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Improving the Reproductive Performance of Domesticated Giant Tiger Shrimp, *Penaeus monodon*

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Abstract

The Giant or Black Tiger Shrimp, *Penaeus monodon*, is an economically significant aquaculture species globally, producing 4.5 million tonnes of product annually at a value of US$ 23.5 billion (FAO, 2016a). Recent innovations in the domestication and selective breeding of *P. monodon* have resulted in significant improvements in growth rate, survival and pathogen tolerance. However, the reproductive performance of domesticated stocks is inferior compared to that of wild-caught broodstock. Significant reductions in the number of females maturing, egg and nauplii production and hatch rates are commonly reported for domesticated stocks relative to their wild-caught counterparts.

The complexities surrounding reduced reproductive performance in domesticated *P. monodon* are underpinned by two critical issues: 1) a poor understanding of the specific nutritional requirements for reproduction in the species and; 2) a lack of clarity as to the characteristics that define a 'good spawner' – particularly on a biochemical and molecular level. The studies that make up this thesis employed a multidisciplinary approach to assess nutritional, biochemical, and molecular factors that relate to broodstock reproductive performance. Primarily this thesis sought to: (1) investigate whether the current constraints to reproductive performance in domesticated stocks could be overcome by including the microbial biomass derived bioactive Novacq™ (Patent #2008201886) within pelleted diets; (2) evaluate whether current broodstock maturation diets are limiting in relation to repeated spawning and; (3) characterise key interactions between micronutrients and regulatory gene(s) and/or pathways linked to reproduction.

A series of reproductive performance trials were undertake to assess the effect of incorporating microbial biomass (Novacq™) within pelleted maturation diets. Preliminary farm-based trials observed significant increases to maturation rate, egg production and nauplii production when domesticated broodstock were fed an experimental pelleted diet containing the Novacq™ ingredient (20% Novacq™ inclusion rate, 2.4% of total diet fed). However, in a subsequent trial conducted under controlled experimental conditions, broodstock fed commercial-grade pelleted diets (30% Novacq™, 5.5% of total diet fed) exhibited a significant decrease in egg hatch rate. Reductions in reproductive performance under controlled experimental conditions were attributed to a decrease in the quality of basal pellet diets, both as a function of increased Novacq™ inclusion and their commercial-based formulation. The above studies suggest the capacity to improve reproductive performance in domesticated *P. monodon*, using biofloc and its substituents, is highly dependent on the quality of the basal maturation diet fed.

In response to the aforementioned studies, a trial was undertaken to identify potential factors limiting reproductive performance within current broodstock maturation diets. The effect of repeated spawning on reproductive performance and tissue biochemistry (ovary and hepatopancreas) was assessed in a population of broodstock fed a typical high performance maturation diet. During the initial two spawning cycles broodstock demonstrated significant variation in aspects of hepatopancreas and ovary biochemistry. Most notably, significant reductions in hepatopancreas and ovarian arachidonic acid (ARA) content were observed, suggesting that the requirement for and/or utilisation of ARA in relation to spawning exceeds quantities provided by current maturation diets. Additionally, a number of hepatopancreas fatty acids were depleted in second spawn, and therefore represent micronutrients likely to become limiting in subsequent spawning cycles.

To further understand the impact of limiting ARA on reproduction, ovarian ARA content was quantified in a homogeneous population of domesticated broodstock. Significant individual variation in ovarian ARA content was observed. RNA-seq analyses was undertaken to investigate the effect of variable ARA content on global gene expression and prostaglandin (ARA derived hormones with significant regulation over reproduction) biosynthesis. Global gene expression analyses identified a total of 757 genes with >2-fold expression difference in relation to ovarian ARA content. Additionally, variation in ovarian ARA content had significant impact on the regulation of prostaglandin biosynthesis genes, particularly those linked to egg production (PGE2) and maturation (PGF2α).

The studies contained in this thesis shed light on the influence of nutritional bioactives, whilst providing a comprehensive framework for the development of high-performance broodstock feed formulations and optimized nutrition strategies.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributor	Statement of contribution
Jake Goodall (Candidate)	Designed experiments (55%) Data Analysis and Interpretation (80%) Wrote the paper (80%)
Nick Wade	Designed experiments (10%) Data Analysis and Interpretation (10%) Wrote and edited the paper (5%)
David Merritt	Wrote and edited the paper (5%)
Melony Sellars	Designed experiments (15%) Wrote and edited the paper (5%)
Kinam Salee	Designed experiments (5%)
Greg Coman	Designed experiments (15%) Data Analysis and Interpretation (10%) Wrote the paper (5%)

Contributions by others to the thesis

Nick Wade, Greg Coman, Melony Sellars and Natasha Botwright contributed to the conception, design and implementation of this research. In addition all contributed extensive comments on the thesis and its associated publications.

David Merritt provided extensive comments and editorial support on the thesis and the publications associated with chapter 3.

Gold Coast Marine Aquaculture contributed all animals used in this thesis, in addition to the on-farm facilities associated with chapters 2 and 3.

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black tiger shrimp, reproduction, domestication, broodstock, microbial biofloc, nutrition, biochemical composition, transcriptome, RNA-seq

Australian and New Zealand Standard Research Classifications (ANZSRC)

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FoR code: 0702, Animal Production, 80%

FoR code: 0704, Fisheries Science, 20%

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List of Abbreviations

Acronym	Definition
DHA	Docosahexaenoic acid
PGES3	prostaglandin E synthase 3
ATP	Adenosine triphosphate
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
ARA	Arachidonic acid
AS	Assembly Samples
AGRF	Australian Genome Research Facility
BFT	Biofloc Technology
BLAST	Basic Local Alignment Search Tool
BUSCO	Benchmarking Universal Single-Copy Orthologs
BIRC	Bribie Island Research Centre
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSIRO	Commonwealth Science and Industrial Research Organisation
cont.	Continued
CTRL	Control diet
C+C	Control grow-out and maturation diet
C+MB	Control grow-out diet, microbial biomass inclusive maturation diet
COX	cyclooxygenase
DNA	Deoxyribonucleic acid
PGE2-d4	Deuterium labelled Prostaglandin E2
DGE	Differential gene expression
DM	Dry matter
EPA	Eicosapentaenoic acid
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
FFF	Fresh frozen food
GC	Gas chromatograph
GO	Gene Ontology
gPGDS	glutathione-dependent prostaglandin D synthase
GCMA	Gold Coast Marine Aquaculture
GSI	Gonadosomatic index
g	Grams
GO	Grow-out
Gdiet	Grow-out diet
GTP	Guanosine triphosphate
HIS	Hepatosomatic index
HUFA	Highly unsaturated fatty acids
Kg	Kilogram
LS	Least squares
LA	Linoleic acid

Acronym (cont.)	Definition (cont.)
LC-MS	Liquid chromatography–mass spectrometry
Mdiet	Maturation diet
MJ	Mega joule
MB	Microbial biomass
MBD	Microbial biomass diet
MB+MB	Microbial biomass inclusive grow-out and maturation diet
MB+C	Microbial biomass inclusive grow-out diet, control maturation diet
MUFA	Monounsaturated fatty acid
MRM	Multiple reaction monitoring
NCBI	National Centre for Biotechnology Information
NIRS	Near-infrared spectroscopy
nsd	No significant difference
cPLA2	Phospholipase A2
PUFA	Polyunsaturated fatty acid
PL	Post-larvae
PC	Pre-conditioning
PGD2	Prostaglandin D2
PGES1	Prostaglandin E synthase 1
PGES2	Prostaglandin E synthase 2
PGE2	Prostaglandin E2
PGFS	Prostaglandin F synthase
PGF2α	Prostaglandin F2α
PGH2	Prostaglandin H2
PTGR1	Prostaglandin reductase 1
REP	Reproductive phase
RT	Retention time
RP	Reverse phase
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SFA	Saturated fatty acid
SPE	Solid phase extraction
SPF	Specific pathogen free
SPR	Specific pathogen resistant
SE	Standard error
SEM	Standard error of mean
TDF	Total diet fed
tblastx	translated BLAST
US	United States

Chapter 1: General Introduction

With the human population predicted to exceed 9.5 billion come 2050, the global requirement for animal protein is set to increase dramatically. The continued development of production systems that yield high quality, yet inexpensive animal-protein is essential to meet global food trajectories. Fish production, which includes finfish, crustacean and mollusc species represent one such commodity. In 2014, an estimated 167.2 million tonnes of fish were consumed globally, accounting for approximately 20% of the global population's intake of animal protein (FAO, 2016a). Capture fisheries represent the predominant source of food-fish production worldwide (93.4 million tonnes produced in 2014) (FAO, 2016a). However, widespread overexploitation of capture fisheries represents a significant impediment to the continued growth of the sector (currently estimated at 0.8% per annum) (FAO, 2016a). Recent modelling suggests that even under strict management regulations the capture sector has little to no capacity to expand in line with global population needs (see Garcia and Rosenberg, 2010). If we are to meet future global food demands any increase in global fish production must be derived primarily from farm-based systems, a practice commonly referred to as aquaculture.

1.1. Aquaculture

The Food and Agriculture Organization of the United Nations (FAO) defines aquaculture as the farming of aquatic organisms including finfish, molluscs, aquatic plants and crustaceans. In 2014, South East Asia represented the largest contributor to aquaculture production, with the top five aquaculture producers (based on total yield) being China (45.5 million tonnes), India (4.9 million tonnes), Indonesia (4.3 million tonnes), Vietnam (3.4 million tonnes), and Bangladesh (2 million tonnes) (FAO, 2016a). In terms of global production volume, annual aquaculture production is primarily comprised of finfish (68% of production, 49.9 million tonnes), molluscs (22% of production, 16.1 million tonnes) and crustaceans (9% of production, 6.9 million tonnes) (FAO, 2016a).

Of the above mentioned groups, crustaceans command the greatest commodity value (US$5,200 per tonne) despite their relatively low production volume (FAO, 2016a). Crustaceans, which include lobsters, crayfish, crabs and shrimp, owe their considerable market value to their status as 'luxury' food items. Shrimp in particular represent the second most valuable aquaculture commodity traded globally (salmon being the first), accounting for 15% of the total value of globally traded fish products in 2014 (FAO, 2016a). Within the shrimp sector, marine *Penaeid* species are of particular

significance, namely *Litopenaeus vannamei* (Pacific White shrimp) and *Penaeus monodon* (Black or Giant Tiger shrimp).

1.2. *Penaeid* Shrimp Aquaculture

The prevalence of disease has been the driving force shaping the development and growth of the shrimp aquaculture industry. Established in the 1980s, the industry saw consistent annual growth of 25% per annum during its founding years (FAO, 2010). At the time, farms focused primarily on the production of *P. monodon*, a species valued for its large size and fast growth. During the industries founder years operations focussed on sourcing broodstock from wild populations to produce high quality seedstock. However, this practice eventually led to the introduction and spread of disease into global farming operations, resulting in considerable slowing of industry growth during the 1990s (5 to10% growth per annum) (FAO, 2010). In response to widespread disease outbreaks, shrimp industries shifted towards the development and production of domesticated lines as a means of reducing industry reliance on wild-sourced seedstock. *Litopenaeus vannamei* quickly emerged as a favourable culture species, due to its rapid growth rate, ability to be cultured under high stocking densities and its readiness to breed in captivity. These traits favoured domestication of the species and lead to the development of genetically-selected specific pathogen free (SPF) lines. As a result, global production of *P. monodon*, which had proved difficult to domesticate, was largely replaced by *L. vannamei* in the early 2000s. The global shift towards domesticated SPF *L. vannamei* stocks dramatically increased industry growth between 2000 and 2006 (43% per annum) (FAO, 2010), before the industry stabilized to the current level of 6.9% growth per annum (Anderson and Valderrama, 2013).

Today, global *L. vannamei* production is derived almost exclusively from domesticated SPF or specific pathogen resistant (SPR) stocks. In stark contrast, progress in the domestication of *P. monodon* has been limited, despite the species retaining its superior market value. The reproductive performance of domesticated *P. monodon* broodstock remains the primary obstacle to the establishment of domesticated SPF and/or SPR lines. Unlike *L. vannamei,* farm-reared *P. monodon* rarely develop mature gonadal tissue without ablation spawn fewer viable offspring than their wild-caught counterparts. As a direct result, *P. monodon* production remains heavy reliant on wild-caught broodstock, exposing the industry to seasonal variability in seedstock quality, precluding opportunities to improve traits through selective breeding and increasing disease risks. If the industry is to replicate the successes made in *L. vannamei,* efforts must first be directed at resolving the poor reproductive performance of *P. monodon* in captivity.

1.3. Commercial Lifecycle of *P. monodon* Broodstock

In recent years the lifecycle of *P. monodon* has been successfully closed, allowing for broodstock to be reared from egg to adult on-farm (Figure 1). Under typical commercial conditions, eggs are broadcast spawned and externally fertilized. The embryos are then hatched within specialized biosecure spawning tanks. Approximately 12 hours following spawning, hatched nauplii are collected and transferred to biosecure nursery facilities. Within nursery facilities nauplii continue to develop though a number of larval stages, including six non-feeding nauplii stages (typically denoted as nauplii I-VI), three feeding protozoea stages (denoted protozoea I-III) and three feeding mysis stages (denoted mysis I-III). Each larval stage molts to progress and is concluded approximately 20 days post-hatch, following one additional molt from mysis III to the juvenile post-larval form. Typically, post-larvae (PL) are reared for an additional 15 days (commonly referred to as PL15) to ensure stability of body proportions, before being transferred from nursery facilities to large earthen seawater ponds (Motoh, 1985). Adolescent broodstock continue to be reared within earthen ponds or dedicated biosecure broodstock ponds or raceways until six months of age (termed the grow-out phase), before being transferred to enclosed biosecure maturation tanks. The now sub-adult broodstock are matured for an additional 1-3 months before reaching sexual maturity at approximately nine months after hatching (Motoh, 1985). Mature broodstock are then conditioned for a further 1-3 months (termed pre-conditioning) before being induced to spawn by ablation, with impregnated gravid females being transferred to biosecure spawning tanks – thus completing the commercial lifecycle.

1.4. Reproductive Performance of Domesticated *P. monodon*

Despite the successful closing of the *P. monodon* lifecycle, the low fecundity of domesticated broodstock represents a significant bottleneck to the broad industry adoption and use of such stocks. Furthermore few companies have mastered the techniques and nuances associated with broodstock husbandry and nutrition. Thus *P. monodon* has not yet undertaken industry wide domestication, with the majority of production still being dependant on the availability of wild sourced broodstock.

One of the primary issues when investigating causal factors of reduced reproductive performance in *P. monodon* is isolating the influences of sex. Given the collection of male spermatophores is non-invasive as compared with the destructive ovary extraction, and that they can be successfully inseminated into a donor female, earlier works tended to focus heavily on male-specific measures of performance. Numerous articles have been published linking spermatophore weight, sperm number,

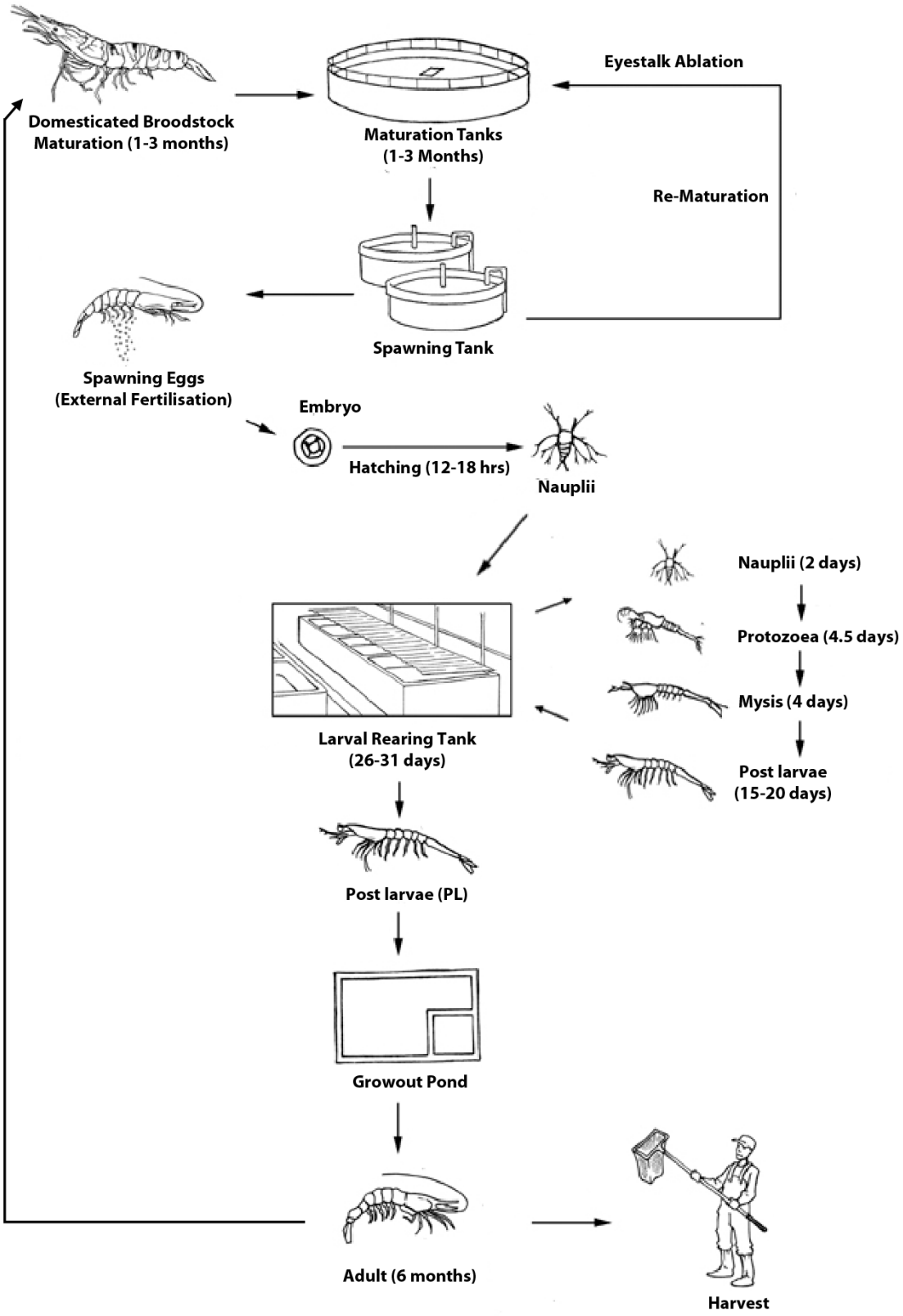


Figure 1. Commercial production cycle of *Penaeus monodon*. Adapted from FAO *Penaeus monodon* factsheet available from http://www.fao.org/fishery/culturedspecies/Penaeus_monodon/en

and quantity of reactive sperm with male reproductive performance and quality (Pongtippatee et al., 2007, Pratoomchat et al., 1993, Jiang et al., 2009, Meunpol et al., 2005). Although not explicitly stated, the bulk of these studies implied that males represented the primary limiting factor to seedstock production in domesticated *P. monodon*. However, more recent works have demonstrated that spermatophore weight and sperm number are not reliable predictors of offspring viability (Arnold et al., 2012). Further, males have far less influence on fertilization rate, hatch rate and subsequent embryo development then originally suggested (Arnold and Coman, 2012, Arnold et al., 2012, Arnold et al., 2013). Certainly, these contemporary studies do not suggest male propagule quality is irrelevant, more so that the mechanisms underlying reduced reproductive performance in domesticated *P. monodon* stocks are predominantly female based.

It is well documented that in a commercial environment, females from domesticated stocks rarely if ever develop fully mature ovaries before spawning unlike their wild-caught counterparts (Coman et al., 2006, Menasveta et al., 1993, Arnold et al., 2013). In addition domesticated *P. monodon* produce far fewer eggs and spawn less frequently than their wild-caught counterparts (Klinbunga et al., 2009, Coman et al., 2006, Hall, 2003, Menasveta et al., 1993, Peixoto et al., 2005, Arnold et al., 2013). Peixoto et al. (2005) noted that the ovaries of mature domesticated broodstock frequently contained high proportions of immature oocytes. The spawning of immature oocytes by domesticated broodstock is likely to lead to low hatch rates (Coman et al., 2005, Hall, 2003, Makinouchi and Hirata, 1995, Preston et al., 2009, Primavera and Posadas, 1981, Arnold et al., 2013). Indeed, in the absence of an observed male effect on fertility, Arnold et al. (2013) concluded that low fertility in domesticated *P. monodon* was due to females spawning with immature ovaries. Taken together, these studies suggest that egg development, quality and quantity represent key target areas for improvement in female domesticated *P. monodon*.

1.5. Current Status of *P. monodon* Selective Breeding in Australia

To date, the bulk of *P. monodon* selective breeding programs in Australia have focused on improving production traits during stock grow-out. Targeted traits include increased growth (Glencross et al., 2013), harvest yields (Arnold et al., 2013) and viral tolerance (Arnold et al., 2013, Coman et al., 2005, Sellars et al., 2015a). Given the prevalence of growth selection in *P. monodon* hatcheries, Macbeth et al. (2007) calculated the degree of genetic correlation between growth and nauplii production. Genetic correlations identified no significant linkage between broodstock growth and nauplii production traits. These results are intriguing given that Arnold et al. (2013) reported increased reproductive performance in eighth-generation selected *P. monodon* lines. Taken

together, these data suggest that, whilst modest improvements in performance have been observed in advanced generation stocks, it is not likely to be due to the current selection pressures imposed (growth, survival and pathogen tolerance). Instead, improvements in Australian domesticated broodstock performance likely reflect improved broodstock husbandry and nutrition. However, Macbeth (2007) noted that egg and nauplii production are heritable traits. Functional genomic studies aimed at identifying gene functions underlying reduced reproductive performance should be a prioritized. Notably, as no annotated genome currently exists for *P. monodon,* transcriptomic techniques such as RNA-seq have the greatest potential to identify genes functionally linked with reproduction. Such genes may serve as future markers for use in selective breeding programs looking to improve reproductive output.

1.6. Broodstock Nutrition

Nutrition is considered as one of the primary factors that constrains the reproductive performance in *Penaeid* shrimp (Arnold et al., 2013, Emerenciano et al., 2013b, Browdy, 1998, Coman, 2014). As such, there is considerable potential to boost stock performance through improved diet formulation or the use of novel feed ingredients. The basic nutritional requirements for shrimp maturation diets have been extensively reviewed in the past (see Wouters et al., 2001a, Harrison, 1990). However, a number of key research areas remain unresolved in relation to broodstock, including quantitative nutritional requirements, nutrient metabolism in relation to spawning and nutrient interaction with broodstock endocrinology (Hoa et al., 2009, Wouters et al., 2001a). Due to our lack of understanding on fundamental broodstock nutrition requirements in *P. monodon*, maturation diets remain heavily reliant on fresh-frozen feed ingredients (i.e. squid polychaete worms, bivalves, *Artemia* biomass, beef liver), which are susceptible to seasonal variations in quality, and provide a potential vector for pathogen transmission (Chimsung, 2014). Compound pelleted diets consistently underperform when compared with fresh feeds and therefore make up a small proportion of typical maturation diet regimes (estimated at 16%) (Meunpol et al., 2005, Harrison, 1990, Bray and Lawrence, 1992, Bray et al., 1990, Wouters et al., 2001a). However, compound maturation diets have a number of advantages over fresh-frozen feeds, including consistent nutritional content, easier management and storage, and reduced risk of pathogenic contamination (Chimsung, 2014, Wouters et al., 2001a, Harrison, 1990). Therefore, the generation of comprehensive reproduction-associated biochemical data to aid in maturation diet formulation represents a key research priority and opportunity.

The superior performance of fresh-frozen feeds has been largely attributed to their optimal fatty acid composition, particularly highly unsaturated fatty acids (HUFA) (Meunpol et al., 2005). Shrimps, as for all crustaceans, have a limited capacity to elongate unsaturated fatty acids or synthesize HUFA *de novo* and therefore these nutrients must be supplied in the diet (Glencross, 2009). In *Penaeid* shrimp a large portion of ovarian HUFA is composed of the essential fatty acids arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Hoa et al., 2009). Broodstock diets rich in ARA, EPA and DHA have been linked to a number of favourable performance traits in *Penaeid* shrimp including: improved nutrient uptake and transfer, improved spawning activity, egg production, hatch rates and nauplii survival (Coman et al., 2011, Cahu et al., 1994, Palacios et al., 2001, Emerenciano et al., 2013b, Xu et al., 1994a). Interestingly, Wouters (2001a) noted that compound diets contain relative low levels of both ARA and EPA when compared with fresh-frozen feeds, and therefore may require further investigation in relation to spawning. The requirement for ARA is of particular interest given ARA serves as the primary substrate for the synthesis of series II prostaglandins, hormones with significant regulatory control over maturation and oocyte development in *P. monodon* (Wimuttisuk et al., 2013).

