm.

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