A novel area of nutrition research is the incorporation of microbial biofloc within broodstock maturation feeding regimes. The term biofloc typically refers to a flocculation of highly-concentrated bacterial and microalgae biomass, which are deployed into commercial grow-out ponds. When made available in-pond, biofloc constitute a significant food source for shrimp (Burford et al., 2004), providing a source of diverse protein (Emerenciano et al., 2012), lipid (Wasielesky et al., 2006), amino acid (Ju et al., 2008) and fatty acid (Izquierdo et al., 2006). The effect of biofloc on reproduction has been examined for a number of *Penaeid* species, with authors reporting improvements in maturation and spawning rates (*Litopenaeus stylirostris*, Emerenciano et al., 2012), total and relative egg production and egg size (*Farfantepenaeus duorarum*, Emerenciano et al., 2013a) and increase egg HUFA content (*Litopenaeus vannamei*, Emerenciano et al., 2013b). The aforementioned studies clearly demonstrated that the biofloc has the potential to improve the reproductive potential in *Penaeid* shrimp. Whether biofloc has the potential to improve reproductive performance in domesticated *P. monodon* warrants further investigation.

1.7. Aims of This Research Thesis

The reduced reproductive of performance of domesticated *P. monodon* broodstock represents the most significant constraint to their widespread domestication and adoption. Distinct knowledge gaps in adequate feed formulation, broodstock nutrition and genetic regulation of maturation and

spawning in *P. monodon* contribute to the ongoing reliance of industry on wild-caught broodstock. This research thesis aims to address these key knowledge gaps by increasing our understanding of the nutritional, biochemical and molecular mechanisms that underlie variations in the reproductive performance of domesticated *P. monodon* broodstock, investigating specifically:

- Whether nutritional intervention using microbial biofloc has the potential to overcome current constraints on reproductive performance
- Whether nutrient requirements and/or utilization are limiting under current high performance maturation feeding regimes in relation to repeat spawning, and
- Whether interactions exist between limiting nutrient(s) and key regulatory genes or gene pathways linked to reproduction.

Answers to these questions will provide a rigorous scaffold for the optimization of maturation diet formulations and further our understanding of the molecular mechanisms regulating reproduction in shrimp. Outcomes from this project will enhance the reproductive productivity of domesticated broodstock, enhance the sustainability of *P. monodon* farming and improve the accessibility of elite domesticated-selected lines to Australian shrimp farmers.

Chapter 2: Effect of microbial biomass supplementation on the reproductive performance of domesticated Penaeus monodon when fed in combination with high quality broodstock diets

2.1. Abstract

The effect of a microbial biomass derived feed additive, Novacq™ (Patent #2008201886), on the reproductive performance of third generation domesticated *Penaeus monodon* broodstock was evaluated across a 20-day period. Reproductive performance was evaluated within a commercial hatchery across two commercial spawning periods (termed experiments), which saw broodstock conditioned for three weeks (experiment one) or 11 weeks (experiment two) prior to ablation. Broodstock were fed a typical fresh-frozen maturation diet throughout both the pre-conditioning and 20-day evaluation period, which included a pelleted diet containing 20% inclusion of the microbial biomass ingredient or a pelleted diet designed to mimic the treatment pellet, without microbial biomass. Pelleted diets made up 12.2% of the total diet fed (based on dry matter) and thus the microbial biomass ingredient constituted 2.4% of the total diet fed within the treatment diet (based on 20% inclusion rate). The reproductive performance of broodstock, across both experiments one and two were evaluated on a 'per female, per day' basis. The proportion of females spawning, the number of eggs produced and nauplii produced were significantly higher ($P<0.05$) in experiment two broodstock fed the treatment pellet. However, no significant differences in performance were observed between experiment one broodstock fed either diet. These results suggest that microbial biomass derived feed additives can enhance reproductive performance in domesticated *P. monodon*, however the effect appears to be dependent on the duration of the pre-conditioning period.

2.2. Introduction

The reproductive performance of domesticated *Penaeus monodon* (giant tiger shrimp) broodstock has routinely been shown to be lower than that of their wild-caught counterparts (e.g. Coman et al., 2006, Arnold et al., 2013). Recent improvements in husbandry and genetic selection have resulted in minor generational improvements to reproductive performance (Coman et al., 2013), yet such improvements have not been comparable with or superior to the reproductive outputs observed in wild-caught broodstock. Whilst many factors influence the reproductive performance in *P. monodon,* an optimal diet is considered critical for 'normal' sexual maturation and spawning (Wouters et al., 2001a). Traditionally, broodstock maturation feeding regimes have consisted of a fresh frozen food (FFF) component (e.g. squid, mussel, bloodworm, *Artemia* and ox liver) supplemented with various pelleted feeds (Wouters et al., 2001a). A number of broodstock nutrition studies have examined the effect of varying quantities of nutrient classes and/or compounds within such broodstock feeding regimes (Alava et al., 1993, Cahu et al., 1994, Xu et al., 1994a, Marsden et al., 1997, Naessens et al., 1997, Hoa et al., 2009, Emerenciano et al., 2013b, Emerenciano et al., 2013a, Coman et al., 2007a, Chimsung, 2014, Wouters et al., 2001a, Wouters et al., 2001b). Recent studies have suggested that biofloc technology (BFT) may help improve reproductive performance in *Penaeid* shrimp (Emerenciano et al., 2012, Emerenciano et al., 2013b, Emerenciano et al., 2013c, Braga et al., 2015). In male broodstock, the use of BFT during pre-conditioning allowed the reduction of dietary protein content from 68.4% to 39.9% whilst maintaining spermatophore and sperm quality in pacific white shrimp (*Litopenaeus vannamei)* (Braga et al., 2015). When conditioned within BFT systems, female pacific blue shrimp (*Litopenaeus stylirostris),* pink shrimp (*Farfantepenaeus duorarum)* and *L. vannamei* broodstock matured and spawned more frequently and produced a greater numbers of eggs (Emerenciano et al., 2012, Emerenciano et al., 2013b, Emerenciano et al., 2013c). However, the ability to improve reproductive performance in female *Penaeid* broodstock using BFT appears to be largely dependent on the length of pre-conditioning prior to ablation, as well as the nutritional quality of the basal maturation diets fed in combination with BFT (Emerenciano et al., 2012, Emerenciano et al., 2013b, Emerenciano et al., 2013c).

Commercially, BFT is largely restricted to the grow-out phase of the production cycle. However, recent attempts have been made to harvest and process the microbial biomass (MB) or biofloc for further use in pelleted maturation diets. This allows MB to be fed across all stages of the production lifecycle, including broodstock conditioning and spawning. This study aimed to evaluate whether a

MB-derived ingredient (Novacq™, Patent #2008201886) influenced the reproductive performance (at the levels fed in this study) of *P. monodon*.

Broodstock were fed the MB ingredient during both pre-conditioning and maturation periods at a rate of 2.4% of the total diet based on dry matter (20% MB inclusion rate, pellet fed at a rate of 12.2% of total diet). The reproductive evaluations within this study were undertaken on-farm (Gold Coast Marine Aquaculture (GCMA), Woongoolba, Queensland, Australia) within commercial maturation and hatchery facilities, as well as across multiple broodstock conditioning periods. This design enabled the assessment of short- and long-term feeding effects of the MB containing diets under commercial hatchery conditions.

2.3. Methods

2.3.1 Manufacture of Supplemental Diets

All raw ingredients were milled separately to 750 μm on a hammer mill prior to use. A single batch of mash was prepared and thoroughly mixed in an upright mixer (Model 60 A-G, Bakemix). The two treatment diets were designated by the addition of dried MB at 20% dry weight basis to one aliquot of mash. The two diets were processed using a laboratory-scale, twin-screw extruder (APV MFP24; APV-Baker, Peterborough, United Kingdom), with intermeshing, co-rotating screws. Measurements were taken during initial running phases with incremental variations in water addition and measurement of the expansion using vernier callipers (TradeToolsDirect, Ormeau, Australia). Feeds were extruded through the 2 mm die and cut into 20 to 25 mm lengths using a two-bladed variable speed cutter and collected on large aluminium oven trays. The diets were dried in fan forced ovens at 60°C overnight until they reached a constant dry matter. Approximately 50 kg batches of each diet were collected and vacuum bagged, stored at -20°C until use.

2.3.2 Stock Origin and Animal Rearing

Experimental broodstock were from a third generation domesticated *P. monodon* line maintained at GCMA. The broodstock were reared within enclosed nursery facilities until postlarval stage (PL) 15 (15 days post-metamorphosis from mysis to postlarval stage 1), before being stocked into earthen grow-out ponds (3,000 $m^{2)}$. After 6 months shrimp were transferred from the grow-out ponds to a series of rectangular 15,000 L indoor maturation tanks (17 m^2) fitted with sub-sand circulation systems. The tanks were stocked with 60 to 80 individual broodstock at a 1:1 sex ratio and density of 3.52 – 4.70 shrimp/ m^2. Seawater flowed through tanks to provide approximately 30 to 50%

water exchange daily maintaining the temperature and salinity at 29 ± 1°C and 35 ± 1 ppt respectively. Lighting within the maturation facilities was low with an ambient photoperiod.

Within the maturation facility, all broodstock were fed on a typical commercial fresh-frozen maturation diet supplemented with the MB-inclusive (MBD) or control (CTRL) pelleted experimental diet (see 2.2.3 Experimental Design). The contribution of pelleted diets to the total maturation diet fed was approximately 12.2% based on dry matter (DM), with the remainder of the total diet fed (based on DM) made up by fresh-frozen feed ingredients: arrow squid (25.2%), green-lip mussel (24.6%), ox liver (12.6%), *Artemia* (10.6%), marine bloodworm (14.8%). All broodstock were fed eight times daily, spread evenly throughout a 24 h period, following standard commercial broodstock protocols at the GCMA hatchery facility, which included a single daily ration of the respective supplemental pellet diet. The total contribution of supplemental pellets, within the complete dietary regime was 12.2%. Therefore, the MBD treatment provided approximately 2.4% of the total diet. During each feeding event the ration was provided to satiation, with excess feed cleaned from tanks using hand nets.

2.3.3 Experimental Design

The study was conducted across two spawning events, designated experiment one and experiment two (Figure 2). The first experiment used stocks at approximately nine months of age, which had been fed their respective diet (MBD or CTRL) for three weeks prior to unilateral eyestalk ablation of females (removal of eyestalk with hot sterile tweezers) and the commencement of reproductive evaluations. The second experiment used stocks of approximately 11 months of age, which had been fed their respective diet (MBD or CTRL) for three months prior to unilateral eyestalk ablation of females and the commencement of spawning evaluations. Across both experiments, all broodstock were fed identical fresh-frozen diets which were supplemented with either the MBD or CTRL experimental pelleted diets.

2.3.4 Reproductive Performance Assessment

The spawning performance in both experiments was assessed for a maximum of 20 days. Following ablation (day 0), broodstock were monitored daily for ovarian development, until the conclusion of the evaluation period (day 20). When ripe ovaries (Stage IV following Tanfermin & Pudadera 1989) were detected, females were removed from maturation tanks and transferred to spawning tanks. The spawning tanks used were 80 L flow-through square spawning tanks (water flow

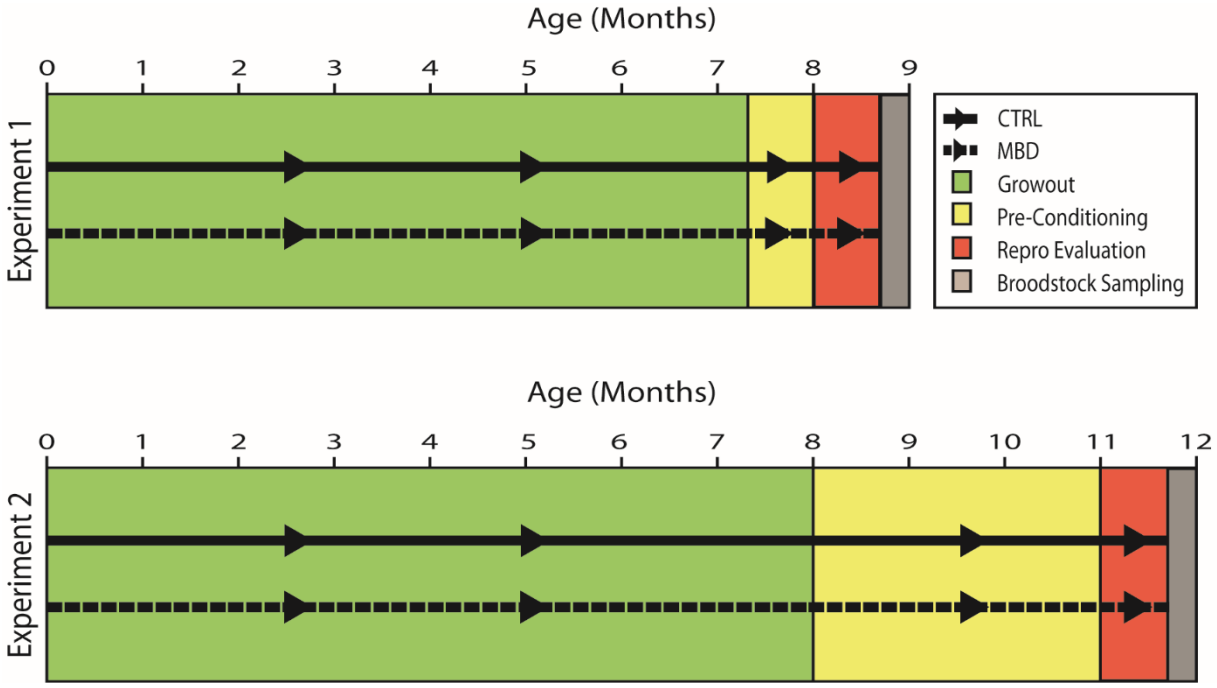


Figure 2. Experimental timeline demonstrating the progression of experiment one and two broodstock. All broodstock were fed identical diets throughout the grow-out period (green). During the pre-conditioning (yellow) and repro (reproductive) evaluation (red) periods broodstock were fed either the control diet (CTRL) or microbial biomass inclusive pelleted diet (MBD)

0.5 L/min, water temperature 29° C, gentle aeration), which were coupled with an overflow egg collection system, which served to separate eggs from the spawning tank soon after spawning. Whilst females were generally stocked into spawning tanks individually, at times multiple females were stocked in individual spawning tanks and left to spawn overnight. The number of females maturing (i.e. developing stage IV ovaries) and thus entering spawning tanks, from each treatment, was recorded each night across the 20-day evaluation period for all experiments and diet treatments

The morning after spawning, eggs within the overflow collector were concentrated and washed thoroughly on a 142 µm mesh, before being transferred into 7 L of fresh seawater for quantification within a standard volume. Total egg production was derived by counting three replicate volumetric 1 mL samples, taken from the 7 L of the egg-seawater solution, under a dissecting microscope. Following quantification, embryos were transferred to a separate nauplii hatching system; which consisted of a 18 L primary vessel coupled with an overflow system which allowed newly hatched, healthy nauplii (strong nauplii display a strong phototactic response) to rise up from the bottom of the primary vessel and be flushed into a 9 L "nauplii boot" (which was screened with 142 µm mesh) (see Supplementary Figure 1). Within the nauplii boot, the healthy nauplii were continually rinsed with clean seawater, until they were counted the following morning, before being transferred onto

the commercial hatchery for rearing. Percentage hatch rate and total nauplii production were derived from three replicate volumetric 1 mL samples taken from the 9 L of the nauplii boot. Percentage hatch rate was calculated from the number of nauplii hatching in the nauplii boot divided by the number of eggs collected within the egg collector. All females were returned to the maturation tanks each morning, regardless of whether spawns were detected.

2.3.5 Broodstock Sampling

Following the 20-day reproductive evaluation period, broodstock were allowed to undergo one additional maturation cycle. When ripe ovaries (Stage IV) were detected females were removed from maturation tanks and anaesthetized via ice-water immersion. Whole ovary, hepatopancreas and muscle tissue (from the first abdominal segment) was excised from anaesthetized females, then frozen on dry ice, before being stored at -80°C awaiting further analysis. Notably, commercial operations did not allow for sampling of broodstock from experiment two and therefore investigations into the effects of diet on ovary, hepatopancreas and muscle tissue were restricted to experiment one broodstock only (i.e. those conditioned for three weeks prior to ablation). Five experiment one broodstock from the CTRL and MBD diet treatments were sampled.

2.3.6 Chemical Analysis of Feeds

Prior to analysis, the feeds were ground into a fine powder and shrimp samples minced frozen, and then freeze-dried in a laboratory freeze dryer (Alpha 1-4, Martin Christ, Germany). A sub-sample of the original frozen mince was analyzed for its moisture content by gravimetric analysis following oven drying at 105˚C for 24 h and this was used as a correction for the freeze-dried material. For the freeze-dried sample, dry matter was calculated by gravimetric analysis following oven drying at 105˚C for 24 h. Gross ash content was determined gravimetrically following loss of mass after combustion in a muffle furnace at 550° C for 12 h. Protein was calculated from the determination of total elemental nitrogen (CHNS-O Flash 2000, Thermo Scientific, USA auto-analyser, based on N x 6.25). Gross energy of freeze-dried diets was determined using adiabatic bomb calorimetry following the manufactures protocols (Parr 6200 Calorimeter, USA). Total lipid extractions and quantification were conducted following Folch (1957). Total lipid content was determined gravimetrically following extraction of the lipids using chloroform/methanol (2:1). Carbohydrates were calculated based on the difference between dry matter content of the feed minus the lipid, ash and protein contents.

2.3.7 Data Analysis

Due to the commercial operations from which the data was collected, individual female performance was unable to be tracked. Moreover, as spawning tanks were at times stocked with multiple females (though always from the same maturation treatment) it was not possible to consistently determine the number of females contributing whole or partial spawnings within each spawning tank. With these constraints to the reproductive evaluations, a novel approach to quantifying reproductive performance was employed.

The reproductive performance of each treatment was analyzed in two ways: 1) averaged across both experiment one and experiment two and; 2) separated across experiment one and two. The first method aimed at discerning the overall effect of MB supplementation, whilst the second method allowed the effect of MB supplementation to be examined after feeding over the two timescales. Reproductive performance measures were assessed on a 'per day' basis, which included diet and run (separated or combined) as main effects, and the covariate term 'female day' (i.e. day following female ablation). The number of female days was simply a function of 'the number of females originally ablated within a tank' totaled up for each treatment based on the number of females from each tank qualifying for evaluation on any particular day. Analysis of co-variance (ANCOVA) (McCullagh and Nelder, 1989) was performed to test for diet type effects (CTRL and MBD) for four reproductive performance measures assessed over the 20-day period post-ablation: the proportion of spawning females (i.e. the number of females transferred into spawning tanks; this being a proxy measure of spawning performance); total egg production; percentage hatch rate and; total nauplii production. Analyses were performed using SAS software using the Generalized Linear Models (PROC GLM; SAS Institute Software, 1999). Levels of significance were defined as $P<0.05$. All biochemical analyses of experiment one broodstock tissues were undertaken using R Studio (R Studio Team, 2015) using Student's T-Test (Zar, 1984a).

2.4. Results

2.4.1 Broodstock Diet Formulation

The biochemical composition of broodstock diets was analyzed. The combined nutritional content of fresh frozen feed ingredients is presented in Table 1. Experimental pelleted diets were analyzed independently, with the CTRL and MBD diet formulation and composition presented in Table 2.

Table 1. Proximate compositional analysis (g/kg unless otherwise stated) of the combined basal fresh-frozen feed (FFF) component of broodstock maturation diets fed during experiments one and two.

	FFF
Composition	
Dry matter	210
Protein	166
Lipid	16
Ash	16
Carbohydrates^	12
Gross Energy (MJ/Kg)	4.6

^Carbohydrates calculated by difference.

2.4.2 Reproductive Performance Measures

Reproductive performance measures in response to diet treatments across the 20-day evaluation period were assessed as a function of the number of original females ablated. When the effect of diet was combined across both experiments one (3-week pre-conditioning) and two (11-week pre-conditioning), a greater proportion of MBD-fed broodstock matured per day (i.e. developed stage IV ovaries and thus entered the spawning facility), in addition to producing greater numbers of total egg and nauplii when compared with CTRL-fed broodstock (P<0.05) (Table 3). Percentage hatch rate did not differ between experimental diet treatments when considered across both experiments one and two (P>0.05) (Table 3). The same data were also separated by experiment to distinguish effects of pre-condition period. The proportion of spawning females , total egg production, percentage hatch rate and total nauplii production did not significantly differ between experiment one broodstock fed MBD or CTRL diets 3-weeks prior to ablation (P>0.05) (Table 3). However, when broodstock were pre-conditioned for 11-weeks prior to ablation (experiment two), a greater proportion of MBD fed broodstock matured per day, in addition to producing greater numbers of total egg and nauplii when compared with CTRL fed broodstock (P<0.05) (Table 3). Percentage hatch rate did not differ between experimental diet treatments when broodstock were pre-conditioned for 11- weeks (P>0.05) (Table 3).

2.4.3 Biochemical Analysis of Broodstock Tissues

Ovary, hepatopancreas and muscle tissue composition was analyzed for experiment one broodstock only, following the 20-day reproductive performance phase. Compositionally, dry matter (%), total protein (%), total lipid (%), ash (%) and gross energy content within hepatopancreas and muscle tissues did not differ significantly between MBD and CTRL-fed broodstock (P>0.05) (Table 4).

Table 2. Ingredient formulation (on a % dry matter (DM) basis) and proximate compositional analysis (g/kg unless otherwise stated) of control (CTRL) and microbial biomass inclusive (MBD) maturation pelleted diets fed during experiments one and two.

	CTRL	MBD
Ingredients (all values presented as % DM)		
Fish meal (anchovetta) [a]	54.5	45
Krill meal [b]	10	10
Wheat gluten [c]	10	15
Wheat flour [c]	19.9	4.4
Fish Oil [a]	1.8	1.8
Astaxanthin (10%) [d]	0.5	0.5
Vitamin premix [e]	1.5	1.5
Arachidonic Acid (40%)® [f]	1.8	1.8
Microbial Biomass [g]	-	20
Composition (all values g/kg unless otherwise stated)		
Dry matter	695	694
Protein	436	362
Lipid	71	72
Ash	52	99
Carbohydrates^	135	162
Gross Energy (MJ/kg DM)	23	20

a Fish (Peruvian anchovetta) meal and oil: Ridley Aquafeeds, Narangba, QLD, Australia. b Krill meal: Qrill™ Aqua, AkerBioMarine, Oksenøyveien, Bærum, Norway. c Wheat gluten and flour: Manildra, Auburn, NSW, Australia. d Carophyll Pink (10%), DSM Nutritional Products, Basel, Switzerland. e Vitamin premix : Rabar, Beaudesert, QLD, Australia; includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 1.25 MIU; Vitamin E, 100 g; Vitamin K3, 10 g; Vitamin B1, 25 g; Vitamin B2, 20 g; Vitamin B3, 100 g; Vitamin B5, 100; Vitamin B6, 30 g; Vitamin B9, 5; Vitamin B12, 0.05 g; Biotin, 1 g; Vitamin C, 250 g; Banox-E, 13 g. f ARASCO®, Martek Biosciences Co., Columbia, MD, USA. g Novacq™ : CSIRO, Cleveland, QLD, Australia, PCT Patent AU 2008201886.
^Carbohydrates calculated by difference

However, the ovarian tissues of MBD fed broodstock contained significantly greater total lipid (%) following the 20-day evaluation period than those fed the CTRL diet (P<0.05). Ovary dry matter(%), total protein (%), ash (%) and gross energy content did not significantly differ between MBD and CTRL fed broodstock (P>0.05).

Table 3. Proportion of spawning females, total egg production, percentage hatch rate and total nauplii performance parameters taken over the 20-day reproductive evaluation period post-ablation. Performance parameters are presented for broodstock fed either a control (CTRL) or microbial biomass inclusive (MBD) diet for either 3-weeks (experiment one), or 12- weeks (experiment two) prior to ablation and during spawning (20-day evaluation period), or averaged across experiments one and two.

		Combined Experiment 1 and 2				Experiment 1				Experiment 2			
Performance Measures	**Diet**	**Reps (n)**	**Mean**	**±SD**	**P-value**	**Reps (n)**	**Mean**	**±SD**	**P-value**	**Reps (n)**	**Mean**	**±SD**	**P-value**
Proportion of Spawning Females	CTRL	216	0.035	0.006	0.019	47	0.021	0.007	nsd	169	0.038	0.006	0.016
	MBD	256	0.042	0.004		42	0.019	0.007		214	0.047	0.004	
Total Egg Production	CTRL	216	10371.61	1822.21	0.018	47	4670.21	1711.30	nsd	169	11742.46	2026.69	0.018
	MBD	256	13152.70	1345.86		42	5369.05	1894.75		214	14731.34	1385.71	
Percentage Hatch Rate	CTRL	216	39.08	4.17	nsd	47	42.90	10.24	nsd	169	38.23	4.33	nsd
	MBD	256	44.76	4.01		42	36.10	10.70		214	45.75	3.91	
Total Nauplii Production	CTRL	216	4017.77	836.94	0.034	47	1500.00	541.23	nsd	169	4649.00	975.51	0.017
	MBD	256	5460.50	695.32		42	1595.24	581.70		214	6289.12	815.37	

All reproductive performance measures are presented on a per female per day basis
SD= Standard Deviation
Reps (n) = replicates (number)
nsd = P>0.05

Table 4. Proximate compositional analysis (expressed on a % dry weight basis unless otherwise stated) of experiment one broodstock hepatopancreas, ovary (stage IV) and muscle tissue sampled one maturation cycle following the conclusion of the 20-day reproductive evaluation period.

Component	CTRL	± SE	MBD	± SE	Significance
Hepatopancreas Tissue					
Dry Matter	97.58	1.00	94.93	0.73	nsd
Protein	44.57	5.16	50.03	3.53	nsd
Lipid	39.48	5.63	38.51	3.36	nsd
Ash	4.23	0.66	4.21	0.40	nsd
Gross Energy	26.46	0.89	26.04	0.49	nsd
Ovary Tissue					
Dry Matter	97.37	1.29	94.59	0.79	nsd
Protein	70.04	1.53	73.37	0.67	nsd
Lipid	19.98	0.77	22.28	0.42	0.032
Ash	6.37	0.53	5.83	0.12	nsd
Gross Energy	25.81	0.62	25.68	0.19	nsd
Muscle Tissue					
Dry Matter	95.04	1.71	92.83	1.27	nsd
Protein	94.12	0.71	97.03	1.29	nsd
Lipid	4.35	0.31	4.32	0.18	nsd
Ash	6.1	0.26	6.11	0.19	nsd
Gross Energy	21.81	0.60	23.23	0.53	nsd

SE= Standard Error
nsd = $P>0.05$

2.5. Discussion

The present study demonstrated that the inclusion of 20% dried MB within broodstock pelleted diets improved reproductive performance of *P. monodon* when fed in combination with high-quality basal maturation feeds. However, such improvements were only observed in experiment two broodstock fed experimental diets over an 11-week pre-conditioning period. A higher proportion of spawning females and greater egg production was observed for experiment two broodstock fed MBD diets relative to those fed the CTRL diet. Whilst nauplii hatch rate was not influenced by experimental diet treatments, the sheer number of eggs spawned and increased maturation rates of MBD fed broodstock resulted in significantly greater nauplii yields. These results are consistent with previous reports demonstrating increased spawning and egg production of *Penaeid* shrimp when reared in live biofloc systems (Emerenciano et al., 2013a, Emerenciano et al., 2013b, Emerenciano et al., 2012).

Therefore it is possible that both live and dried bioflocs contain similar assemblages of microbial biomass or that the bioactive mechanism by which biofloc improves performance remains similar between dried and live strains. Further research is required to elicit the mechanism by which biofloc acts upon reproductive performance in *Penaeid* shrimp and to understand whether such mechanisms in biofloc differ between live and dried variants.

In addition to investigating dried MB, the current experiment highlights the significance of broodstock pre-conditioning. Whilst improvements in reproductive performance were observed in experiment two broodstock, the performance of experiment one broodstock did not differ across diet treatments. Certainly, the type of diet (selected FFF and/or percentage of dry pellet applied) fed during pre-conditioning may affect broodstock performance (Emerenciano et al., 2013b, Emerenciano et al., 2013a). However, for the current study the pre-conditioning diet regime was identical between experiments one and two, with the exception of the length of the pre-conditioning period. Compositional analysis of the experimental diet pellets highlighted reduced protein content within the MBD diet. However, when the tissue composition of experiment one broodstock was analyzed no significant difference in protein content were observed for hepatopancreas, ovary or muscle tissues. Experiment one broodstock fed the MBD diet displayed significantly greater ovary lipid content than the CTRL fed animals, suggesting that the total MBD feeding regime (which includes fresh-frozen ingredients) did not result in nutrient deficiencies in MBD broodstock relative to CTRL. Tissue samples could not be obtained from the commercial broodstock used in experiment two, so the effect of the MBD on broodstock composition over extended timescales could not be determined. However, it is possible that 3-weeks of pre-conditioning simply did not provide sufficient level of exposure to the MB ingredient to elicit the level of improvement that was observed in experiment two broodstock. I also cannot exclude the possibility that individual shrimp simply did not consume much of the available pellets during experiment one or that the age of broodstock between experiments one and two may have also contributed to variation in spawning performance. Future studies may be needed to optimize the duration of exposure to MB supplemented diets, in addition to optimization of pre-conditioning period.

The present study suggests that, in addition to promoting growth in juvenile shrimp (Glencross et al., 2014, Glencross et al., 2013), the inclusion of dried MB within pelleted diets may enhance reproductive performance in domesticated *P. monodon* broodstock.

However, the ability to improve reproductive performance appears highly dependent on quality of the entire broodstock diet (which includes both the fresh frozen and basal pellet formulation) as well as the duration of the pre-conditioning period. Currently, the exact nature of the bioactive constituents within biofloc and dried MB that are influencing reproduction remain unknown. Notably, the reproductive performance evaluations within this study were taken on-farm within two commercial spawning runs. Commercial environments impose unique challenges such as, in these experiments, the inability to track and monitor individuals. Therefore, future works should aim to investigate the mechanism by which MB influences reproductive performance under 'commercial-like' experimental conditions where individuals can be tracked and more control over the production system can be imposed.

Chapter 3: The effects of adding microbial biomass to grow-out and maturation feeds on the reproductive performance in *Penaeus monodon*

3.1. Abstract:

A 40-day reproductive performance trial was conducted to assess the effect of targeted supplementation of *Penaeus monodon* broodstock grow-out and maturation diets with microbial biomass (MB; Novacq™, Patent #2008201886). Over a seven month grow-out period, shrimp were fed a typical pelleted grow-out diet with or without 10% MB. Broodstock were then transferred to a maturation facility and a subset of animals from each grow-out diet fed on a typical fresh-frozen maturation diet that included a pellet ration with or without 30% MB. The pelleted diet constituted 18.5% of total diet (based on dry matter) and therefore the MB ingredient was fed at an approximate rate of 5.5% of their total diet fed (based on a 30% inclusion rate). At nine months of age, all broodstock were unilaterally eyestalk-ablated and reproductive assessments commenced. No significant difference in ovarian maturation, hepatosomatic index, spawning and egg and nauplii production parameters were found between diet treatments ($P \geq 0.05$). However, females originating from control ponds displayed a higher gonadosomatic index at first spawn, whilst the percentage of embryos that hatched was lower in females fed a MB-inclusive maturation diet ($P < 0.05$). These results indicate that the inclusion of MB within broodstock grow-out and maturation diets (at the rates presented in this study) did not enhance reproductive performance of domesticated broodstock. When MB supplementation was limited to the broodstock grow-out phase only, diets containing 10% MB did not impact on the reproductive performance of stocks. Breeders therefore have the potential to include MB within grow-out diets, at levels optimal for improving growth and health benefits, without impacting on potential reproductive performance.

3.2. Introduction:

To date, significant advances have been made in the domestication and genetic improvement of *Penaeus monodon* (giant tiger shrimp). These include superior growth and feed utilization (Glencross et al., 2013, Glencross et al., 2014), increased harvest yields (Preston et al., 2009) and increased viral tolerance (Sellars et al., 2015a). Despite these improvements, the reproductive performance of domesticated broodstock remains inferior when compared with wild-caught broodstock (Menasveta et al., 1993, Peixoto et al., 2005, Coman et al., 2006, Arnold et al., 2013). As a result, progress in widespread commercial domestication of *P. monodon* has been slow, leaving most farming operations to rely on stocks produced from wild-caught broodstock. Continued reliance on the progeny of wild-caught broodstock is unfavourable as farmers risk the introduction of wild diseases and pathogens into the farming system, as well as precluding the opportunity for genetic improvements via selective breeding. While reproductive performance in domesticated broodstock has been shown to improve over successive generations in captivity (Coman et al., 2013, Preston et al., 2009), there remains significant scope and economic merit to improving reproductive output and seedstock production from domesticated stocks (Arnold et al., 2013).

Broodstock nutrition is regarded as one of the primary factors that constrains the reproductive performance of domesticated *Penaeid* shrimp (Arnold et al., 2013, Emerenciano et al., 2013b, Browdy, 1998, Coman et al., 2007a, Coman et al., 2007b). In general broodstock diets can be classified into two broad categories: 'grow-out diets' designed to facilitate rapid and sustained crop growth in juvenile shrimp; and 'broodstock conditioning and maturation diets' (henceforth referred to simply as maturation diets) designed to provide mature broodstock with the nutrients required for high larval output over successive spawnings. Inadequacies in broodstock diets are known to impact on the reproductive performance of broodstock either by negatively affecting egg formation and development (resulting in poor offspring viability) or through the stunting or inhibition of spawning activity (Wouters et al., 2001b, Clarke et al., 1990, Harrison, 1990, Wouters et al., 2001a). The nutritional status of females prior to maturation can have significant implications for subsequent performance. For example, Marsden et al. (1997) demonstrated that maturation diets fed to wild *P. monodon* broodstock after capture and during maturation significantly influenced spawning frequency and larval quality. However, these authors also noted that seasonal or individual variation, presumably reflected a large-part in the nutritional condition of the stocks, could not necessarily be

eliminated by short-term dietary changes, and that the diet consumed in the period prior to maturation significantly influenced subsequent reproductive performance. This has led some authors to speculate that variation in diet prior to capture may explain the disparate reproductive performance between wild and domesticated broodstock (Coman et al., 2006, Arnold et al., 2013).

A particularly promising area of shrimp nutrition research is the use of concentrated microbial aggregates, known as bioflocs. The growth promoting effects of microbial biomass on shrimp have been reported in a number of studies (Burford et al., 2004, Kuhn et al., 2008, Kuhn et al., 2009). Nutritional studies have demonstrated that the growth enhancing effects are critically dependent on meeting the overall nutritional demands of shrimp (Glencross et al., 2013, Glencross et al., 2014).

A number of studies have examined the effects of microbial biomass on reproductive performance of farmed shrimp. Comparison of the reproductive performance showed that female *Farfantepenaeus duorarum* (pink shrimp) reared in a biofloc system had higher spawning activity than females reared in clear water (Emerenciano et al., 2013a). However, as demonstrated for growth rates in shrimp, the effects of microbial biomass on reproductive performance appear to be strongly co-dependent on other nutritional factors. For example, fresh food supplementation enhanced the reproductive performance of *Litopenaeus vannamei* (pacific white shrimp) reared under biofloc conditions (Emerenciano et al., 2013b). Similar improvements in reproductive performance were observed in *P. monodon* when fed 20% MB-inclusive pelleted diet (2.4% of total diet), however these improvements in egg production maturation frequency and nauplii production were largely dependent on the length of pre-conditioning period (see chapter 2).

The purpose of the current study was to assess the effects of including microbial biomass (Novacq™, Patent #2008201886) in the diet of domesticated *P. monodon* during a seven month grow-out phase and the subsequent three month maturation phase. Broodstock grow-out was conducted on a commercial farm, before stocks were transferred to a research facility where they were pre-conditioned, matured and spawned. Maturation and spawning of broodstock off-farm allowed for increased precision and control when assessing individual broodstock response to diet treatments.

3.3. Methodology

3.3.1 Stock Origin and Rearing

The experimental stocks used in this trial were from a fourth generation commercially domesticated *P. monodon* line maintained by Gold Coast Marine Aquaculture (GCMA, Woongoolba, Queensland, Australia). These stocks were spawned over two commercial spawning events in 2013, using the company's commercial maturation, hatchery and broodstock nursery protocols. Upon reaching postlarval stage (PL) 15 (15 days post-metamorphosis from mysis to postlarval stage 1), juvenile shrimp were stocked into three earthen grow-out ponds at a density of 2-3 shrimp/m^2 (Table 5). At this time, juvenile broodstock began receiving experimental grow-out diets. One group received the control diet that was a commercially-produced pellet that did not contain the MB ingredient, whilst the other group received a commercially-produced pellet formulated similarly to the control diet, but including 10% MB (10% of total diet; crude analysis of the two grow-out diets provided below: Table 6). In accordance with GCMA's commercial grow-out protocols, broodstock were fed their respective grow-out diets four times per day in preparation for maturation and spawning. During grow-out, average female weight for each grow-out treatment was estimated on a monthly basis. Average weight per treatment was derived from 20 individual female broodstock sampled (via cast net) per pond, per month, averaged across all ponds within a given diet treatment.

When animals reached sexual maturity at approximately seven months, a total of 80 male and 80 female domesticated broodstock from each respective grow-out treatment were randomly selected from the grow-out ponds via cast net. All shrimp were transported 2 h by road to

Table 5. Commercial pond and stocking parameters for broodstock reared at Gold Coast Marine Aquaculture. Broodstock were fed on either a control (pond C) or microbial biomass (MB) inclusive (pond A and B) pelleted diet throughout the grow-out period (7 months).

	Pond Size (m^2)	**No. Shrimp Stocked**	**Stocking Density**	**Grow-out Diet**
Pond A	2000	6000	3 shrimp/ m^2	MB Pellet
Pond B	3000	5000	2 shrimp/ m^2	MB Pellet
Pond C	3000	5000	2 shrimp/ m^2	Control Pellet

Table 6. Proximate compositional analysis (on a % dry matter (DM) basis) of control and microbial biomass (MB)-inclusive pelleted diets fed during broodstock grow-out and maturation (includes both conditioning and spawning periods) phases.

Diet	Component	Control	MB
Grow-out	Protein	47.9	47.4
	Fat	7.9	8.4
	Moisture (air)	8.7	9.1
	Ash	9.0	12.6
	Crude Fibre	2.6	3
	Carbohydrate^	35.2	31.6
	Energy (kJ/g)[1]	19.5	18.9
Maturation	Protein	36.9	30.7
	Fat	8.5	8.3
	Moisture (air)	31.3	34.6
	Ash	9.3	9.9
	Crude Fibre	0.4	2.1
	Carbohydrate^	45.3	51.1
	Energy (kJ/g)[1]	19.1	18.8

All results are reported on a % weight per weight basis unless otherwise stated^ Carbohydrates calculated by difference

[1] Energy calculated as 21.3 kJ/g for protein, 17.6 kJ/g for carbohydrate and 39.5 kJ/g for lipid (Cuzon and Guillaume, 1997)

CSIRO's research facility at the Bribie Island Research Centre (BIRC, Woorim, Queensland, Australia). Animals were stocked into 10,000 L circular maturation tanks containing a fine layer of sand substrate (Crocos and Coman, 1997). Seawater flowed through the tanks at 4 L min^{-1} (57% water exchange per day) at an average water temperature of 27°C, and 35 ± 1 ppt salinity. Photoperiod was maintained at 14 h light: 10 h dark with an artificial light system.

At BIRC, tanks were allocated a maturation diet as described in Figure 3. Broodstock that had previously been reared on a control grow-out diet were either allocated to a control maturation diet (C+C) or switched to a MB-inclusive maturation diet (C+MB). Broodstock previously reared on a MB-inclusive grow-out diet were either allocated to a MB-inclusive maturation diet (MB+MB) or switched to a control maturation diet (MB+C). Control maturation diets consisted of a fresh-frozen invertebrate based maturation diet supplemented with a high-quality commercial broodstock maturation pellet. MB-inclusive maturation diets consisted of the same fresh-frozen invertebrate based diet supplemented with a high-quality broodstock maturation pellet that comprised of 30% MB prepared on a similar base as to the control diet. The total contribution of pelleted feeds within maturation diets was

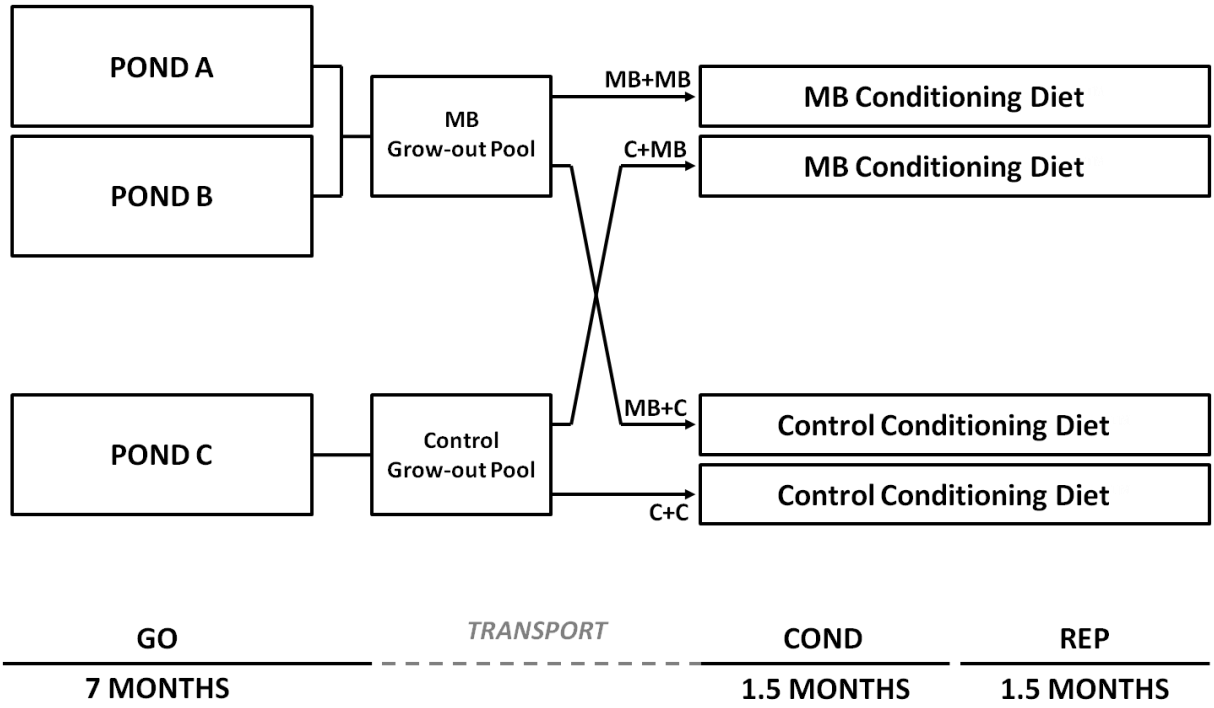


Figure 3. Diagrammatic representation of experimental stock and diet allocations. All stocks were reared on-farm under controlled pond grow-out (GO) conditions for a total of seven months. During the GO (grow-out) phase shrimp stocked in ponds A and B were fed on a MB inclusive grow-out diet, whilst those stocked in pond C were fed on a control grow-out diet. At seven months of age broodstock from ponds A and B were combined (n=160 shrimp/ treatment, 1 ♂:1 ♀) to form the MB grow-out pool. A second independent control grow-out pool was created by sampling broodstock from pond C only (n=160 shrimps/ treatment, 1 ♂:1 ♀). Both pools were then transported off-farm to maturation tanks and randomly allocated to a maturation diet. Half of the broodstock within the MB grow-out pool were allocated a MB inclusive maturation diet (MB+MB) whilst the remaining half were switched to a control maturation diet (MB+C). Similarly, half of the broodstock within the control grow-out pool were allocated a control maturation diet (C+C) whilst the remaining half was switched to a MB- inclusive maturation diet (C+MB). Two independent replicate tanks were used per treatment, with each tank containing 40 individuals (4 shrimp/ m^2, 1 ♂:1 ♀). All broodstock were fed their allocated broodstock diet for the entirety of the pre-maturation (PC) and reproductive (REP) phases.

approximately 18.5%, with the remainder made up by fresh-frozen ingredients: *Artemia* biomass (5.1% of total diet fed); bloodworm (16.6% of total diet fed); mussel (15.8% of total diet fed); ox liver (13.5% of total diet fed); squid (30.6% of total diet fed). Therefore, within the MB-inclusive maturation diets the MB ingredient was provided to broodstock at approximately 5.5% of their total diet. A crude analysis of the two pelleted maturation diets is provided below in Table 6. Broodstock were fed to slight excess five times daily which included a single daily ration of the experimental pelleted diets. Animals were fed their respective maturation diets over a three week acclimation period (post-stocking) followed by

a four week conditioning period in preparation for reproductive assessments, and throughout the assessment period.

3.3.2 Reproductive Performance Trial

At 7.5 months of age, all female broodstock were captured from tanks and eye-tagged for individual identification. At 8.5 months of age, all female broodstock were molt-tagged and subsequently monitored daily for evidence of molting. Four days post-molt, females were unilaterally eyestalk ablated using sterile forceps to induce ovarian development. Ablated females were examined daily for ovarian maturation by shining a torch through their dorsal exoskeleton (Coman et al., 2005). When ripe ovaries (Stage IV: Tanfermin & Pudadera 1989) were detected, females were removed from maturation tanks and transferred to 80 L spawning tanks maintained with water flow of 0.7 L min-1 and 29˚C water temperature.

The reproductive performance of each ablated female was assessed for a maximum of two molt cycles (approximately 40 -days) or until sacrificed according to the sampling schedule (see 3.3.3 Measures of Broodstock Performance below). The morning after spawning, or if the ovaries had regressed, females were weighed and returned to maturation tanks. All spawnings were designated ‘normal’ or ‘partial’ based on whether females presented with fully (Stage V) or partially (Stage I-III) spent ovaries post-spawn. The total number of eggs per spawn was estimated by taking egg counts from three 250 mL samples from the spawning tank water after thoroughly mixing to ensure eggs were homogenous within the water column (note that eggs, whether they are fertilized and technically embryos or unfertilized eggs, will be referred to as eggs from herein for simplicity). The remaining eggs were left to hatch within the spawning tanks. Approximately 2 to 3 h after the observation of first hatching, larvae were mixed thoroughly and nauplii per spawn estimated by counting viable nauplii from three 250 mL representative samples. The percentage of nauplii hatching per spawn was estimated from the total number of nauplii divided by the number of eggs that spawned.

3.3.3 Measures of Broodstock Performance

Over the course of the reproductive trial, samples were collected from individual broodstock for evaluation of broodstock performance across two maturation cycles. Within all feed treatments groups, a subset of broodstock (n=10) were anaesthetized and sacrificed at first maturation (i.e. upon reaching ovary stage IV for the generation of gonadosomatic index

(GSI) and hepatosomatic index (HSI). Each shrimp was weighed (g) before the hepatopancreas and ovaries were removed via dissection. The ovary and hepatopancreas were weighed and GSI/ HSI calculated as follows:

GSI = [Gonad weight/ Total female weight] x 100

HSI = [Hepatopancreas weight/ Total female weight] x 100

The remaining broodstock were allowed to spawn before completing one additional maturation cycle. When ripe ovaries were detected in second order spawners broodstock were again culled for the generation of somatic indexes (n=10).

The size of female broodstock during the maturation period was expressed as weight (g) at first maturation and weight (g) at second maturation. The number of days elapsed between molt tagging and the detection of first molt and subsequent ablation was expressed as days to first molt and days until ablation, respectively. Survival was assessed as percentage of natural female deaths (i.e. not as a result of culling) post-ablation and post-ablation prior to first maturation. For all ablated females, broodstock maturation was expressed as the percentage of females maturing at least once, percentage of females maturing to stage 3 ovaries at least once, days from ablation to first maturation, days from maturation to first spawning (excluding those that were culled) and days to second maturation from first maturation.

3.3.4 Statistical Analysis

Time to first molt and subsequent ablation, survival of females throughout the trial and the difference in individual reproductive and broodstock performance parameters were analyzed using Two-way ANOVA (Zar, 1984b) (Generalized linear model; PROC GLM; SAS Institute Software, 1999) which included an interaction term;

Model I $$Y_{ijk} = \mu + G_{dieti} + M_{dietj} + G_{dieti} \times M_{dietj} + eijk$$

where Y is the performance of the kth female which had been fed on the ith grow-out diet and the kth conditioning diet; μ is the overall mean; Gdieti is the effect of the ith grow-out diet; Mdietj is the effect of the jth maturation diet; e is the random error. Where significant interactions were found between grow-out and maturation diets, parameters were analyzed by

Student's T-Test (Zar, 1984a) for each maturation and grow-out diet separately (SAS Institute Software, 1999).

3.4. Results

3.4.1 Broodstock Grow-out Weight

Female weight in response to grow-out diets varied throughout the grow-out period (Supplementary Figure 2). At three months of age female weight did not significantly differ between grow-out diets ($P \geq 0.05$). However, female weight at four ($P < 0.001$), five ($P < 0.001$), six ($P < 0.001$), and seven ($P < 0.001$) months of age was significantly higher in females reared within control ponds, relative to their MB reared counterparts (Supplementary Figure 2).

3.4.2 Molt Period, Ablation And Broodstock Survival Post-Ablation

The interval between molt tagging and first molt did not significantly differ in response to grow-out or maturation diets ($P \geq 0.05$), with females molting 12.8 to 14.4 ± 0.8 days following molt tagging (Table 7). Similarly, broodstock grow-out and maturation diet did not affect time to ablation with females being ablated 15.6 to 18.4 ± 0.8 days following molt tagging ($P \geq 0.05$). Mortality following ablation was not significantly impacted by grow-out or maturation diets ($P \geq 0.05$). Similarly, mortality of females (of those ablated) prior to first maturation did not differ in response to grow-out or maturation diets ($P \geq 0.05$).

3.4.3 Broodstock Maturation And Spawning

The percentage of females maturing to stage III or stage IV did not significantly differ in response to grow-out or maturation diets ($P \geq 0.05$) (Table 8). The interval between ablation and first maturation was not significantly affected by grow-out or maturation diets ($P \geq 0.05$); with females maturing 12.2 to 13.9 ± 0.9 days post-ablation. Similarly, the interval between ablation and first spawn did not significantly differ between diets ($P \geq 0.05$); with females spawning 10.9 to 13.2 ± 1.2 days post-ablation. The capacity for repeat spawns was similar in response to grow-out and maturation diets ($P \geq 0.05$) with females completing a second maturation cycle 4.2 to 10.2 ± 1.0 days following first maturation.

3.4.4 Somatic Indices

The body weight of females at first and second maturation differed significantly in regards to

Table 7. Molt, ablation and broodstock survival parameters of female *P. monodon* broodstock in response to broodstock grow-out and maturation diet regime, following the completion of the broodstock pre-conditioning (3-weeks acclimation period followed by 4 weeks pre-conditioing) phase at Bribie Island Research Centre.

	Measure	C+C	C+MB	MB+C	MB+MB	Pooled SEM	Gdiet	Mdiet	Gdiet x Mdiet
Molt period	Days to first molt from stocking	12.8	13.5	14.2	14.4	0.8	nsd	nsd	nsd
Ablation	Days to ablation	15.6	16.3	17.3	18.4	0.8	nsd	nsd	nsd
Survival	% female deaths post-ablation	23.5	13.2	10.3	5.1	2.9	nsd	nsd	nsd
	% female deaths (of those ablated) prior to first maturation	14.7	10.5	7.7	6.1	2.5	nsd	nsd	nsd

Treatment combinations are represented based on diet treatments fed, where: C+C= control grow-out and maturation diet; C+MB= control grow-out diet with MB maturation diet; MB+C= MB grow-out diet with control maturation diet; MB+MB= MB grow-out and maturation diet; Gdiet = grow-out diet; Mdiet= maturation diet

nsd = no significant difference (P>0.05)

Table 8. Maturation parameters and somatic indices of female *P. monodon* broodstock in response to broodstock grow-out and maturation diet regime, sampled across first-order and second-order maturation cycles.

	Measure	C+C	C+MB	MB+C	MB+MB	Pooled SEM	Gdiet	Mdiet	Gdiet x Mdiet
Development	% of females maturing at least once	70.6	68.4	76.3	71.9	3.8	nsd	nsd	nsd
	% of females maturing to stage III at least once	85.3	89.5	94.7	90.6	2.5	nsd	nsd	nsd
1st Maturation	Time from ablation to 1st maturation (days)	13.9	12.3	12.2	12.4	0.9	nsd	nsd	nsd
	Female weight at first maturation (g)	167.1^{A}	170.7^{A}	142.8^{B}	150.9^{B}	2.1	$p<0.0001$	nsd	nsd
	Gonadosomatic index at 1st maturation	8.7^{A}	7.6^{A}	7.2^{B}	6.6^{B}	0.32	$p=0.026$	nsd	nsd
	Hepatosomatic index at 1st maturation	2.9	3.0	3.3	3.2	0.09	nsd	nsd	nsd
	Time from first ablation to 1st spawning (days)	13.2	10.9	11.1	12.8	1.8	nsd	nsd	nsd
2nd Maturation	Time to 2nd maturation (days)	7.9	9.4	4.2	10.2	1.0	nsd	nsd	nsd
	Female weight at 2nd maturation (g)	170.6^{A}	163.1^{A}	145.1^{B}	158.0^{B}	3.1	$p=0.021$	nsd	nsd
	Gonadosomatic index at 2nd maturation	6.9	6.8	7.5	7.5	0.28	nsd	nsd	nsd
	Hepatosomatic index at 2nd maturation	2.9	2.8	3.0	3.1	0.07	nsd	nsd	nsd

Treatment combinations are represented based on diet treatments fed, where: C+C= control grow-out and maturation diet; C+MB= control grow-out diet with MB maturation diet; MB+C= MB grow-out diet with control maturation diet; MB+MB= MB grow-out and maturation diet; Gdiet = grow-out diet; Mdiet= maturation Diet; nsd = no significant difference ($P>0.05$).

Superscripts marked on individual treatment combinations (in each row) refer to ANOVA results for the diet treatment comparison (i.e. the A superscript is noted on both individual treatments comprising the diet treatment that is significantly larger, and the B superscript on both treatments comprising the diet that is significantly smaller).

grow-out treatments (P<0.05) (Table 8). At first maturation, broodstock reared in control ponds were significantly larger (167.1 to 170.7 ± 2.6 g) than those reared in MB ponds (142.8 to 150.9 ± 2.4 g) (P<0.0001). Likewise, female body weight at second maturation was significantly greater when females were reared in control ponds (163.1 to 170.6 ± 3.6 g) relative to MB ponds (145.1 to 158.0 ± 4.4 g) (P=0.021). Female weight at first and second maturation was not affected by maturation diet (P≥0.05).

GSI at first maturation differed significantly in response to grow-out diet (P<0.05). The proportion of gonad weight to overall female body weight was significantly greater in females reared on the control grow-out diet (7.6 to 8.7 ± 0.45) relative to those reared on the MB grow-out diet (6.6 to 7.2 ± 0.44) (P=0.026). Maturation diet had no significant effect on GSI at first maturation (P≥0.05). At second maturation GSI did not differ in response to grow-out or maturation diets (P≥0.05). HSI at first and second maturation was unaffected by grow-out and maturation diets (P≥0.05); with females presenting a hepatopancreas to body weight ratio of 2.9 to 3.3 ± 0.09 and 2.8 to 3.1 ± 0.07 respectively.

3.4.5 Egg and Nauplii Production

The body weight of females sampled at first spawn differed significantly in regards to grow-out treatments (P<0.05) (Table 9). When females were weighed post-spawn, broodstock reared in control ponds (164.6 to 167.6 ± 2.9 g) were significantly larger than those reared in MB ponds (142.9 to 146.3 ± 3.1 g) (P<0001).

Female weight at first spawning was not affected by maturation diet (P≥0.05). The percentage of females spawning at least once did not significantly differ in response to grow-out or maturation diet treatments (P≥0.05) with 58.3 to 65.5 ± 4.8% of the females allowed to spawn, spawning at least once. The number of eggs per spawn was not significantly different between broodstock diet treatments (P≥0.05). Total egg production was similar across grow-out and maturation diets (P≥0.05) with females producing 225,541 to 230,644 ± 15,252 eggs per spawn. Similarly, total eggs spawned per gram female body weight was unaffected by grow-out and maturation diets (P≥0.05) with females spawning 1,457 to 1,611 ± 89 eggs/ g body weight. The percentage of spawnings that hatched did not significantly differ between grow-out and maturation diet treatments (P≥0.05). However, whilst the mean hatch rate (of the spawning that hatched) was unaffected by grow-out diet (P≥0.05), maturation diets

Table 9. Spawning, egg and nauplii production parameters for first order female *P. monodon* spawners in response to broodstock grow-out and maturation diet regime.

	Measure	C+C	C+MB	MB+C	MB+MB	Pooled SEM	Gdiet	Mdiet	Gdiet x Mdiet
Spawning	Female weight at first spawning (g)	167.6^{A}	164.6^{A}	142.9^{B}	146.3^{B}	2.5	*P<0.0001*	nsd	nsd
	% of females spawning at least once	58.3	61.5	65.5	60.9	4.8	nsd	nsd	nsd
Egg Production	No. of eggs spawned '	249 687	255 535	230 644	225 541	15 252	nsd	nsd	nsd
	No. of eggs spawned/ g female body weight '	1 457	1 531	1 611	1 480	89.0	nsd	nsd	nsd
Hatching	% of first spawnings hatching ' *	100	100	94.5	92.3	2.5	nsd	nsd	nsd
	Hatch Rate (%) ' *	44.2^{A}	29.2^{B}	38.2^{A}	28.4^{B}	3.0	nsd	*P*=0.041	nsd
Nauplii Production	No. nauplii produced '	111 565	70 092	80 533	65 261	8 477	nsd	nsd	nsd
	No. nauplii produced/ g female body weight '	653	416	554	417	50.0	nsd	nsd	nsd

Treatment combinations are represented based on diet treatments fed, where: C+C= control grow-out and maturation diet; C+MB= control grow-out diet with MB maturation diet; MB+C= MB grow-out diet with control maturation diet; MB+MB= MB grow-out and maturation diet; Gdiet = grow-out diet; Mdiet= maturation Diet; nsd = no significant difference (*P*>0.05)

Superscripts marked on individual treatment combinations (in each row) refer to ANOVA results for the diet treatment comparison (i.e. the A superscript is noted on both individual treatments comprising the diet treatment that is significantly larger, and the B superscript on both treatments comprising the diet that is significantly smaller).

' excludes partial spawns

* excludes non-valid eggs and nauplii

significantly impacted hatch rate (P= 0.043). The percentage of eggs hatched within each spawn was significantly lower when broodstock were fed a MB maturation diet (28.4 to 29.2 ± 3.2% hatch rate) compared with the control maturation diet (38.2 to 44.2 ± 4.8% hatch rate). Despite the difference in hatching, total nauplii production did not differ significantly in response to grow-out and maturation diet treatments (P≥0.05) with females producing 65,261 to 111, 565 ± 8, 477 nauplii per spawn. Nauplii production per gram female body weight was unaffected by grow-out and maturation diets (P≥0.05) with broodstock producing 416 to 653 ± 50 nauplii per gram body weight.

3.5. Discussion

In the present study, no significant improvement or reduction in nauplii production was conferred to domesticated broodstock via the inclusion of MB in broodstock diets. Similar responses have been observed in *Litopenaeus vannamei*, whereby no significant variation in growth performance was observed between control groups and animals fed MB-inclusive pelleted diets (Bauer et al., 2012). However, these results contrast to the positive effects on reproductive performance reported for *Litopenaeus stylirostris* and *Farfantepenaeus duorarum* (Emerenciano et al., 2013a, Emerenciano et al., 2012) and those reported for *P. monodon* in chapter 2. The pelleted maturation diets used in this study contained 30% MB yet the reproductive performance of females was similar to those fed on the commercial control diet (with the exception of percent hatch rate). The results of this study may indicate a negative MB-specific dose-response, where the relationship between observed performance benefits relative to MB inclusion rate is not linear. Similar dose-responses have been reported for biofloc supplementation; Anand et al. (2014) reported that supplementation of shrimp diets with 4 and 8% biofloc improved both growth and digestive enzyme activity in juvenile *P. monodon*. However, 12% biofloc supplementation did not result in proportionate growth and enzyme activity, with shrimp performing on par with control groups (Anand et al. 2014). Similarly, Kuhn et al. (2010) reported significant improvements to growth performance in *Litopenaeus vannamei* at 10 and 15% dietary biofloc inclusion levels, yet non-significant growth at 21 and 30% inclusion. Whether MB has a similar dose-dependent influence on reproductive performance remains undetermined, however at 30% inclusion rate, no improvements were observed in this study. A point of contrast with several previous studies is that the MB used in the present study was a dried product. Live bioflocs have been shown to contain various compounds including carotenoids, chlorophylls, phytosterols, bromophenols, amino sugars and anti-bacterials, all of which potentially play a role in bioactivity (Ju et al., 2008, Crab et al., 2010, Anand et al., 2014).

However, the bioactive components of live bioflocs or bioflocs that have been harvested and dried have yet to be comprehensively characterized.

Several factors influence the number of spawned eggs that hatch to nauplii including mating success, egg fertilization, and subsequent development of the fertilized embryo (Coman et al., 2007a). In this study, the maturation diet containing MB impacted broodstock performance by significantly reducing the percentage of eggs hatching. Mating success did not appear to be a significant constraint in this study as >95% of spawnings hatched irrespective of diet treatment. Rather, the distinctive hatch rates suggest that nutritional differences between the control and MB maturation diets produced eggs or sperm of differing quality, contributing to the lower hatching of spawns from females fed the MB maturation diet. It is well established that the maturation diet fed immediately prior to spawning can drastically impact on egg quality and thus egg hatching in shrimp (Millamena, 1989, Bray and Lawrence, 1992, Cahu et al., 1995a, Cahu et al., 1994, Xu et al., 1994a, Wouters et al., 2001a, Wouters et al., 1999, Coman et al., 2007a, Coman et al., 2011, Hoa et al., 2009). The proximate analysis demonstrated that the diet containing MB had lower protein content than the control diet. Reproductive development and maturation is a period of intense protein biosynthesis, whereby the parent must sustain not only its own metabolic needs, but also stock the oocytes with the nutritional requirements for the developing eggs once spawned and fertilized (Harrison, 1990, Wouters et al., 2001a). It is known that the availability and storage of dietary protein plays a critical role in the reproductive biology of decapod crustaceans (Castille and Lawrence, 1989, Harrison, 1990, Wouters et al., 2001a, Rodriguez-Gonzalez et al., 2013, Rodriguez-Gonzalez et al., 2006, García-Guerrero et al., 2003, Saoud et al., 2012). As such, the reduced protein content of the MB maturation diet may have contributed to the reduced hatch rates. Given the known effects of maturation diet on egg quality, it is plausible that changes in egg quality associated with the 5.5% inclusion of MB within the maturation diet may have compromised either, or both, egg fertilization and subsequent embryo development.

The failure of the MB diet to improve broodstock performance in the present study contrasts the results reported in chapter 2. When fed a pelleted diet including 20% MB (2.4% of total diet) broodstock demonstrated significant increases in maturation frequency, egg production or nauplii production, with no significant reductions in egg hatching. The disparity between chapter 2 and the present study likely reflects the quality of the pelleted diets, which contrast experimental pellets designed for optimal performance (chapter 2) and the commercially formulated diets of the present study. Notably, the diet fed in the present study contained 10% more MB, which may have made the pelleted diet less palatable overall, reducing uptake of the pelleted maturation diets. In addition,

astaxanthin (derived from Carophyll Pink), arachidonic acid (ARASCO®) and phospholipids (in the form of krill meal) are known to improve reproductive performance and embryonic and larval development (Alava et al., 1993, Coman et al., 2011, Wouters et al., 2001a), and thus their inclusion within chapter 2's maturation diets may have served to satisfy or offset nutritional requirements which were lacking in the current experiments commercial formulation.

One notable outcome of this study was the slower growth of broodstock fed diets containing MB, compared to broodstock fed the control diet, in the grow-out phase (see Supplementary Figure 2). This result is inconsistent with former studies which have reported improved growth of juvenile shrimp when fed diets including MB (Glencross et al., 2014, Glencross et al., 2013, Anand et al., 2014, Glencross et al., 2015). There are several factors that may have contributed to the growth rates observed in the present study. Firstly, differences in stocking densities among ponds within the two treatments may have contributed. Certainly, broodstock rearing density is known to significantly affect growth rate in shrimp (Jackson & Wang 1998; Coman et al. 2004; Coman et al. 2007b). Secondly, the inherent pond to pond variation which results from each pond being its own ecosystem, and which is impossible to control completely even through consistent management, may also have impacted on observed growth outcomes. The possible impact of pond to pond variation on differences in growth among the grow-out treatment diets is heightened by lack of pond replication used in the study. Thirdly, the present study was conducted in low density pond environments, which provide much more natural forage for the shrimp than semi-intensive ponds or experimental tank systems. It is possible that the 10% inclusion rates of MB used within this studies grow-out diets were not optimized for the growth of *P. monodon* broodstock in low density rearing (as could be the case for reproductive performance). Whilst certainly one explanation for the observed pond growth result was that the MB grow-out diet constrained growth under these low-density pond conditions, it is also possible that any growth enhancing effects of including MB in the grow-out diet may have been masked by other variables within the study (i.e. stocking density). Future research should aim to assess potential interactions (if any) between rearing of broodstock within low density ponds and the dietary inclusion of MB (ideally across multiple dosage rates).

Certainly, the variation in stocking densities among broodstock ponds, and limited numbers of broodstock ponds used, are acknowledged as weaknesses of the study. These design weaknesses reflect the practical constraints of running such trials in commercial environments where access to low-density *P. monodon* broodstock ponds are very limited. However, it should be noted that this study was not focused on assessing growth, but rather on assessing reproductive performance. Growth rates in all ponds were very high, with mean 6-monthly weights of females in both

treatments of greater than 100 g. These high growth rates reflect both the genetic quality of the domesticated stocks originating from the company's breeding program, but also the quality management of the ponds and the favorable seasonal conditions that existed throughout the production cycle. Certainly, broodstock from both grow-out treatments had grown well in ponds, and as such, were expected to be produce good quality broodstock and to allow a reliable evaluation of the impact of the MB during the grow-out period on subsequent reproductive performance.

Finally, the results of this study demonstrated that MB supplementation during broodstock grow-out did not significantly affect the subsequent reproductive performance when broodstock were fed a commercial maturation diet. Limiting MB supplementation to the broodstock grow-out phase may be advantageous as breeders have the potential to increase juvenile crop growth, energy utilization and resilience to disease (Sellars et al., 2015b, Glencross et al., 2014, Glencross et al., 2013), without reducing the reproductive potential of mature broodstock.

To date, the causes of constraints to reproduction in *P. monodon* remain multifaceted and complex. The exact nature of the bioactive constituents within MB influencing a range of commercially relevant traits has not been specified. Results of the present study demonstrated that adding MB to the diet of domesticated *P. monodon* broodstock did not improve their reproductive performance. Despite this, use of MB in broodstock grow-out may still have value as breeders have the potential to improve upon juvenile crop growth and disease tolerance without impinging upon reproductive potential. Further research into the specific nutritional requirements of broodstock, particularly in response to spawning, is critical for the continued development and optimization of broodstock nutritional diets.

Chapter 4: Interactions between repeated spawning and tissue biochemistry in domesticated *Penaeus monodon*

4.1. Abstract:

Reliable production of seedstock remains a major constraint on the *Penaeus monodon* aquaculture industry, both through inability to stock production ponds and inefficient resource utilization maintaining poor performing broodstock. The present study investigated the effect of consecutive spawning events on the reproductive performance and tissue biochemistry in a homogeneous population (152.5 g mean initial weight) of domesticated *P. monodon*. A multidisciplinary approach was undertaken by which the following were investigated in parallel: a) comparative reproductive performance across first and second spawn; b) changes in biochemistry between first and second spawn; and c) the correlation between reproductive performance at first spawn and second spawn biochemistry. Broodstock demonstrated significant variation in aspects of hepatopancreas and ovarian tissue biochemistry in response to successive spawning. Notably, a significant decrease in hepatopancreas total saturated fatty acid (SFA), polyunsaturated fatty acids (PUFA), n-3 and n-6 fatty acids was observed in second order spawners ($P<0.05$). Conversely, ovary tissues at second spawn contained significantly greater quantities of total lipid, monounsaturated fatty acid (MUFA), PUFA, n-3 and n-9 fatty acids relative to first order spawners ($P<0.05$). When lipid classes were investigated independently, significantly lower levels of hepatopancreas C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:4n-6(ARA), C20:5n-3 (EPA), C22:6n-3 (DHA), as well as ovarian C20:4n-6(ARA) were observed ($P<0.05$), suggesting the utilization and/or requirement for said fatty acids may exceed that which is provided by current broodstock feeding regimes. Significant positive correlations were observed for latency period and hepatopancreas PUFA, C20:5n-3, C22:6n-3 and n-3 content, relative egg production and hepatopancreas C18:0 content, and hatch rate and ovarian C14:0 content ($P<0.05$). Combined, results of this study suggested that lipid composition has a greater influence on reproductive performance than total lipid content, and that the availability and utilization of specific fatty acids are critical to maintaining broodstock reproductive performance. In particular, the consistent depletion of arachidonic acid in hepatopancreas and ovarian tissues in response to repeat spawning requires further investigation.

4.2. Introduction:

Penaeus monodon (Giant Tiger Shrimp), is an economically significant aquaculture species producing 4.5 million tonnes product annually, at a value of US$23.5 billion (FAO, 2016b). Whilst *P. monodon* remains a popular species, maximizing production potential through the development of domestication and breeding programs has been problematic. Considerable advances have been made with respect to growth potential (Glencross et al., 2014, Glencross et al., 2013, Preston et al., 2009), yet the reproductive performance of domesticated stocks remains far from optimal. Domesticated stocks show significantly lower numbers of females maturing, lower egg production and lower hatch rates compared to their wild-caught counterparts (Arnold et al., 2013, Coman et al., 2006, Menasveta et al., 1993, Peixoto et al., 2005, Hall, 2003, Wen et al., 2015). Furthermore, the proportion of domesticated broodstock that contribute to nauplii yields can be greatly skewed – with a small proportion of high-performing females contributing the significant majority of offspring (Parnes et al., 2007).

Disparity in *P. monodon* reproductive quality carries substantial economic cost for breeders. Farms may waste substantial resources on conditioning poor-performing individuals, which provide proportionally few viable nauplii in return, and limits the ability to stock production ponds. Alternatively, breeders may be forced to supplement their breeding programs with seedstock spawned from wild-caught broodstock, which are expensive to source, dilute gains made through selective breeding programs (via the introduction of non-selected alleles), and represent a potential vector for the introduction of pathogens. The development of predictive measures of reproductive performance may facilitate the identification high performing individuals, whilst also allowing for early detection and/or removal of sub-optimal broodstock.

The reduced reproductive performance observed in domesticated stocks is thought to be derived largely from female body condition prior to ablation and spawning (Arnold et al., 2013, Coman et al., 2006). The relationship between optimal nutrition and spawning performance has been investigated extensively in shrimp (Bray and Lawrence, 1992, Cahu et al., 1994, Cahu et al., 1995b, Coman et al., 2007a, Coman et al., 2011, Hoa et al., 2009, Millamena, 1989, Wouters et al., 2001a, Xu et al., 1994b, Chimsung, 2014). However, it is worth noting that many of these studies did not assess the condition of the ovaries directly; instead the biochemical composition of ovulated eggs or tissues at the completion of spawning were used as a proxy measure (e.g. Cahu et al., 1994, Cahu et al., 1995b, Coman et al., 2011, Xu et al., 1994b). This is due to the non-complementary nature of assessing reproductive performance and tissue biochemistry for a single individual. It is not possible

to obtain both pre-spawning tissue samples (i.e. ovarian and hepatopancreas tissues) along with complementary measures of broodstock production performance (i.e. hatch rates and egg/ nauplii production) from the same individual over a single spawning event (i.e. spent ovaries are not representative of mature ovaries).

The present study analyses reproductive performance and tissue biochemistry (ovary and hepatopancreas) in response to consecutive spawns within a homogeneous, domesticated *P. monodon* population (152.5 g mean initial female weight), reared under typical commercial conditions. In a bid to overcome the incompatibility of assessing reproductive performance and tissue biochemistry, a multidisciplinary approach was undertaken by which: a) comparative reproductive performance across first and second spawn was assessed based on historical spawning data for individuals reared under similar commercial conditions; b) changes in biochemistry between first and second spawners were assessed for a cohort of broodstock held under identical conditions and; c) correlation between productive performance at first spawn and second spawn biochemistry were assessed for a single cohort (see Figure 4). We aimed to identify variables with significant influence on reproductive performance that represent priority traits for improvement during broodstock maturation.

4.3. Materials and Methods:

4.3.1 Acquisition Of Historic Spawn Data

Comparative reproductive performance measures total egg number, hatch rate and total nauplii production were sourced from historic *P. monodon* spawning data (CSIRO unpublished data). Data were selected only if they met the following conditions: 1) rearing was undertaken under typical farm conditions; 2) females were mated and spawned under typical farm conditions; 3) females had been fed a typical commercial control diet throughout rearing and maturation; 4) concurrent spawning data existed for each individual across the first two spawning events post-ablation. A total of 42 females met the above requirements and were available for statistical analysis of performance from first spawn (SP1) to second spawn (SP2, Figure 4). Spawn performance measures were determined as outlined below.

4.3.2 Live Broodstock Origin And Rearing

To assess variation in biochemistry across subsequent spawns, *P. monodon* broodstock were sourced from Gold Coast Marine Aquaculture (Woongoolba, Queensland, Australia). All

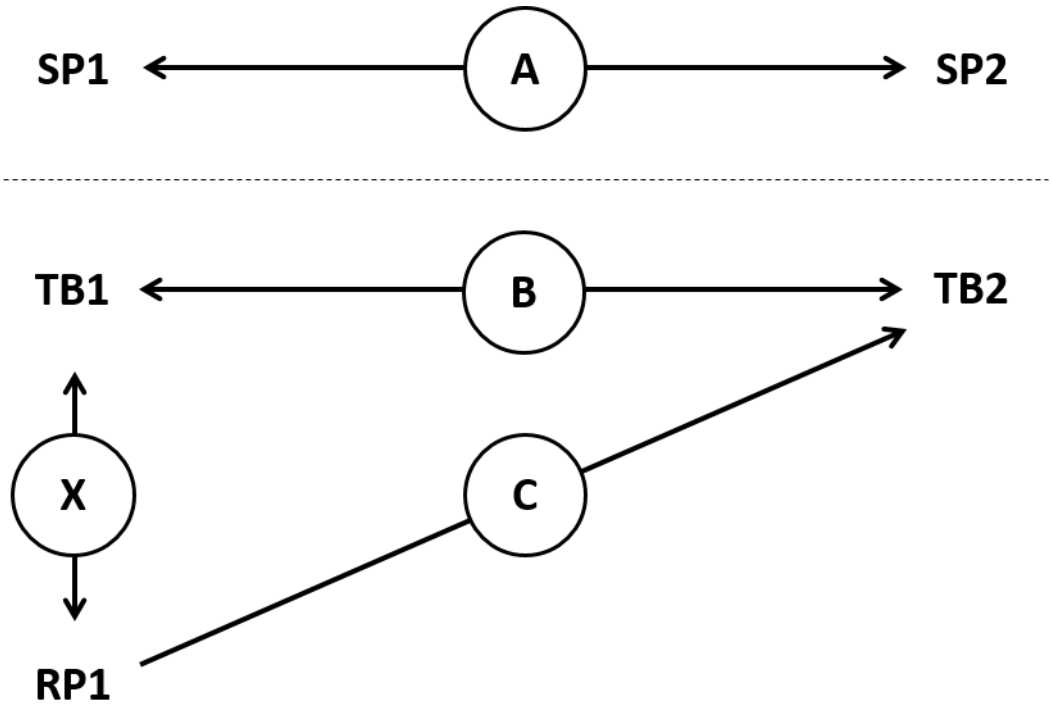


Figure 4. Representation of the three key comparative lines of investigation in this study: a) spawning performance across first (SP1) and second spawn (SP2) derived from historic spawning data; b) tissue biochemistry across first (TB1) and second spawn (TB2) derived from live animals and; c) correlations between first spawn reproductive performance (RP1) and second spawn tissue biochemistry (TB2). Dashed lines indicate the separation of datasets between historic data and data collected from live animals. The notation 'X' indicates which it is not possible to obtain both forms of data from a single time point, from a single individual.

broodstock were reared within earthen grow-out ponds under the company's commercial broodstock management protocols. At seven months of age, 160 broodstock were caught via cast net and transported to CSIRO's research facility, Bribie Island Research Centre (BIRC, Woorim, Queensland, Australia).

Broodstock were held in four 10,000 L circular maturation tanks (40 shrimp per tank, stocking density of 4 shrimp/ m^2, sex ratio1 ♂:1 ♀), as per the conditions described by Goodall et al. (2016). In brief tanks contained a fine layer of sand substrate, seawater was maintained at 27°C, at a flow rate of 4 L min^{1} (57% water exchange per day) and average salinity of 35 ± 1 ppt, whilst photoperiod was maintained at a ratio of 14 h light: 10 h dark. All broodstock were fed on a typical composite maturation diet consisting of fresh-frozen feedstuffs: squid (30.6% of total diet fed (TDF), bloodworm (16.6% of TDF), mussel (15.8% of TDF), ox liver (13.5% of TDF), *Artemia* biomass (5.1% of TDF) supplemented with a high quality broodstock pellet (Ridley Aqua Feed™: MR Broodmax, 18.5% of TDF, Narangba, Queensland, Australia). All feed ingredients were fed

separately *ad libitum*, with five rations provided daily, which included a single daily ration of the pelleted diet. All animals were fed the maturation diet for seven weeks post-stocking at BIRC (pre-conditioning period), and throughout the reproductive assessment period which followed (40-days total).

4.3.3 Performance Trial Design And Sampling

In preparation for spawning, all females were eye-tagged for individual identification (plastic, numerically-coded open-split bird bands on the eyestalk) at 7.5 months of age as previously described by Goodall et al. (2016). At 8.5 months of age all females were molt tagged (water-proof numerically coded labels glued to the carapace) to allow for standardization ablation to the molt cycle (Goodall et al., 2016). All females were unilaterally eyestalk ablated (using sterile forceps) four days following the detection of molt (152.5 g mean initial female weight). The ovarian maturation of ablated females was monitored daily as performed previously (Coman et al., 2005). When mature ovaries (Stage IV: Tanfermin and Pudadera, 1989) were detected, females were alternately allocated to one of two groups, as outlined in Figure 4. In group 1 (TB1; n=20) females were anaesthetized via ice-water immersion and sampled for biochemical analysis of tissues (see 4.3.5 Biochemical Analysis of Animal Tissues) just prior to their first spawn (i.e. following the detection of stage 4 ovaries) (termed first order spawners). The second group (TB2, n=19) females were moved to 80 L spawning tanks and allowed to spawn overnight. The following morning, reproductive performance measures were determined (RP1) as outlined below, and ovulated females were weighed then returned to maturation tanks. Ovulated females were allowed to complete one additional maturation cycle, before being anaesthetized and sampled for biochemical analyses just prior to second spawn (i.e. following the detection of stage IV ovaries). For all groups, anaesthetized females were first patted dry and weighed, before whole ovary and hepatopancreas were removed via dissection. Whole ovary and hepatopancreas tissues were weighed to the nearest mg, frozen in liquid nitrogen and stored at -80°C awaiting further analysis.

4.3.4 Reproductive Performance Measures

Two sets of independently assessed reproductive performance measures were taken during this experiment. For the comparison of historic spawning data (SP1 vs. SP2) total egg production, percentage hatch rate and total nauplii production were sourced from historic data. For the comparison of first spawn reproductive performance (RP1) and second spawn biochemistry (TB2), relative (per gram female weight) egg and nauplii production, percentage hatch rate and latency

period (days till the detection of stage IV ovaries post-spawn) were measured. For all RP1 broodstock, females were removed from spawning tanks and weighed before being returned to maturation tanks. Relative egg production was derived by dividing total egg production — quantified from three 250 mL representative water samples taken from spawning tanks following thorough mixing of the water column — by total female weight. The remaining eggs were monitored for hatching. Relative nauplii production was similarly derived by dividing total nauplii production — quantified from three 250 mL representative water samples taken from spawning tanks three hours after the detection of first hatching, following thorough mixing of the water column — divided by total female weight. Percentage of nauplii hatching was estimated from total number of nauplii production divided by the total number of eggs produced. Latency period was defined as the number of days elapsed between spawning and subsequent sampling upon re-maturation.

4.3.5 Biochemical Analysis of Animal Tissues

To assess tissue biochemistry, all TB1 and TB2 hepatopancreas and ovary tissues were minced frozen using a blender. A sub-sample of the original frozen mince was analyzed for its moisture content by gravimetric analysis following oven drying at 105˚C for 24 h and this was used as a correction for the freeze-dried material. The remaining frozen mince was freeze-dried to completion in a laboratory freeze dryer (Alpha 1-4, Martin Christ, Germany), re-homogenized in a blender and used for subsequent analyses. Protein was calculated from the determination of total elemental nitrogen (CHNS-O Flash 2000, Thermo Scientific, USA, auto-analyser, based on N x 6.25). Total lipid extractions and quantification were conducted following Folch (1957). Total lipid content was determined gravimetrically following extraction of the lipids using chloroform/methanol (2:1). Fatty acid composition was determined following published best-practice methods (Christie (2003b). A known quantity of lipids was esterified by an acid-catalyzed methylation and 0.3 mg of an internal standard was added to each sample (21:0 Supelco, PA, USA). The fatty acids were identified relative to the internal standard following separation by gas chromatography using an Agilent Technologies 6890N GC system (Agilent Technologies, California, USA) fitted with a DB-23 (60m x 0.25mm x 0.15 μm, cat 122-2361 Agilent Technologies, California, USA) capillary column and flame ionization detector. Individual fatty acids were expressed on a mg/g of total lipid basis.

4.3.6 Data Analysis and Statistics

A sample set of 42 domesticated *P. monodon* broodstock for which repeated spawning information (SP1 and SP2) was available was obtained from historic CSIRO spawning data. Unfertilized spawns, i.e. those with zero hatching and zero nauplii production values, were removed from hatch rate and nauplii production analyses reducing sample sizes from n=42 to n=22 for those measures. A Shapiro-Wilk normality test (Wilks, 1946) identified the dataset as non-normally distributed. As a result, individual differences in hatch rate, egg and nauplii production across first and second spawns were assess using a Wilcoxon Signed Rank Test (Siegel, 1956).

The biochemical composition of TB1 and TB2 groups was compared. For all factors, normality testing was conducted using Shapiro-Wilk normality testing (Wilks, 1946). Outliers were identified and removed using modified Thompson's Tau Test. Changes in biochemical composition between TB1 and TB2 for all factors were assess using Student's T-Test (Zar, 1984a). Potential correlations between RP1 and TB2 were investigated (n=19). Normality testing and outlier detection were conducted using Shapiro-Wilk normality testing (Wilks, 1946) and modified Thompson's Tau Test, respectively. Significant correlations between factors were identified using Spearman's Rank correlation coefficient (Sokal and Rohlf, 1981).

4.4. Results:

4.4.1 Changes in Spawning Performance (Historic Data)

Variation in spawning performance was assessed across first (SP1) and second (SP2) order spawning events (Table 10). Total egg production decreased in successive spawnings, however this

Table 10. Comparison of egg production, hatch rate and nauplii production parameters between first (SP1) and second (SP2) order spawners, sourced from historic *P. monodon* spawning data. SE= standard error

	SP1	SP2	Significance
Egg Production (number of eggs; n=42)			
Mean	185,446	158,500	0.177
±SE	18.759	16,662	
Hatch Rate (percent; n=22)			
Mean	24.2	23.4	0.949
±SE	3.8	4.4	
Nauplii Production (number of nauplii; n=22)			
Mean	59,500	40,393	0.337
±SE	13,742	9,079	

decrease was not significant ($P \geq 0.05$). Similarly, hatch rate and nauplii production were reduced in second order spawners, but the decrease was also not significant.

4.4.2 Tissue Biochemistry In Relation To Subsequent Spawns

Variation in biochemical parameters between first (TB1) and second (TB2) order spawners was assessed. Within hepatopancreas tissues, no significant changes in hepatosomatic index (HSI), total protein or total lipid were detected ($P \geq 0.05$) (Table 11). When considering fatty acid content broadly, significantly lower saturated fatty acid (SFA), polyunsaturated (PUFA), n-3, and n-6 fatty acid content (mg/g^{-lipid}) was observed in second order spawns relative to first order broodstock ($P < 0.05$). However, no significant variation in total monounsaturated fatty acid (MUFA), n-9 content and n-3:n-6 ratios were observed between successive spawns. When hepatopancreas fatty acid composition was separated further, significantly lower C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:4n-6(ARA), C20:5n-3 (EPA), C22:6n-3 (DHA) and minor PUFA ($Other^{3}$) content was observed in the tissues of second order spawners (TB2) relative to first order spawners (TB1) ($P < 0.05$). Significantly higher C18:3n-3 fatty acid content was observed in second order (TB2) relative to first order (TB1) spawners ($P < 0.05$).

Within ovarian tissues, no significant changes in gonadosomatic index (GSI) or total protein were detected ($P \geq 0.05$) between spawns (Table 12). However, ovarian total lipid was comparatively higher ($P < 0.05$) in second-order spawners (TB2) relative to first-order spawners (TB1). When considering fatty acid content broadly, significant higher total MUFA, PUFA, n-3 and n-9 content (mg/g^{-lipid}) was observed in second-order spawns relative to first order broodstock ($P < 0.05$). However, no significant variation in total SFA, n-6 content and n-3:n-6 ratios were observed in response to spawn order. When ovarian fatty acid composition was separated further, significantly higher C14:0, C16:0, C19:0, C16:1n-7, C18:1n-9, C18:1n-7, C20:1n-9, C18:3n-3, C20:2n-6, C22:6n-3(DHA), and minor SFA ($Other^{1}$) and MUFA ($Other^{1}$) fatty acid content (mg/g^{-lipid}) were observed in second-order spawners (TB2) relative to first order (TB1) ($P < 0.05$). Meanwhile, there was a significant lower ovarian C20:4n-6(ARA) content in second-order spawners (TB2) relative to first order (TB1) ($P < 0.05$).

4.4.3 Correlation Between Spawning Performance And Biochemistry

Correlations between first order reproductive performance (RP1) and second order biochemistry (TB2) were investigated for hepatopancreas and ovary tissues. Relative egg production at first spawn was significantly positively correlated with second spawn hepatopancreas C18:0

Table 11. Proximate composition analysis of female *P. monodon* hepatopancreas biochemistry between first (TB1) and second (TB2) order spawners. Abbreviations within this Table are defined as: HSI= hepatosomatic index; SFA= saturated fatty acid; MUFA= mono unsaturated fatty acid; PUFA= poly unsaturated fatty acid; LA= linoleic acid; ARA= arachidonic acid; EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid; nsd= no significant different (*P*>0.05).

	TB1		TB2		
Parameter	Mean	±SE	Mean	±SE	p-value
HSI	**3.12**	**0.10**	**2.95**	**0.09**	**nsd**
Protein (%)	**31.63**	**1.59**	**32.36**	**2.18**	**nsd**
Total Lipid (%)	**51.39**	**1.68**	**50.90**	**1.92**	**nsd**
Sum SFA '	**298.18**	**4.04**	**276.53**	**4.75**	**0.001**
14:0 '	10.90	0.44	11.24	0.37	nsd
15:0 '	5.13	0.09	4.43	0.09	<0.001
16:0 '	211.22	3.39	198.69	5.10	nsd
17:0 '	7.38	0.24	6.60	0.17	0.012
18:0 '	51.10	3.52	48.16	2.46	nsd
19:0 '	1.44	0.05	1.39	0.05	nsd
20:0 '	1.43	0.05	1.54	0.06	nsd
Other ' [1]	6.10	0.24	5.59	0.17	nsd
Sum MUFA '	**223.49**	**5.71**	**207.87**	**5.59**	**nsd**
16:1n-7 '	24.50	1.17	22.00	0.58	nsd
18:1n-9 '	130.38	3.10	115.76	4.95	0.019
18:1n-7 '	32.96	1.09	32.61	1.00	nsd
20:1n-9 '	25.40	1.15	26.89	0.94	nsd
Other ' [2]	10.76	0.35	10.33	0.21	nsd
Sum PUFA '	**127.32**	**23.03**	**60.10**	**11.15**	**0.014**
18:2n-6 (LA) '	31.89	3.42	21.11	2.19	0.013
18:3n-6 '	0.00	0.00	0.03	0.03	nsd
18:3n-3 '	0.83	0.06	1.19	0.08	0.001
20:2n-6 '	9.19	0.79	8.45	0.79	nsd
20:3n-6 '	4.58	0.90	2.19	0.36	0.022
20:4n-6 (ARA) '	7.03	1.54	3.06	0.61	0.025
20:5n-3 (EPA) '	12.42	2.93	3.91	1.13	0.012
22:6n-3 (DHA) '	38.28	10.29	12.14	4.22	0.027
Other ' [3]	11.35	2.36	4.62	0.87	0.014
Sum n-3 '	**65.42**	**15.50**	**21.36**	**6.29**	**0.014**
Sum n-6 '	**57.92**	**7.85**	**36.66**	**4.00**	**0.023**
Sum n-9 '	**185.97**	**4.56**	**173.87**	**4.99**	**nsd**
n-3:n-6	**0.70**	**0.12**	**0.46**	**0.06**	**nsd**

' $mg/g^{-total\ lipid}$

[1] Sum C8:0, C10:0, C11:0, C12:0, C13:0, C22:0, C23:0, C24:0

[2] Sum C14:1n-5, C15:1, C17:1, C20:1n-7, C22:1n-9, C24:1n-9

[3] Sum C18:4n-3, C20:3n-3, C22:2, C22:4n-6, C22:5n-3

Table 12. Proximate composition analysis of female *P. monodon* ovary biochemistry between first (TB1) and second (TB2) order spawners. Abbreviations within this table are defined as: GSI= gonadosomatic index; SFA= saturated fatty acid; MUFA= mono unsaturated fatty acid; PUFA= poly unsaturated fatty acid; LA= linoleic acid; ARA= arachidonic acid; EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid; nsd= no significant different (*P*>0.05).

	TB1		TB2		
Parameter	Mean	±SE	Mean	±SE	p-value
GSI	**7.69**	**0.41**	**7.25**	**0.29**	**nsd**
Protein (%)	**67.16**	**0.17**	**67.78**	**0.29**	**nsd**
Total Lipid (%)	**20.73**	**0.17**	**22.06**	**0.28**	**<0.001**
Sum SFA '	**251.57**	**5.57**	**274.76**	**12.65**	**nsd**
14:0 '	11.59	0.36	13.48	0.48	0.003
15:0 '	3.52	0.08	3.61	0.08	nsd
16:0 '	175.18	4.38	200.44	6.08	0.002
17:0 '	6.94	0.11	6.99	0.20	nsd
18:0 '	54.89	1.23	54.71	1.97	nsd
19:0 '	0.56	0.11	0.99	0.03	0.001
20:0 '	3.18	0.07	3.27	0.12	nsd
Other ' [1]	5.10	0.13	5.80	0.16	0.002
Sum MUFA '	**229.95**	**3.94**	**260.97**	**7.20**	**0.001**
16:1n-7 '	32.60	1.06	40.73	1.75	<0.001
18:1n-9 '	152.82	2.90	167.29	5.39	0.025
18:1n-7 '	25.36	1.04	29.50	1.05	0.008
20:1n-9 '	11.38	0.50	15.06	0.53	<0.001
Other ' [2]	10.12	0.49	12.68	0.71	0.006
Sum PUFA '	**317.96**	**8.40**	**349.69**	**10.31**	**0.023**
18:2n-6 (LA) '	44.78	1.27	45.20	2.20	nsd
18:3n-6 '	0.00	0.00	0.00	0.00	nsd
18:3n-3 '	2.13	0.08	2.51	0.15	0.033
20:2n-6 '	8.81	0.37	10.88	0.42	0.001
20:3n-6 '	8.58	0.78	8.40	0.78	nsd
20:4n-6(ARA)'	32.17	1.62	26.56	1.91	0.032
20:5n-3(EPA)'	57.91	1.49	63.64	2.58	nsd
22:6n-3(DHA)'	149.30	5.79	177.45	8.09	0.008
Other ' [3]	21.11	0.95	21.92	0.96	nsd
Sum n-3 '	**225.82**	**6.61**	**260.66**	**9.62**	**0.006**
Sum n-6 '	**99.63**	**3.56**	**97.04**	**5.35**	**nsd**
Sum n-9 '	**179.46**	**3.92**	**202.50**	**6.23**	**0.004**
n-3:n-6	**2.26**	**0.13**	**2.65**	**0.17**	**nsd**

' $mg/g^{-total\ lipid}$

[1] Sum C8:0, C10:0, C11:0, C12:0, C13:0, C22:0, C23:0, C24:0

[2] Sum C14:1n-5, C15:1, C17:1, C20:1n-7, C22:1n-9, C24:1n-9

[3] Sum C18:4n-3, C20:3n-3, C22:2, C22:4n-6, C22:5n-3

fatty acid content (Figure 5; p=0.02). Furthermore, significant positive correlations existed between latency period and hepatopancreas total PUFA (P=0.003), C20:5n3 (EPA, p=0.0014), C22:6n-3 (DHA, p=0.001), total n-3 fatty acid content. No significant correlations between first order spawn hatch rate and naupii production were observed for second order hepatopancreas biochemistry (P>0.05). For ovary tissues, a significant positive correlation was existed between first spawn hatch rate (%) and second spawn C14:0 (Figure 6, p=0.04). No significant correlations between first order relative egg production, relative nauplii production and latency period were observed for second spawn ovary biochemistry (P>0.05).

4.5. Discussion

Whilst the degree to which reproductive performance declines over successive spawning events may reflect species-specific variation in nutritional requirements, an adequate broodstock diet remains a critical factor in maintaining spawn quality in *Penaeid* shrimp (Wouters et al., 2001a, Chimsung, 2014). The sustained production of high quality spawns requires shrimp to continuously absorb and assimilate nutrients via the hepatopancreas, as well as efficiently transfer such nutrients to the developing ovary. However, endocrine manipulation resulting from unilateral eyestalk ablation forces females to mature and molt more frequently, causing broodstock to allocate greater energy reserves over a shorter periods (Racotta et al., 2003). Physiological exhaustion as a result of repeated spawning and the subsequent impact on reproductive traits such as gonadosomatic index, hatch rate, fecundity and offspring viability has been well documented in *Penaeids* (Lumare, 1979, Emmerson, 1980, Beard and Wickins, 1980, Browdy and Samocha, 1985, Bray et al., 1990, Marsden et al., 1997, Mendoza, 1997, Wouters et al., 1999, Arcos et al., 2005). Certainly, there is evidence to suggest that declines in reproductive performance as a result of physiological exhaustion can be mitigated through optimal diet (Marsden et al., 1997, Wouters et al., 1999). However, as the specific nutrient requirements with respect to repeated spawning remain unknown, at least for *P. monodon*, the formulation of optimal maturation diets remains difficult.

All broodstock maintained under the present study were fed a high-quality composite diet, which included both commercial broodstock pellets and fresh-frozen feeds. Despite this, broodstock displayed significant variation in hepatopancreas and ovary fatty acid composition between first and second spawns. For second order spawners (TB2), a significant higher ovarian total lipid was observed, however, no significant correlation between first order performance (RP1) and total lipid (TB2) was detected. With the exception of latency period and hepatopancreas total PUFA and n-3

	Relative Egg Production	Hatch Rate (%)	Relative Nauplii Production	Latency Period
HSI	-0.17	-0.27	-0.30	0.14
Protein (%)	0.18	0.34	0.30	-0.52
Total Lipid (%)	-0.13	-0.29	-0.24	0.43
Sum SFA	0.18	0.23	0.21	-0.38
14:0 '	-0.03	-0.20	-0.23	-0.39
15:0 '	-0.04	0.17	0.17	-0.07
16:0 '	-0.13	0.20	0.09	-0.39
17:0 '	0.19	0.07	0.08	0.34
18:0 '	**0.54**	-0.05	0.15	0.07
19:0 '	-0.07	-0.05	0.01	-0.07
20:0 '	-0.01	-0.21	-0.20	-0.15
Other ' [1]	0.41	0.26	0.45	0.26
Sum MUFA '	0.08	-0.38	-0.22	-0.35
16:1n-7 '	-0.17	-0.08	-0.10	-0.43
18:1n-9 '	-0.09	-0.44	-0.25	0.37
18:1n-7 '	-0.34	-0.15	-0.25	-0.50
20:1n-9 '	-0.21	0.01	-0.04	-0.52
Other ' [2]	-0.07	-0.54	-0.33	-0.44
Sum PUFA '	-0.09	-0.29	-0.27	**0.14**
18:2n-6 (LA) '	-0.13	-0.32	-0.30	-0.12
18:3n-6 '	-0.25	0.26	0.07	0.39
18:3n-3 '	0.33	0.16	0.17	-0.33
20:2n-6 '	0.02	0.12	0.15	0.22
20:3n-6 '	0.04	-0.16	-0.12	0.00
20:4n-6 (ARA) '	-0.01	-0.06	-0.09	-0.08
20:5n-3 (EPA) '	-0.09	-0.28	-0.28	**0.05**
22:6n-3 (DHA) '	-0.11	-0.28	-0.28	**0.05**
Other ' [3]	0.03	-0.20	-0.16	-0.12
Sum n-3 '	-0.07	-0.28	-0.28	**0.01**
Sum n-6 '	-0.16	-0.25	-0.27	-0.12
Sum n-9 '	0.10	-0.37	-0.17	-0.34
n-3/n-6	0.16	-0.22	-0.28	-0.21

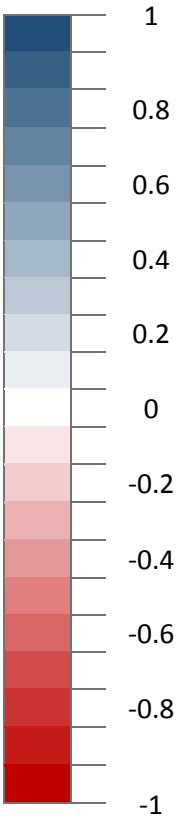


Figure 5. Heatmap showing correlations (R^2) between reproductive performance measures at first spawn (RP1) and hepatopancreas biochemistry at second spawn (TB2). Values greater than zero (i.e. blue) indicate positive correlation between traits, whilst values less than zero (i.e. red) indicate negative correlation. Where values appear in bold, significant correlations occur (P<0.05). Abbreviations within this table are defined as: HSI= hepatosomatic index; SFA= saturated fatty acid; MUFA= mono unsaturated fatty acid; PUFA= poly unsaturated fatty acid; LA= linoleic acid; ARA= arachidonic acid; EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid

' $mg/g^{-total\ lipid}$

[1] Sum C8:0, C10:0, C11:0, C12:0, C13:0, C22:0, C23:0, C24:0

[2] Sum C14:1n-5, C15:1, C17:1, C20:1n-7, C22:1n-9, C24:1n-9

[3] Sum C18:4n-3, C20:3n-3, C22:2, C22:4n-6, C22:5n-3

	Relative Egg Production	Hatch Rate (%)	Relative Nauplii Production	Latency Period
GSI	0.45	0.46	0.32	0.24
Protein (%)	-0.37	-0.37	-0.41	0.07
Total Lipid (%)	-0.02	0.04	-0.08	0.05
Sum SFA	-0.05	-0.23	-0.22	0.17
14:0 '	-0.04	**0.46**	0.46	0.20
15:0 '	0.17	0.05	-0.09	0.05
16:0 '	-0.25	-0.10	-0.18	0.12
17:0 '	-0.02	0.12	0.06	0.00
18:0 '	0.21	0.15	0.24	0.08
19:0 '	-0.24	-0.15	-0.09	0.22
20:0 '	-0.02	-0.27	-0.31	0.16
Other ' [1]	-0.02	0.31	0.16	0.37
Sum MUFA '	-0.55	-0.27	-0.40	0.15
16:1n-7 '	-0.17	-0.21	-0.28	0.05
18:1n-9 '	-0.06	0.28	0.17	0.21
18:1n-7 '	-0.11	-0.20	-0.22	0.13
20:1n-9 '	-0.39	-0.21	-0.19	0.39
Other ' [2]	-0.04	-0.25	-0.29	0.01
Sum PUFA '	-0.26	-0.13	-0.09	0.23
18:2n-6 (LA) '	-0.25	-0.28	-0.33	0.18
18:3n-6 '	0.00	0.00	0.00	0.00
18:3n-3 '	0.14	-0.07	0.05	-0.02
20:2n-6 '	-0.31	0.01	-0.14	0.26
20:3n-6 '	0.22	-0.11	-0.16	-0.14
20:4n-6 (ARA) '	0.40	-0.02	0.06	0.05
20:5n-3 (EPA) '	-0.09	0.25	0.21	0.32
22:6n-3 (DHA) '	-0.32	-0.06	-0.06	0.15
Other ' [3]	0.05	0.04	-0.04	-0.04
Sum n-3 '	-0.21	-0.02	-0.02	0.16
Sum n-6 '	0.03	-0.11	-0.13	-0.07
Sum n-9 '	-0.22	-0.14	-0.24	0.14
n-3:n-6	-0.05	-0.22	-0.10	-0.23

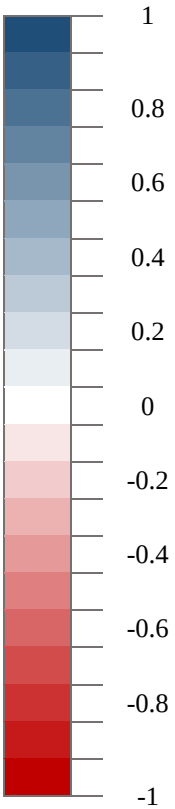


Figure 6. Heatmap showing correlations (R^2) between reproductive performance measures at first spawn (RP1) and ovary biochemistry at second spawn (TB2). Values greater than zero (i.e. blue) indicate positive correlation between traits, whilst values less than zero (i.e. red) indicate negative correlation. Where values appear in bold, significant correlations occur (P<0.05). Abbreviations within this table are defined as: GSI= gonadosomatic index; SFA= saturated fatty acid; MUFA= mono unsaturated fatty acid; PUFA= poly unsaturated fatty acid; LA= linoleic acid; ARA= arachidonic acid; EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid

' $mg/g^{-total\ lipid}$

[1] Sum C8:0, C10:0, C11:0, C12:0, C13:0, C22:0, C23:0, C24:0

[2] Sum C14:1n-5, C15:1, C17:1, C20:1n-7, C22:1n-9, C24:1n-9

[3] Sum C18:4n-3, C20:3n-3, C22:2, C22:4n-6, C22:5n-3

content, no significant correlations were detected in either hepatopancreas or ovary tissues for broad level fatty acid groups, i.e. total SFA, MUFA, PUFA, n-3, n-6 and n-9.. It appears that simply increasing total lipid content and/or availability in feeds is unlikely to improve reproductive performance in *P. monodon*, instead targeting specific formulations may have a greater impact.

In the present study, significantly less hepatopancreas SFA, PUFA, n-3 and n-6 fatty acids were present per gram^{-lipid} in second order spawners, largely reflecting a decrease in C15:0, C17:0, C18:2n-6, C20:3n-6, C20:4n-6(ARA), C20:5n-3 (EPA), C22:6n-3 (DHA) content within available lipid. Significantly less C18:1n-9 MUFA was also observed within the hepatopancreas tissues of second order spawners. The reduction of fatty acid content likely reflects the transfer of nutrients from the hepatopancreas to the developing ovary. In *P. monodon,* nutrient stores within hepatopancreas tissues decrease to meet the energetic needs for rapid development of ovary tissues (Marsden et al., 2007). However, disproportionately large reductions in fatty acid content within lipid, such as those noted for C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:4n-6(ARA), C20:5n-3 (EPA), C22:6n-3 (DHA), may indicate the preferential utilization of, or requirement for, such fatty acid classes during the maturation and spawning process. Significant reductions in fatty acid content suggest that where such fatty acids are being utilized, such demands are in excess of amounts supplied by the broodstock diet. Interestingly, no significant correlations were detected between hepatopancreas C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:4n-6(ARA), fatty acid content and reproduction performance measures. Still, where such fatty acids have essential roles in the maturation of ovarian tissue or the future development of nauplii, nutritional shortfalls within hepatopancreas tissues may have significant impact on the continued reproductive success of broodstock.

In addition to declines in hepatopancreas arachidonic acid (C20:4n-6(ARA)), ovarian tissues showed decreases in ARA content between first and second order spawners. Consistent declines in both tissues suggest that utilization, and therefore the requirement for ARA in *P. monodon*, is high in relation to multiple spawnings. The potential for limiting ARA content within both hepatopancreas and ovary tissues is of particular concern given the role of ARA in maturation and spawning. A study conducted by Coman et al. (2011) demonstrated a positive association between ARA availability and increased spawn frequency and egg production. In addition to content, the ratio of ARA:EPA has also been suggested to influence reproduction by modulating the production of prostaglandins (Coman et al., 2011). Prostanoids derived from ARA (i.e. series II prostaglandin) have significant roles in the onset of maturation, stimulation and sequestration of egg yolk and development of essential cellular machinery such as cortical rods in *P. monodon* (Wouters et al.,

2001a, Meunpol et al., 2010, Wimuttisuk et al., 2013). It has previously been reported that the ARA:EPA ratio in mature ovaries of wild *P. monodon* range from 0.3-0.6 (Crocos, 1997, Marsden et al., 1991, D'Souza and Kelly, 2000, Wouters et al., 2001a, Huang et al., 2008), whilst the diet used by Coman et al. (2011) contained a ARA:EPA ratio of 0.5 and resulted in a ratio of 0.57 in spawned eggs. In the present study, the ARA:EPA ratio within ovarian tissue declined from 0.55 to 0.41 between first and second spawn, suggesting that the production and utilization of ARA was limiting in this study, despite the use of current 'best practice' feeding regimes. Given that maturation rate and egg viability are common areas of concern for domesticated broodstock production, potential deficiencies in the ARA-prostaglandin synthesis cascade as a response to current feeding regimes require further investigation. Similarly, further investigation of ARA utilization in domesticated *P. monodon* is required with particular emphasis on interactions between prostaglandin synthesis and spawning.

Whilst ovarian ARA declined in second order spawners, this trend was not reflected in the remaining fatty acids. Comparatively higher MUFA, PUFA, n-3, n-9, C14:0, C16:0, C19:0, C16:1n-7, C18:1n-9, C18:1n-7, 20:1n-9, C18:3n-3, 20:2n-6, and 22:6n-3(DHA) content were all observed in second order spawners, relative to first order broodstock. The results of this experiment suggest that unlike hepatopancreas, at least for the initial two spawns, many ovarian fatty acids were not limiting. These results may explain, to a degree, why reproductive performance did not decline between first and second spawns for historic spawning data. However, we cannot exclude that analyses of historic spawning simply did not have sufficient power to detect declines in performance observed in previous works (see Hansford and Marsden, 1995, Makinouchi and Hirata, 1995, Primavera and Caballero, 1992). Similarly, it is possible the reproductive evaluation period (40-days, maximum of two spawning cycles) within the present study was simply too short to observe significant declines in ovarian FA content. Still, whether increasing accumulation of lipid within ovary tissues continues across subsequent spawning events (i.e. third, fourth and fifth order spawns) requires further investigation, particularly given the significant depletion of various hepatopancreas fatty acids between first and second spawn. Given that relatively few correlations were observed between ovarian biochemistry and reproductive performance traits, future work should consider assessing changes in biochemistry across all tissues associated with the translocation of nutrient to ovary tissues (i.e. hepatopancreas and haemolymph) across an extended number of spawns (i.e. third, fourth, fifth order spawns).

Improvement of reproductive performance for domesticated *P. monodon* broodstock requires the continued analysis of reliable repeat spawning data across variable timescales. In the current study,

broodstock demonstrated significant variation in aspects of hepatopancreas and ovarian tissues within the initial two spawns. Notably, whilst not limiting in ovary tissues, a number of fatty acids appear to be depleted rapidly within hepatopancreas tissues (i.e. C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:4n-6(ARA), C20:5n-3 (EPA), C22:6n-3 (DHA)), and therefore represent candidates for further investigation with respect to utilization in relation to spawning. The utilization and availability of hepatopancreas and ovarian ARA in particular requires further investigation in relation to spawning and prostaglandin synthesis. Notably, the current experiment failed to identify significant correlations between broad-level lipid and fatty acid components (i.e. total lipid, SFA, MUFA, PUFA, etc.), with the exception of latency period and hepatopancreas total PUFA and n-3 content. The results of this study suggest that lipid composition of broodstock diets has a greater influence on reproductive performance than total lipid content. Continued optimization of fatty acid content within broodstock maturation diets is critical for the improvement of broodstock reproductive performance, with particular emphasis on FAs likely to be limiting in hepatopancreas and ovary tissues.

Chapter 5: Regulating reproduction: RNA-seq analysis of variation in ovarian arachidonic acid levels in domesticated *Penaeus monodon*

5.1. Abstract:

Recent works have demonstrated that current high-performance feeding regimes are not sufficient to sustain the high arachidonic acid (ARA) requirements for spawning in *Penaeus monodon* broodstock. Deficiencies in ARA content may impact egg production and maturation rates directly, or indirectly by limiting the production of downstream ARA-derived molecules with significant control over reproduction, such as prostaglandin. The current study investigated the availability, production and regulation of ARA and prostaglandin within fourth generation domesticated *P. monodon* broodstock (n = 19) fed an identical high-performance maturation diet. Broodstock demonstrated considerable variation in ovarian ARA content from 1.4 – 5.0%. To further inform biological conclusions drawn from biochemical data analyses, a gene expression study was undertaken. For this, a reference *P. monodon* ovarian tissue transcriptome was assembled using 100 bp paired-end sequences generated on an Illumina HiSeq 2500. Differential gene expression analysis identified a total of 757 genes with greater than 2-fold expression change in response to variable ARA content. Of these genes, 677 could be annotated as unique homologs. However, a paucity of genomic resources for *P. monodon* and crustaceans generally, resulted in only 19 genes which could be assigned functional gene ontology (2.5% of all genes identified). An alternative approach was undertaken, where a subset of the 40 most differentially expressed genes between the LOW and HIGH groups were manually annotated through a search of gene keywords in the UniProt database to assess putative gene function. A specific study of prostaglandin synthesis genes from this dataset indicated a significant positive correlation between ovarian ARA content and cPLA2 and COX expression (P<0.05). Broodstock also demonstrated significant variation in COX, PGFS, PGE1 and PGE3 as a function of varying levels of ovarian ARA (P<0.05). This study is the first demonstration of population level variation in ovarian ARA content, despite broodstock being fed identical high-performance maturation diets. Differential gene expression analysis demonstrates that variation in ARA has direct impact on the synthesis of key downstream prostaglandin synthesis genes, which have potent roles in broodstock egg production and maturation and elucidates a suite of currently uncharacterized genes for *P. monodon*.

5.2. Introduction:

Continued improvement of reproductive performance in domesticated *Penaeus monodon* is essential to reducing industry reliance on wild-caught broodstock. Whilst incremental improvements have been via selective breeding (Coman et al., 2013), the fecundity of domesticated stocks is the key bottleneck to broader industry adoption. Recent analyses of domesticated *P. monodon* broodstock fed high-performance broodstock diets revealed significant reductions in hepatopancreas and ovarian arachidonic acid (ARA) content in response to repeat spawning (Goodall, J *unpublished data*, see chapter 4). Shortfall in the availability of ARA has the potential to impact reproductive performance either directly or through the disruption of downstream pathways. For many fish and crustacean species, ARA forms a significant fraction of the lipid present within reproductive tissues. Its presence has been linked to variation in maturation frequency, oocyte development, egg production and larval health (Millamena et al., 1993, Cahu et al., 1995b, Furuita et al., 2003, Mazorra et al., 2003, Meunpol et al., 2005, Huang et al., 2008, Coman et al., 2011, Ginjupalli et al., 2015, Ogata et al., 2004, Salze et al., 2005). Within most fresh-frozen maturation shrimp broodstock maturation diets, polychaete worms (e.g. *Marphysa sp., Perineries sp.*) form an essential component they contain relatively high levels of ARA when compared with other common fresh-frozen ingredients (i.e. squid, mussel)(Coman et al., 2011). Coincidently, polychaete worm-based diets have been demonstrated to have significant influence on spawning performance and larval production in shrimp (Naessens et al., 1997, Lytle et al., 1990, Browdy, 1992). In *P. monodon*, ARA-supplemented diets have been demonstrated to increase broodstock egg production, maturation and spawning frequency (Coman et al., 2011).

In addition to the direct utilization, ARA serves as the primary substrate for the synthesis of series II prostaglandin, bioactive lipid mediators with significant regulatory control over reproduction in invertebrates (Rowley et al., 2005, Stanley, 2006, Stanley and Howard, 1998). The prostanoid pathway is characterized by the release of cellular/ intracellular membrane bound ARA by phospholipase A2 (cPLA2), which is subsequently cyclized and reduced to prostaglandin H2 (PGH2) by the cyclooxygenase (COX) (see Figure 7) (Stanley, 2006, Funk, 2001). Class specific synthases then convert PGH2 to various prostaglandins, such as prostaglandin E2 (PGE2), prostaglandin D2 (PGD2) and prostaglandin F2α (PGF2α) (Wimuttisuk et al., 2013). In crustaceans the presence or absence of prostaglandin class varies. PGE2, PGD2 and PGF2α have been detected in the fresh water crab, *Oziotelphusa senex senex* (Sreenivasula Reddy et al., 2004), PGE2 and

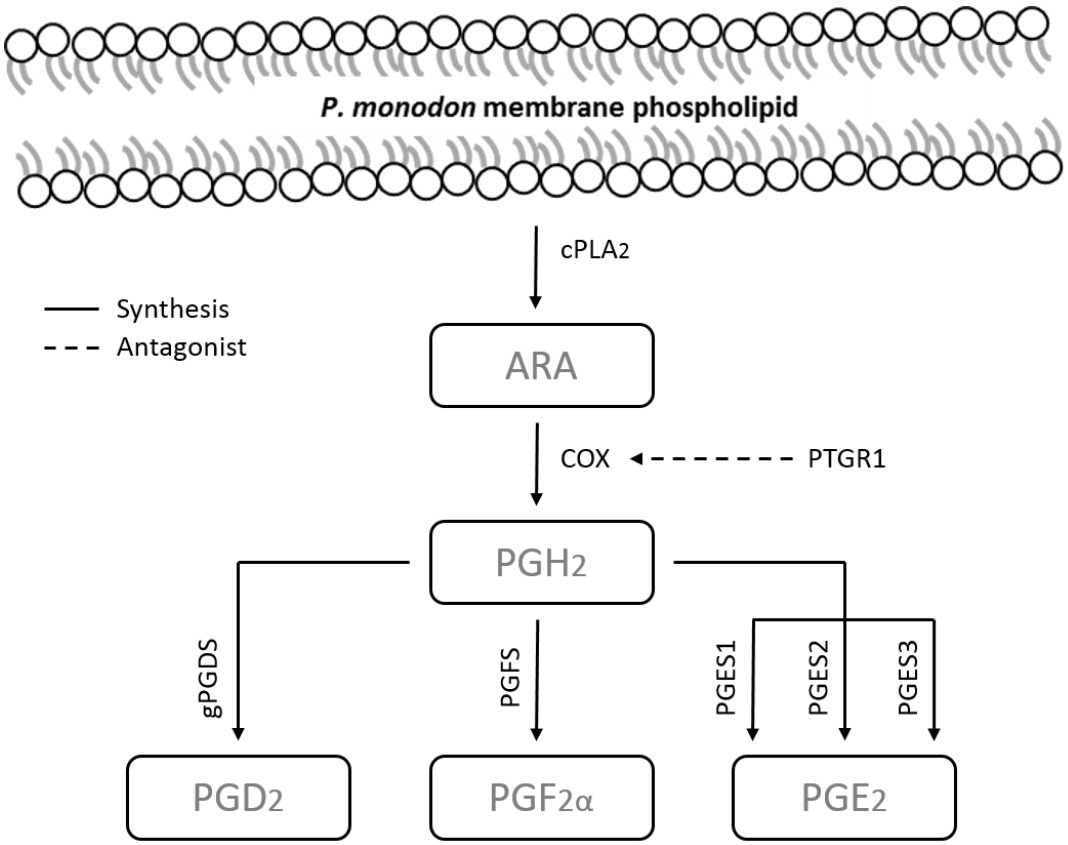


Figure 7. Predicted *Penaeus monodon* prostanoid biosynthesis pathway. Modified from Wimuttisuk et al. (2013)

PGF2α in the Florida crayfish *Procambarus paeninsulanus* (Spaziani et al., 1995, Spaziani et al., 1993), Kuruma shrimp *Marsupenaeus japonicus* (Tahara and Yano, 2004), and *P. monodon* (Wimuttisuk et al., 2013), whilst only PGE2 has been reported in the shore crab *Carcinus maenas* (Hampson et al., 1992) and acorn barnacle *Balanus Amphitrite* (Knight et al., 2000). In *P. monodon*, PGF_2 acts as a regulator of maturation (Wimuttisuk et al., 2013), whilst PGE2 promotes the development of yolk globules and cortical rods in *P. monodon* oocytes during early, and late stage development respectively (Meunpol et al., 2010).

Given the significant role of ARA and prostaglandin in *P. monodon* reproduction, the impact of variable ARA availability as a result of current feeding strategies forms the basis of this investigation. Among a larger set of *P. monodon* broodstock samples (Goodall, J. *unpublished data*, see chapter 4) that showed variable ovarian ARA content, the current study aimed to investigate the impact of ovarian ARA content on the regulation of key *P. monodon* prostaglandin biosynthesis genes using RNA-seq. In addition, the global regulatory gene expression response was compared between ovary tissues of different individuals that contained high or low levels of ARA.

5.3. Materials and Methods:

5.3.1 Broodstock and Sampling

All broodstock used in the current study (n=19) were derived from a fourth generation domesticated population maintained at Gold Coast Marine Aquaculture (GCMA; Woongoolba, Queensland, Australia). Juvenile broodstock were reared within earthen grow-out ponds as per GCMA's broodstock management protocols (3,000 m^2). A total of 40 female broodstock (7 months of age) were transferred from GCMA to CSIRO's Bribie Island Research Centre (BIRC; Woorim, Queensland, Australia). At BIRC, broodstock were maintained in two 10,000 L circular maturation tanks at 40 shrimp per tank (20 male: 20 females, 4 shrimp/ m^2). Tanks were fitted with flow through seawater systems at 4 L min^{-1} and 57% exchange per day to maintain seawater at an average salinity of 35 ± 1 ppt) and 27°C. Photoperiod was maintained at 14 h light: 10 h dark (Goodall et al., 2016). Broodstock were fed a typical broodstock maturation diet consisting of fresh-frozen feed (squid, bloodworm, mussel, ox liver, *Artemia* biomass) supplemented with a commercial pelleted maturation diet (Ridley Aqua Feed™: MR Broodmax) (Goodall, J. *unpublished data*, see chapter 4*)*. Feeds were provided separately, *ad libitum*, five times daily including a single ration of the pelleted diet.

Broodstock were conditioned for seven weeks, before being unilaterally eyestalk ablated four days post-molt (Goodall et al., 2016). Following ablation, broodstock were monitored daily for ovarian maturation (by shining a torch through the dorsal carapace). Broodstock were allowed to spawn once before completing one additional maturation cycle. When stage IV mature ovaries were detected (Tanfermin and Pudadera, 1989) broodstock were removed from maturation tanks, anaesthetized via ice-water immersion and ovary tissues sampled via dissection. Dissected ovary tissues were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

5.3.2 Biochemical Analysis of Ovarian Arachidonic Acid Content

Prior to analysis, all ovarian samples were minced frozen using a blender. The frozen mince was freeze-dried to completion in a laboratory freeze-dryer (Alpha 1-4, Martin Christ, Germany), re-homogenized in a blender and used for subsequent analyses. Total lipid was extracted following Folch (1957), esterified by an acid-catalyzed methylation and 0.3 mg of an internal standard added to each sample (21:0 Supelco, PA, USA). Arachidonic acid contents were identified relative to the internal standard following separation by gas chromatography using an Agilent Technologies 6890N GC system (Agilent Technologies, California, USA) fitted with a DB-23 (60m x 0.25mm x

0.15 µm, cat 122-2361 Agilent Technologies, California) capillary column and flame ionisation detector (Christie, 2003a).

To reduce the impact of potential biological variation and aid in further downstream transcriptome assembly and analyses, all samples were plotted based on the percentage ovarian ARA content, and mean percentage of ARA content with 95% confidence intervals. The four samples with the lowest and highest percentage of ovarian ARA content were termed 'LOW' and 'HIGH' groups respectively (total n = 8). In addition, the sample with the median percentage of ovarian ARA content for all 19 was termed 'MEDIAN'.

5.3.3 Quantification of Ovarian Prostaglandins

Ovary prostaglandin extraction and purification was conducted using a modified ELISA assay protocol as per the manufacturer's instructions (see Sapphire Bioscience, Kookaburra Prostaglandin E2 Enzyme Immunoassay Kit, Cat# 133-16359). Optimization of extraction method was conducted using a 10-fold increasing input series (i.e. 0.5 mg, 5.0 mg, 50 mg freeze dried tissue used in original extraction). Prior to extraction all samples were spiked with deuterated prostaglandin E2 standard (PGE2-d4, Caymann Chemical, item# 14010) (volume equal to 1µg/mL of final volume). Samples were acidified with 1.0 M HCl to a pH < 4.0. Following sample acidification, a C-18 SPE cartridge was conditioned by passing 5 mL of methanol, followed by 5 mL of Millipore water through the column. The sample was then washed through to the conditioned column, followed by 5 mL Millipore water and 5 mL of hexane. The column was allowed to dry. Eluted prostaglandin were then washed from the column by passing 5 mL of ethyl acetate containing 1% methanol through the column into a collection reservoir. Prostaglandin extracts were dried to completion under a stream of nitrogen.

Analysis of prostaglandin extracts was conducted using methods adapted from Prasain et al. (2013). All analyses were conducted using a Shimadzu LC-MS system consisting of a Nexera X2 LC and an 8030 LC-MS using electrospray ionization in negative ion mode. Prior to analysis, both dried extracts and a mixed standard containing PGE2-d4, prostaglandin E2 (PGE2, Caymann Chemical, item# 16010) and prostaglandin F2α (PGF2α, Caymann Chemical, item# 314010) (1 µg/mL) standards were eluted in 2 mL of methanol:water (8:2) and mixed thoroughly. Sample solutions and standards were stored within the 1.5 mL vial rack prior to analysis, which was maintained at 4°C. The mobile phase consisted of two solutions: 0.1%formic acid in Millipore water (A) and acetonitrile containing 0.1% formic acid (B).

Samples were injected (25 µL) into a Synergy hydor RP-C18 column under a gradient elution, starting with 12% B, increasing to 85% B from 0 to11 minutes, 85-100% B from 11 to 14 minutes, and returning to 12% B at 16 minutes (total run time 20 min). Detected peaks for the standards and biological samples were quantified based on retention time (RT) and MRM transitions ([M-H]-m/z) (PGE2-d4: RT=6.75 mins, [M-H]-m/z=355>275; PGE2: RT=7.05 mins, [M-H]-m/z=351>271/289; PGF2α RT=6.4 mins, [M-H]-m/z=353>309/193) (Prasain et al., 2013). Nitrogen was used as a nebulizer and drying gas. The collision energy was optimised for each compound and the collision gas pressure set at 230 kpa, with a heat block and desolvation line temperatures of 400°C and 250°C respectively. Analytical data was processed using LabSolutions LC-MS version 5.82.

5.3.4 De Novo Transcriptome Assembly

RNA was extracted from all 19 individuals using the Qiagen™ RNeasy® Plus mini kit, as per manufacturer's instructions (Qiagen™, Australia). To ensure high quality RNA was obtained prior to sequencing, RNA quantity and quality was assessed using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Australia) and Agilent 2100 bioanalyzer (Agilent Technologies, Australia). The Australian Genome Research Facility (AGRF, Melbourne, Australia) completed 100 base pair library preparation and paired end sequencing on an Illumina HiSeq 2500. Raw reads were processed following the Oyster River Protocol (MacManes, 2016. Available from http://oyster-river-protocol.readthedocs.io/en/latest/). Data was subjected to quality control by checking raw reads for Illumina sequencing adaptors, trimming low quality reads (<30 Phred) with Trimmomatic (version 0.32, Bolger et al., 2014), error correction using Rcorrector (Song and Florea, 2015) and completing quality assessment with FastQC (Andrews, 2010. Available from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Three samples were used to construct the reference transcriptome (assembly samples denoted with AS in Figure 8A). The first two represented those samples with the highest total sequence read count from the LOW and HIGH groups respectively. The third sample, MEDIAN, was included to ensure that all transcripts across the spectrum of low to high percent ovarian ARA content were represented. The preliminary transcriptome was *de novo* assembled using Trinity (version 2.0.6, Haas et al., 2013). The raw reads used to construct the preliminary assembly were mapped back against the transcriptome using Transrate (version 1.0.3, Smith-Unna et al., 2015) to filter and remove poorly supported transcript artefacts which are commonly retained by Trinity. The resultant transcriptome and the preliminary assembly were assessed for structural integrity and genic completeness using BUSCO (version 1.1b1, Simão et al., 2015) and Transrate respectively.

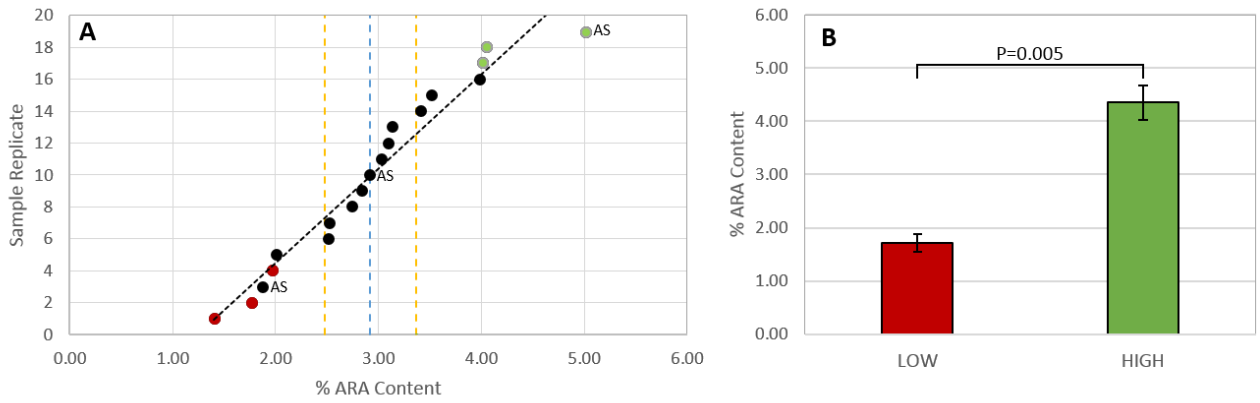


Figure 8. Total percentage ovarian arachidonic (ARA) acid content for *P. monodon* broodstock fed an identical maturation diet. All individuals were ranked by ARA content and designated a sample replicate number between 1 and 19. Data is displayed as: A) total percentage ARA content for all nineteen individuals surveyed and; B) Individuals designated as members of LOW (red), HIGH (green) or MEDIAN (sample replicate 10) groups respectively. The average percentage ARA content for all surveyed individuals is represented by a broken blue line, whilst samples within the broken yellow lines fall within the 95% confidence interval. Replicates used in assembly construction are denoted with an AS.

5.3.5 Differential Gene Expression and Functional Analyses

Differential gene expression analysis was undertaken by indexing trimmed and error-corrected reads against the transcriptome using Bowtie (version 2.2.4, Langmead and Salzberg, 2012, Langmead et al., 2009). Read counts were quantified using Corset (version 1.04, Davidson and Oshlack, 2014). The Corset output was assessed for potential batch effects using Harman (Oytam, Y., 2014. Harman. Available online at http://bioinformatics.csiro.au/harman) and poorly clustering samples removed, reducing biological replicates for each of the LOW and HIGH groups to three (previously four as determined by biochemical analyses above). Differential gene expression (DGE) statistical analysis was undertaken using EdgeR (version 3.3, Robinson et al., 2010).

To determine putative identity, transcripts with greater than 2-fold DGE between LOW and HIGH groups were subject to tblastx against the NCBI nucleotide database (BLAST 1.0, accessed July 2016) and two crustacean species, *Daphnia pulex* (accessed July 2016, Colbourne et al., 2011) and *Parhyale hawaiensis* (accessed July 2016, Zeng et al., 2011). Sequence identity was not available for the crustacean transcriptome datasets therefore these datasets were also subject to tblastx to generate gene identities. The resultant two-step tblastx analysis dataset was manually curated for sensible gene annotation. However, a paucity in functional gene ontology (GO) of crustacean species was generally prohibitive in using conventional Blast2GO methods.

To overcome this, gene ontology was derived manually following two methods. The first method aimed to derive GO terms for all DGE by extracting the sequences of homologous genes from the NCBI database and tblastx followed by the gene identity using the gene2accession.gz database (source ftp.ncbi.nlm.nih.gov/gene/DATA/). Where possible, the GO terms where then extracted from the gene2go.gz database (source ftp.ncbi.nlm.nih.gov/gene/DATA/). The second method used the EdgeR DGE fold change data to identify 20 transcripts for each group (LOW and HIGH) with the greatest positive and negative (total $n = 40$) fold change. For each of these sequences gene identity keywords were searched against the UniProt database (accessed September 2016, available from http://www.uniprot.org/) to inform the functional annotation of the manually extracted homologous genes.

5.3.6 Prostaglandin Gene Expression Pathway Analysis

A second analysis was undertaken to specifically investigate expression levels of known genes associated with the series II prostanoid synthesis pathway (see Figure 7). The transcriptome assembly was imported into CLC Genomics Workbench (v 9.0.1) and converted to a BLAST database. The homologous putative *P. monodon* prostaglandin pathway and synthesis genes were identified by tblastx analysis of the transcriptome against the NCBI nucleotide database including phospholipase A2 (*PmcPLA2*), cyclooxygenase (*PmCOX*), glutathione-dependent prostaglandin D synthase (*PmgPGDS*), prostaglandin F synthase (*PmPGFS*), prostaglandin E synthase 1 (*PmPGES1*), prostaglandin E synthase 2 (*PmPGES2*), prostaglandin E synthase 3 (*PmPGES3*) and prostaglandin reductase 1 (*PmPTGR1*) (Supplementary Table 1).

To assess gene expression of these prostaglandin genes, the total read counts for the *PmcPLA2*, *PmCOX*, *PmgPGDS*, *PmPGFS*, *PmPGES1*, *PmPGES2*, *PmPGES3*, *PmPTGR1* homologs were extracted from the corset output and analyzed in two ways: 1) total reads for prostanoid genes were correlated with total ARA content on an individual basis using Spearmann's rank correlation coefficient (Sokal and Rohlf, 1981) and; 2) differential gene expression for prostanoid genes between LOW and HIGH groups ($n = 3$) were compared using EdgeR.

5.4. Results:

5.4.1 Quantification of Ovarian Arachidonic Acid Content

Percentage ovarian ARA content was assessed for nineteen mature (ovary stage IV) broodstock fed identical diets. Broodstock ranked by percentage ARA content are displayed in Figure 8A. Mean

percentage ARA content within the surveyed cohort was 2.92 (SE ± 0.21), whilst the outer limits of the 95% confidence interval ranged from 3.37 to 2.48. A total of five and six samples respectively had an ovarian ARA content which fell outside the lower and upper 5% confidence intervals respectively, suggesting variation in percentage ARA content was high within the sampled population. Individual broodstock were designated either LOW or HIGH based on the significant difference in ovarian ARA content ($P < 0.005$, Figure 8B). This disparity in ARA content for groups LOW and HIGH was investigated further using comparative RNA-seq gene expression analyses.

5.4.2 Quantification of Ovarian Prostaglandin Content

PGE2 and PGF2α levels were quantified from LOW and HIGH ARA groups against a mixed standard using LC-MS. For the mixed standard, detection peaks and retention times for prostaglandin standards PGE2-d4, PGE2 and PGF2α fell within expected ranges, and therefore could be accurately quantified (Supplementary Figure 3). For tissue samples, PGE2-d4 retention times fell within expected ranges, however, the observed peaks were on average four to 22 times less than those detected in the mixed standard (despite equal concentrations of 1µg/mL). This suggests a considerable reduction in potential prostaglandin yield most likely due to reduced extraction efficiency. In addition, PGE2 and PGF2α detection peaks could not be identified in tissue samples and therefore could not be accurately quantified. A typical tissue sample chromatographs derived from 0.5 mg of tissue is presented in Supplementary Figure 4, and chromatographs of 5.0 mg, 50.0 mg tissue input showed a similar profile (data not shown). Given prostaglandin could not be accurately detected in tissues samples further attempts to quantify PGE2 and PGF2α via LC-MS were discontinued.

5.4.3 De Novo Transcriptome Assembly

Illumina HiSeq 2500 sequencing produced a total of 98,292,911 paired end reads from nine *P. monodon* ovary samples. Structural completeness and accuracy were assessed for both preliminary and final transcriptome assemblies using Transrate and BUSCO respectively (Supplementary Table 3). Both assemblies were of high quality as evidenced by Transrate (preliminary= 0.39; final =0.61) and BUSCO (preliminary= 91% complete BUSCOs; final =90% complete BUSCOs) scores. Notably remapping of raw reads improved Transrate score by approximately 1.5× (a score of 0.61 is three times greater than the minimum assembly score of 0.2), with only marginal reductions in complete BUSCOs. Data demonstrated that an accurate and

complete ovarian transcriptome had been assembled, and thus the final assembly was used for all further analyses that compared the expression levels between groups of varying ARA content.

5.4.4 Differential Gene Expression and Functional Analyses

LOW and HIGH samples were mapped against the transcriptome assembly to quantify read counts and clustered against the first and second principle component using Harmann. One sample was removed from further analysis for each group (LOW and HIGH) due to poor clustering of samples. DGE analysis identified a total of 1,805 genes with significantly different expression between LOW and HIGH groups (Table 13). Transcripts with less than 2-fold expression change were removed from analyses. The remaining 757 transcripts were queried against the NCBI database using tblastx. A total of 754 transcripts were assigned a blast match, of which 677 hits were unique and 77 represented isoforms.

Given that automated methods of assigning gene ontology (i.e. Blast2GO) could not be undertaken for this study, all gene ontology assignments were conducted manually. For all transcripts with >2-fold expression change, gene identity and accession number were extracted from tblastx results. This resulted in identification of 677 unique transcripts with a total of 440 transcripts matching known homologs within the NCBI gene2accession.gz database enabling annotation. A total of 1,108 associated GO terms were extracted from the NCBI gene2go.qz database resulting in functional GO annotation of 19 unique transcripts (2.5% of all entries). An alternate functional analysis identified the 20 (n = 40) most positively (Table 14) and negatively (Table 15) expressed transcripts, between LOW and HIGH groups based on log-fold change. For all 40 transcripts, GO terms were extracted from homologues genes contained within the UniProt database based on the

Table 13. Summary of differential gene expression analyses and manual extraction of gene ontology (GO) terms from National Centre Biotechnology Information databases between arachidonic acid groups LOW and HIGH.

Parameter	n
Significant expression change between LOW and HIGH	1805
> 2-fold expression change	757
Assigned a Blast match	754
Assigned a unique accession number	677
Isoforms assigned non-unique accession number	77
Assigned a GeneID based on accession and BlastID	440
Assigned GO terms based on GeneID	1108
Unique sequences with associate GO terms	19

Table 14. List of differential expressed genes with the greatest positive LogFC between arachidonic acid groups LOW and HIGH, and their corresponding homologs based on tblastx results against the National Centre Biotechnology Information database. Positive LogFC indicated genes are upregulated in group HIGH relative to group LOW. For all genes differential expression were significantly different between LOW and HIGH (P<0.05) based on EdgeR. PRED=Predicted.

P. monodon Query	Accession	Description	LogFC
TR8839-c0_g1_i1	XM_013314116	PRED: Papilio xuthus 28S ribosomal protein S23, mitochondrial (LOC106119202), mRNA	5.96
TR25321-c0_g1_i1	XM_008199456	PRED: Tribolium castaneum kinase D-interacting substrate of 220 kDa (LOC100141654), transcript variant X12, mRNA	5.79
TR30211-c0_g1_i1	XM_012881566	PRED: Fundulus heteroclitus tumor necrosis factor receptor superfamily member 16-like (LOC105939421), mRNA	5.78
TR1196-c0_g1_i1	U66319	Homarus americanus beta-II tubulin mRNA, complete cds	5.52
TR25386-c0_g1_i1	XM_016862475	PRED: Gossypium hirsutum proline-rich receptor-like protein kinase PERK2 (LOC107930757), mRNA	5.45
TR12891-c0_g1_i1	XM_014752225	PRED: Polistes canadensis longitudinals lacking protein, isoforms A/B/D/L-like (LOC106788730), transcript variant X2, mRNA	5.43
TR20169-c0_g1_i1	XM_009909863	PRED: Picoides pubescens zinc finger, CCHC domain containing 7 (ZCCHC7), partial mRNA	5.35
TR21764-c0_g1_i1	KM280384	Litopenaeus vannamei vascular endothelial growth factor receptor precursor (VEGFR) mRNA, complete cds	5.32
TR11481-c0_g1_i1	XM_013921346	PRED: Limulus polyphemus protein Smaug homolog 1-like (LOC106461518), transcript variant X3, mRNA	5.32
TR20787-c0_g1_i1	XM_009970265	PRED: Tyto alba collagen, type IV, alpha 5 (COL4A5), partial mRNA	5.24
TR29794-c0_g1_i1	XM_017001213	PRED: Homo sapiens potassium voltage-gated channel subfamily A member 2 (KCNA2), transcript variant X6, mRNA	5.24
TR15314-c0_g1_i1	XM_013536924	PRED: Lingula anatina U6 snRNA-associated Sm-like protein LSm5 (LOC106160346), transcript variant X2, mRNA	5.01
TR8397-c0_g1_i1	XM_014443496	PRED: Microplitis demolitor nipped-B-like protein A (LOC103573670), mRNA	5.01
TR11699-c1_g1_i1	XM_013929102	PRED: Limulus polyphemus histamine H2 receptor-like (LOC106468667), mRNA	5.24
TR3671-c0_g1_i1	XM_012398145	PRED: Athalia rosae ribosome biogenesis protein TSR3 homolog (LOC105684564), transcript variant X2, mRNA	5.23
TR18791-c1_g1_i1	XM_013289875	PRED: Papilio polytes N-acetylglucosaminyl-phosphatidylinositol de-N-acetylase (LOC106108619), mRNA	5.21
TR6181-c0_g1_i1	XM_011499038	PRED: Ceratosolen solmsi marchali uridine phosphorylase 1-like (LOC105361772), transcript variant X2, mRNA	5.13
TR24207-c0_g1_i1	XM_001348671	Plasmodium falciparum 3D7 transcription factor with AP2 domain(s), putative (ApiAP2) mRNA, complete cds	5.13
TR3775-c0_g1_i1	XM_003729901	PRED: Strongylocentrotus purpuratus insulin-like growth factor-binding protein 2 (LOC100892499), mRNA	5.12
TR29562-c0_g1_i1	AY965681	Eptatretus stoutii nonfunctional variable lymphocyte receptor B (VLRB) gene, complete sequence	5.04

Table 15. List of differentially expressed genes with the greatest negative LogFC between arachidonic acid groups LOW and HIGH, and their corresponding homologs based on tblastx results against the National Centre Biotechnology Information database. Negative LogFC indicated genes are downregulated in group HIGH relative to group LOW. For all genes differential expression were significantly different between LOW and HIGH (P<0.05) based on EdgeR. PRED=Predicted.

P. monodon Query	Accession	Description	LogFC
TR19100-c0_g1_i1	XM_011669812	PRED: Strongylocentrotus purpuratus ras-related protein Rab-6A (LOC586726), transcript variant X2, mRNA	-2.97
TR8190-c0_g3_i1	XM_013938698	PRED: Limulus polyphemus actin-binding protein anillin-like (LOC106478176), mRNA	-3.01
TR607-c0_g1_i1	XM_014406041	PRED: Cimex lectularius protein Star-like (LOC106673783), mRNA	-3.02
TR11878-c0_g1_i1	XM_012406958	PRED: Athalia rosae protein spinster (LOC105689720), transcript variant X1, mRNA	-3.10
TR13636-c0_g1_i1	FN298876	Blattella germanica mRNA for Dicer-1 (dcr1 gene)	-3.13
TR23229-c0_g1_i1	XM_013924824	PRED: Limulus polyphemus zinc finger protein 423-like (LOC106464665), mRNA	-3.16
TR1331-c0_g1_i1	DQ455050	Penaeus monodon prophenoloxidase activating factor (PPAF) mRNA, complete cds	-3.25
TR29302-c1_g1_i1	XM_016062965	PRED: Parasteatoda tepidariorum DNA replication complex GINS protein PSF3-like (LOC107447926), transcript variant X3, mRNA	-3.26
TR18858-c1_g1_i1	XM_012424614	PRED: Orussus abietinus mucin-1 (LOC105699533), mRNA	-3.27
TR29674-c0_g1_i1	XM_006756458	PRED: Myotis davidii programmed cell death 11 (PDCD11), transcript variant X3, mRNA	-3.42
TR7908-c0_g1_i1	XM_014432904	PRED: Halyomorpha halys collagen alpha-1(I) chain-like (LOC106688446), transcript variant X2, mRNA	-3.52
TR500-c0_g1_i1	XM_006024794	PRED: Alligator sinensis NUAK family, SNF1-like kinase, 2 (NUAK2), mRNA	-4.65
TR9023-c0_g1_i1	XM_011696734	PRED: Wasmannia auropunctata ATP-dependent RNA helicase DDX24 (LOC105454235), mRNA	-4.96
TR30138-c0_g1_i1	XM_012375094	PRED: Linepithema humile serine hydroxymethyltransferase, cytosolic (LOC105676883), transcript variant X1, mRNA	-5.11
TR4692-c0_g1_i1	DQ667142	Callinectes sapidus hemolectin mRNA, partial cds	-5.36
TR28007-c0_g1_i1	XM_015256557	PRED: Diachasma alloeum zinc finger protein 608 (LOC107037804), mRNA	-5.42
TR11167-c1_g1_i1	XM_008552877	PRED: Microplitis demolitor retinoblastoma-binding protein 5 homolog (LOC103573691), mRNA	-5.63
TR15716-c0_g2_i8	DQ205425	Fenneropenaeus chinensis putative thrombospondin mRNA, complete cds	-5.93
TR11443-c0_g1_i1	XM_015660048	PRED: Neodiprion lecontei regulator of telomere elongation helicase 1 homolog (LOC107221150), mRNA	-6.70
TR8169-c0_g1_i1	XM_013275923	PRED: Oreochromis niloticus piggyBac transposable element-derived protein 3-like (LOC102079904), mRNA	-7.60

gene identity inferred by tblastx. With respect to cellular component ontology, nucleus, membrane and cytoplasm associated genes were the most highly represented for both LOW and HIGH groups (Figure 9). However with respect to molecular function, upregulation of protein, poly(A) RNA, RNA, GTP binding and potassium channelling associated genes were observed in the LOW group relative to the HIGH group (Figure 10A). Conversely, upregulation of ATP, metal ion, DNA, actin binding and hydrolaze activity associated genes was observed for the HIGH group, relative to the LOW group (Figure 10B). Biological process ontology highlighted upregulation in regulators of transcription (DNA templated), potassium ion transmembrane transport, mitotic sister chromatid cohesion, axon midline recognition and guidance associated genes relative to the LOW group (Figure 11A). In contrast, upregulation of ventricular system deployment, mitotic cytokinesis, DNA repair and cellular senescence associated genes were observed in the LOW group relative to the HIGH group (Figure 11B).

5.4.5 Analysis of Prostaglandin Pathway Genes

A searchable nucleotide database derived from the transcriptome assembly was generated. Eight putative *Penaeus monodon* genes: *PmcPLA2*, *PmCOX*, *PmgPGDS*, *PmPGFS*, *PmPGES1*, *PmPGES2*, *PmPGES3*, and *PmPTGR1* were queried against the nucleotide database using tblastx and homologs were derived from the transcriptome assembly (Supplementary Table 1). Read counts for predicted *PmcPLA2*, *PmCOX*, *PmgPGDS*, *PmPGFS*, *PmPGES1*, *PmPGES2*, *PmPGES3*, and *PmPTGR1* transcripts were extracted from the Corset output and either: analyzed for correlation with ARA content across all 19 samples or; compared between groups LOW and HIGH only. Significant positive correlations were identified across all 19 samples between *PmcPLA2* (R^2 = 0.540, p = 0.013) and *PmCOX* (R^2 = 0.478, p = 0.012) transcript expression and percentage ovarian ARA content (Figure 12). No significant correlations between *PmgPGDS*, *PmPGFS*, *PmPGES1*, *PmPGES2*, *PmPGES3*, *PmPTGR1* gene expression and percentage ovarian ARA content were identified (P>0.05) (Figure 12 and Figure 13).

Total read counts for *PmgPGDS* did not meet the minimum requirements for the transcript to be retained during Corset analysis (>10 total reads). Therefore *PmgPGDS* was excluded from the final analysis. Total read count was significantly higher on average within the HIGH group for *PmCOX*, *PmPGFS* and *PmPGE1* genes, when compared to the LOW group (P<0.05) (Figure 12 and Figure 13). For the LOW group, total read count for *PmPGES3* was significantly higher on average when compared with the HIGH group (P<0.05) (Figure 13). No significant difference in total read count

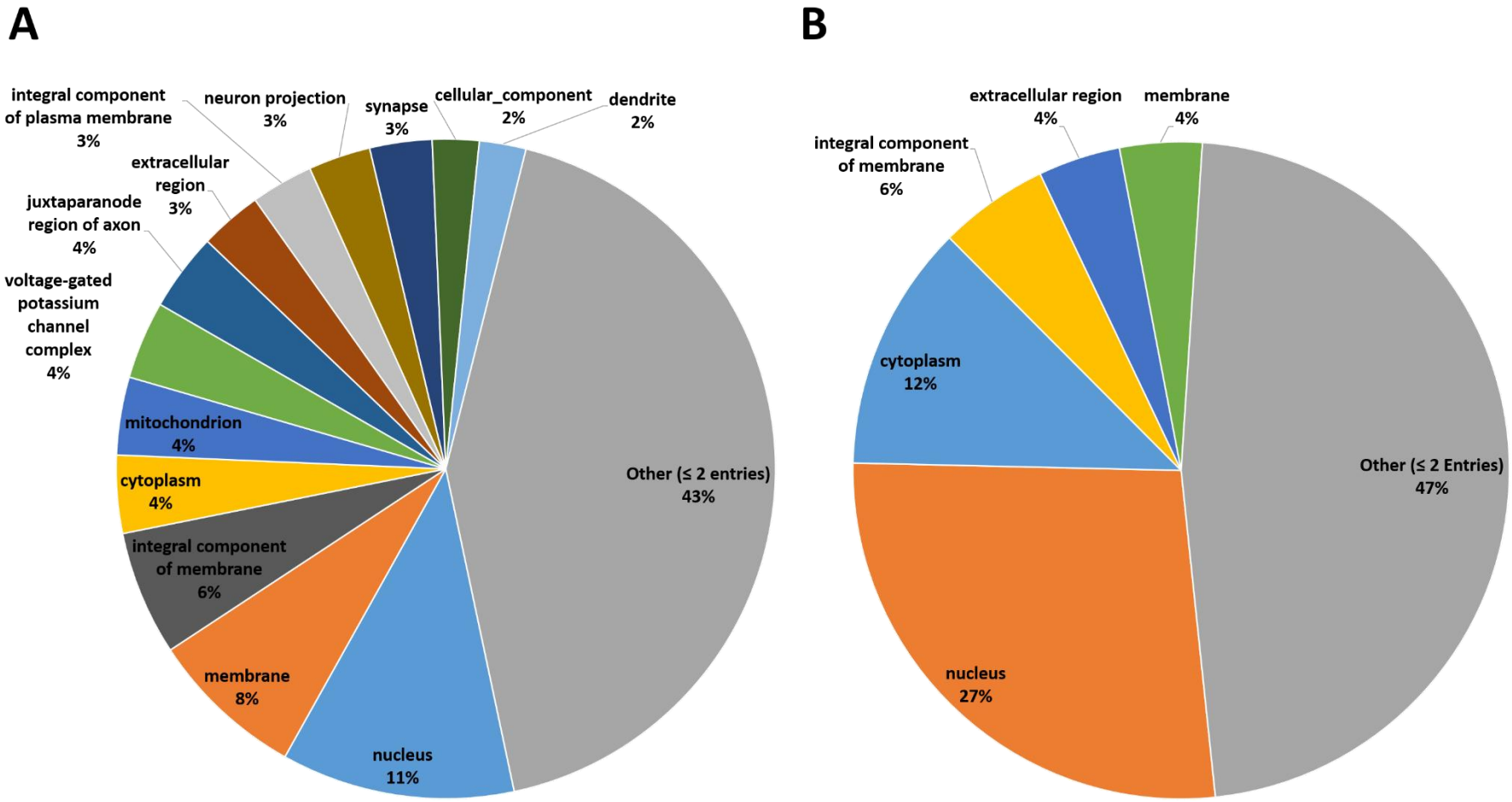


Figure 9. Summary of cellular component ontology for sequences with the highest positive (A) and negative (B) log-fold change, derived from UniProt database. Terms which appeared ≤ 2 times were grouped as "Other".

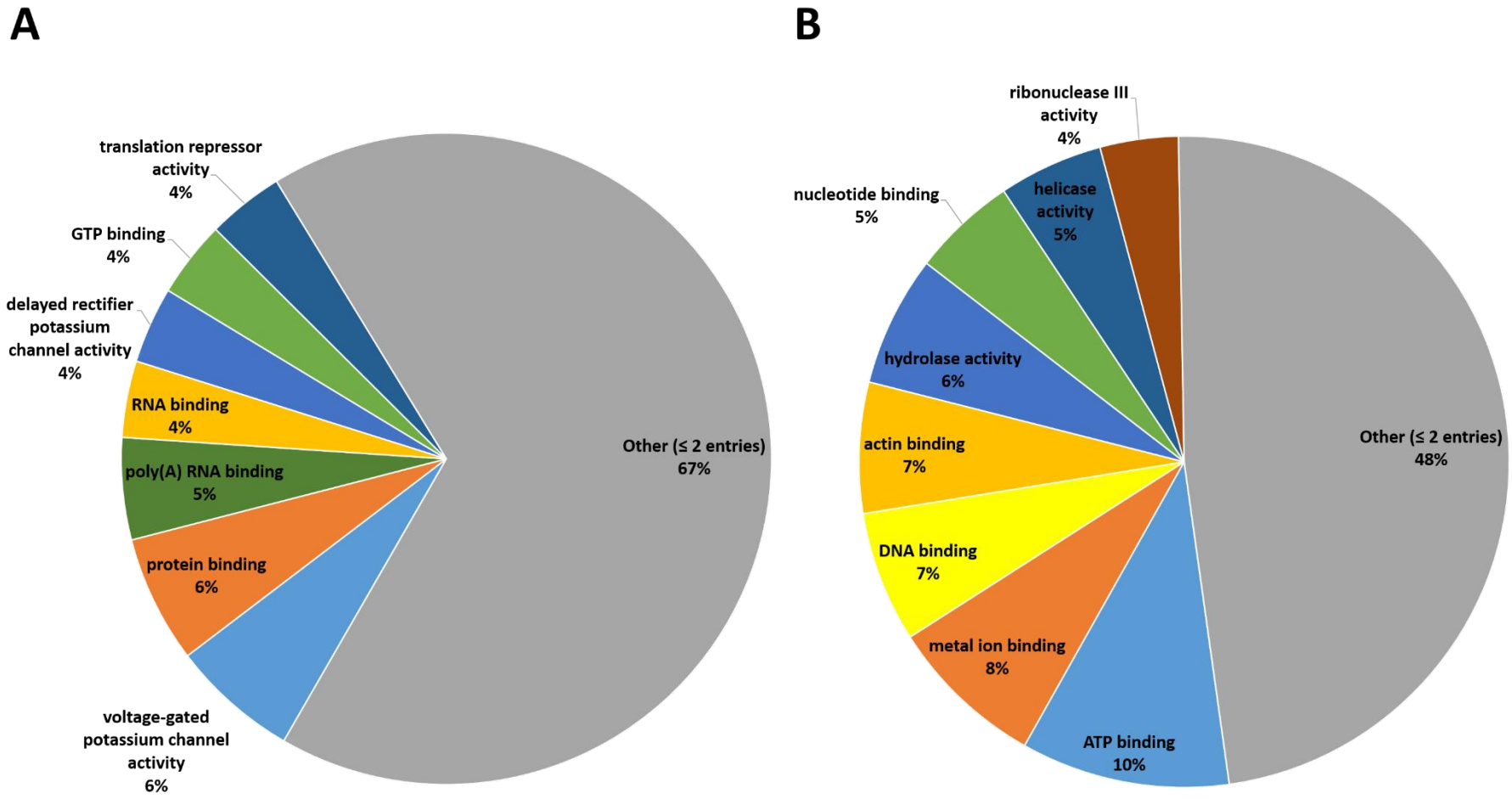


Figure 10. Summary of molecular function ontology for sequences with the highest positive (A) and negative (B) log-fold change, derived from UniProt database. Terms which appeared ≤ 2 times were grouped as "Other".

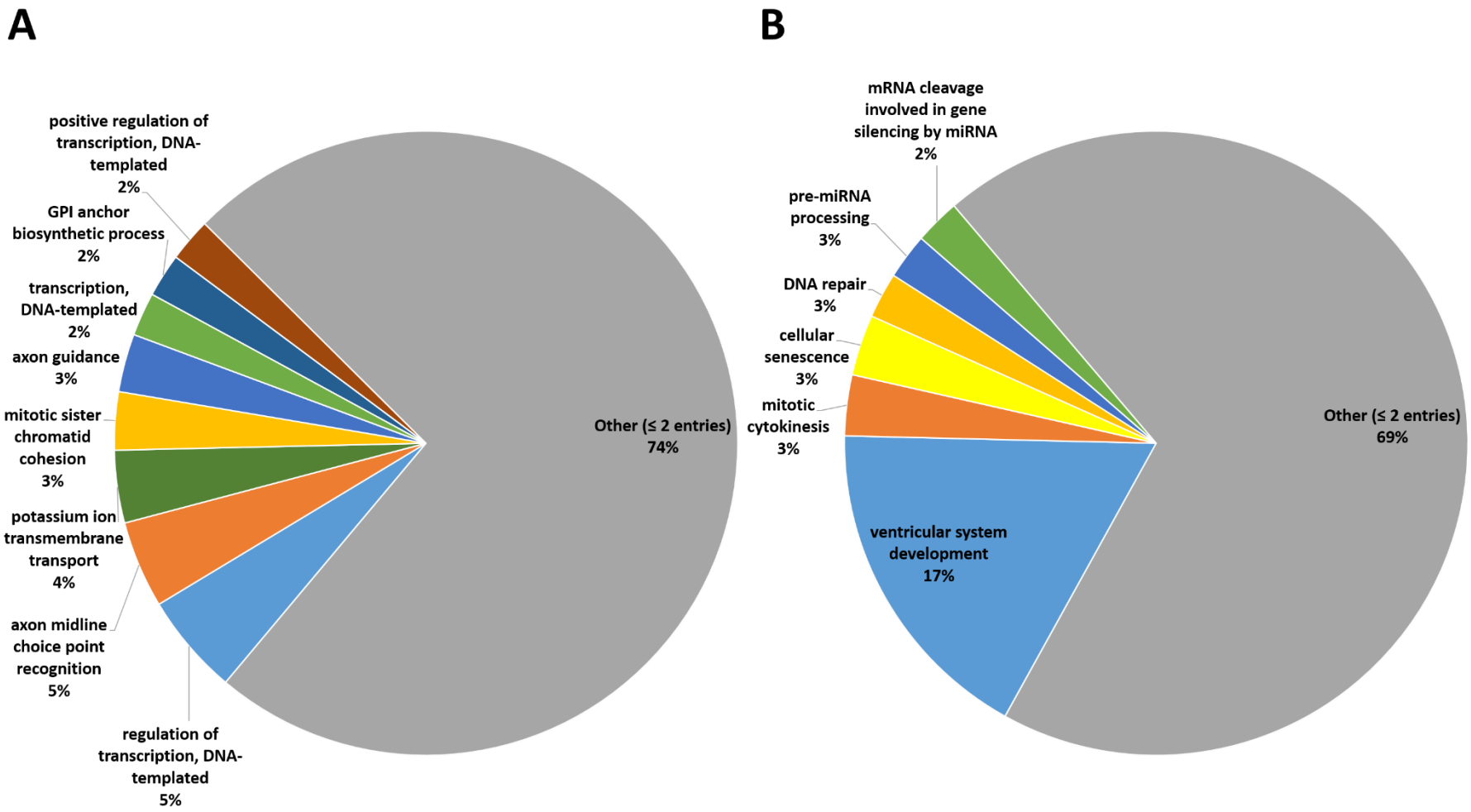


Figure 11. Summary of biological process ontology for sequences with the highest positive (A) and negative (B) log-fold change, derived from UniProt database. Terms which appeared ≤ 2 times were grouped as "Other".

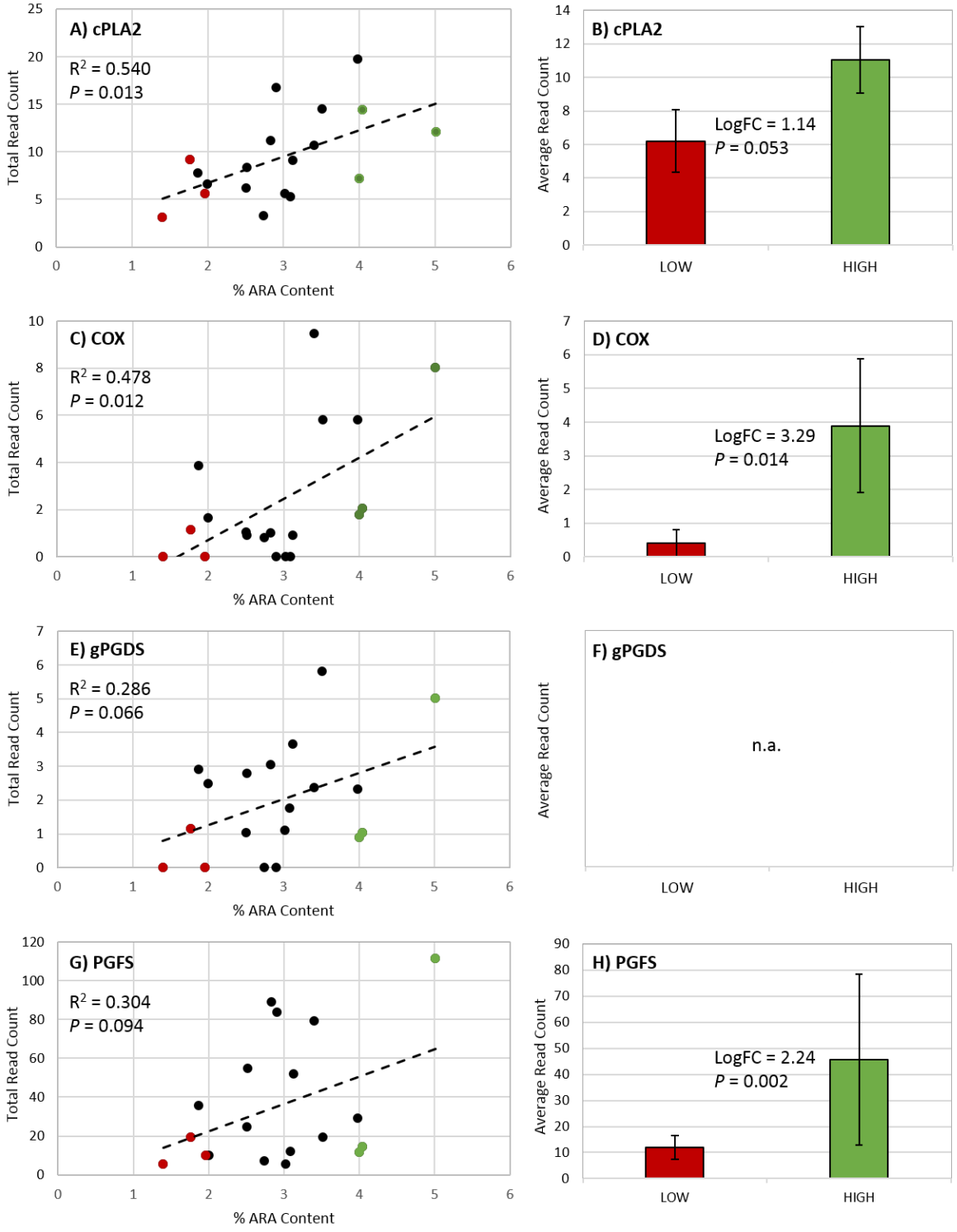


Figure 12. Analysis of prostanoid pathway genes in relation to variable ovarian arachidonic acid (ARA) content. Genes were analyzed in two ways. Prostanoid gene expression (total read counts) were correlated (R^2) with % ovarian ARA content for all n=19 samples (A, C, E, G). Alternatively, prostanoid gene expression was compared between LOW (red) and HIGH (green) groups only (B, D, F, H). Analysis of the gPGDS gene (F) could not be completed as samples did not achieve minimum read counts required for comparison. Other genes of the prostanoid pathway are continued in Figure 13.

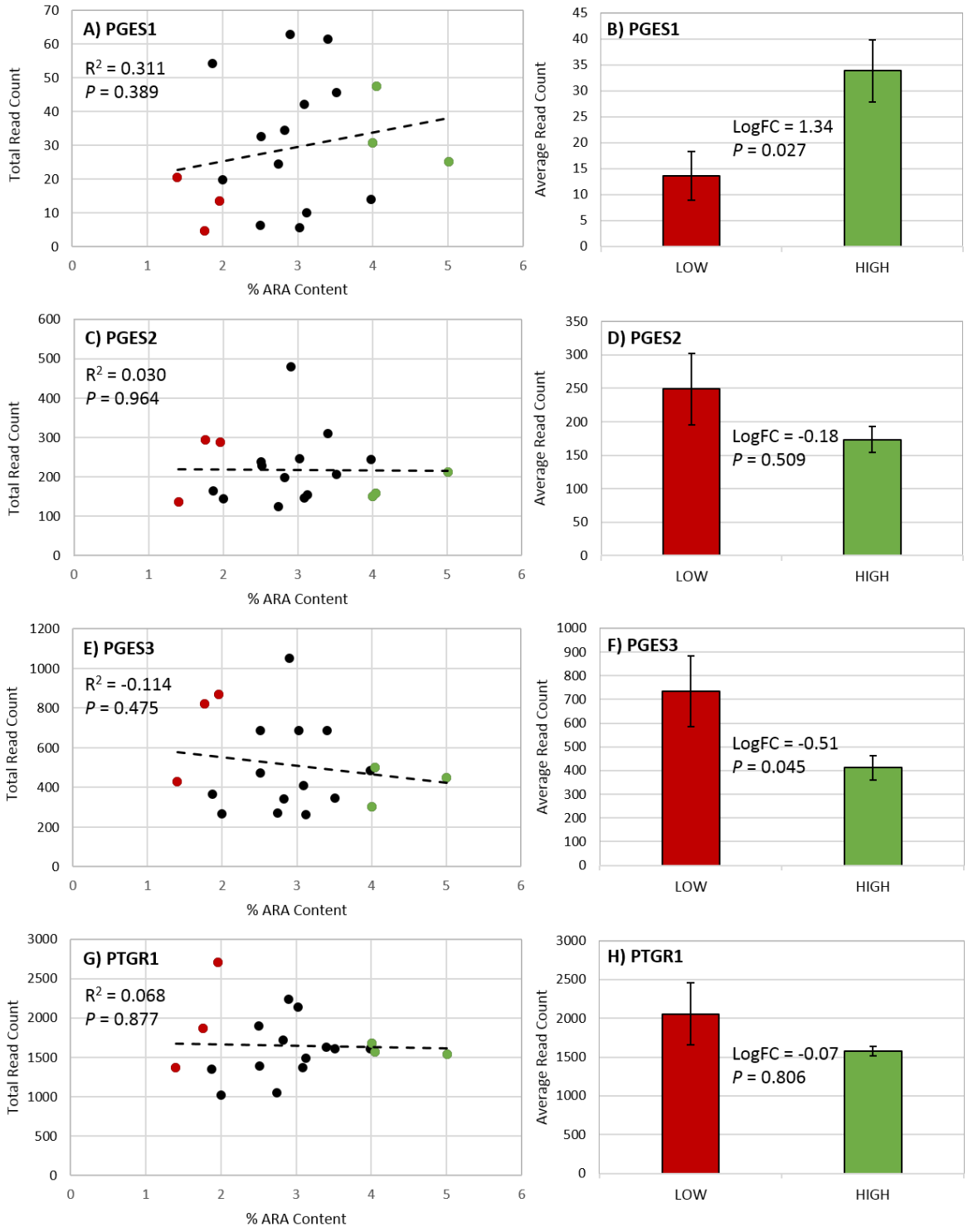


Figure 13. Analysis of prostanoid pathway genes in relation to variable ovarian arachidonic acid (ARA) content, continued from Figure 12. Genes were analyzed in two ways. Prostanoid gene expression (total read counts) were correlated (R^2) with % ovarian ARA content for all n=19 samples (A, C, E, G). Alternatively, prostanoid gene expression was compared between LOW (red) and HIGH (green) groups only (B, D, F, H).

for *PmcPLA2*, *PmPGE2* and *PmPTGR1* genes was observed between LOW and HIGH groups (P>0.05).

5.5. Discussion

The present study investigated the impact of variable ARA on the regulation of global ovarian and prostaglandin biosynthesis pathway genes. Notably, samples were derived from a homogeneous population of domesticated *P. monodon* broodstock (fourth generation) reared and conditioned under identical experimental conditions and diets. Despite this, broodstock demonstrated significant variation in percentage ovarian ARA content at a population level. In addition, the percentage of ovarian ARA content demonstrated significant positive correlation with *PmcPLA2* and *PmCOX* gene expression. Previous studies have shown that increasing the dietary levels of ARA promoted broodstock maturation and egg production (see Coman et al., 2011). The results of this study complement those of previous works, whereby the observed variation in ovarian ARA levels may contribute to the variable reproductive output commonly reported in domesticated broodstock. The reduction in ovarian ARA content may also have the secondary effect of limiting the release of bound phospholipid by cPLA2 and conversion of free ARA to PGH2 by COX. This has the potential to further reduce reproductive potential by limiting the availability of substrate essential for the production of prostaglandin E2 and F2α. Combined, these results suggest that an increase of ARA content within broodstock maturation diets may improve reproductive performance, and that a systematic review of ARA requirements in *P. monodon* broodstock may be warranted. Dietary ARA supplementation should be carefully considered given the well-documented effects of ARA overdose in aquaculture species (Furuita et al., 2003, Glencross and Smith, 2001, Tveiten et al., 2004).

We identified several effects of variable ARA levels within ovarian tissues. Significant differences in the expression of specific prostanoid biosynthesis genes were observed between the LOW and HIGH percentage ovarian ARA content groups. Significant up-regulation of *PmCOX*, *PmPGFS*, and *PmPGES1* gene expression was observed in the HIGH group, whilst *PmPGES3* was significantly up-regulated in the LOW group. The up-regulation of *PmPGFS* in the HIGH group is noteworthy, given its role in regulating the onset of maturation and the previously demonstrated reduction in *PmPGFS* gene expression in ovaries mature (Wimuttisuk et al., 2013). Within *P. monodon* ovaries, oocytes migrate radially away from the site of proliferation and therefore it is possible for oogonia, primary oocytes and mature oocytes to be present simultaneously within mature ovaries (Tanfermin and Pudadera, 1989). Therefore, a minimum level of PGFS expression

may be required to ensure the continued development and proliferation of oocytes even within mature ovaries. The up-regulation of *PmPGFS* expression in the HIGH relative to the LOW group may therefore reflect more optimal conditions for continued oocyte development to occur as a function of greater ARA availability.

We identified three *PmPGES* isoforms, and each exhibited variable expression profiles in response to ARA. For the HIGH group significant up-regulation of *PmPGES1* or *membrane-associated PGES 1* was observed, whilst the LOW group saw significant up-regulation of *PmPGES3* or *cytosolic PGES 3*. Unfortunately, prostaglandin quantification via LC-MS was unsuccessful in this study and therefore the significance of up-regulation of membrane- or cytosolic-associated *PmPGES* on total PGE2 could not be determined. The regulation of key prostanoid synthesis genes further suggests variation in ARA availability may directly impact the regulation of prostaglandin within *P. monodon* ovary tissues.

Finally, the differential expression of all ovary genes in comparison between LOW and HIGH ovarian ARA content was investigated using RNA-seq. A total of 757 transcripts between LOW and HIGH groups (>2-fold expression change) were identified, the majority of which were up-regulated (n=675) compared with down-regulated (n=82). However, the identification of only 19 of these differential genes expressed could be functionally annotated (2.5% of genes identified). To date, high-quality genomic and functional annotation for many marine invertebrate species, particularly shrimp, remains scarce and this impacted on the ability to attribute potential function to differentially expressed genes.

A manual annotation approach was undertaken for 40 of the most significantly expressed transcripts between the LOW and HIGH groups using comparative UniProt annotation and associated gene ontologies. Functional analysis of the transcripts significantly up-regulated in the HIGH group suggested an overrepresentation of genes associated with the proliferation and development of oocytes. Ontology analyses of molecular function and biological processes were predominately associated with transcriptome regulation, hydrolysis of ATP, neural development and chromosome segregation. Interestingly, transcripts significantly up-regulated in the LOW group were associated with cellular division, cellular senescence and binding of G-protein coupled receptors. Up-regulation of transcripts associated with G-protein coupled receptors have a putative link with prostaglandin binding and cellular senescence, and although requiring experimental confirmation may indicate a level of prostaglandin modulated oocyte atresia when ovarian ARA levels are reduced. Cellular component ontology was similar between both LOW and HIGH groups (nucleus,

membrane and cytoplasm), suggesting variation in ARA primarily impacts nucleus, membrane and cytoplasm.

Taken together, the results of the present study suggest that variations in the availability of dietary ARA may contribute to reduced reproductive performance either directly, or by limiting the substrate available for downstream pathways such as prostaglandin. Significant variation in the regulation of several prostanoid biosynthesis genes in response to ovarian ARA content was observed. In particular the expression of *PmCOX*, which converts free ARA to the prostaglandin intermediate PGH2 demonstrated significant correlation to percentage of ovarian ARA content. Further research is required to optimize ARA content within broodstock maturation diets particularly given the population-level variation observed here, despite broodstock being fed identical diets. Similarly, the continued development of detailed genomic and functional annotation is essential to the long-term advancement of economically important aquaculture species such as *P. monodon*.

Chapter 6: General Discussion

The ideal farming model for shrimp industries involves production of disease-free, selectively-bred stocks, derived independently from wild seedstock. To date, the reduced reproductive performance of domesticated broodstock has been the primary constraint to the establishment of this model in *P. monodon*. Continued industry reliance on wild-caught seedstock is perpetuated by a distinct lack of knowledge of reproductive performance in domesticated broodstock. Most notably, our current understanding of the nutritional, biochemical and molecular mechanisms that influence maturation and spawning in domesticated *P. monodon* is lacking. If at a minimum we are to achieve reproductive parity between domesticated and wild-caught stocks, key relationships between reproduction, nutrition and gene regulation must be identified and investigated.

The research presented in this thesis examined a number of key areas in domesticated female *P. monodon* reproductive biology. The exclusive focus on female broodstock reflects recent research suggesting that male quality has far less influence on fertilization, hatch rates and offspring viability than previously estimated (Arnold and Coman, 2012, Arnold et al., 2012, Arnold et al., 2013). Aspects relating to male reproductive biology fall outside the scope of this thesis, which is not to suggest male *P. monodon* reproductive biology is not important. As is the case in females, a number of factors relating to male reproductive biology remain unresolved (Leelatanawit et al., 2011). Still, improvements to female broodstock maturation and conditioning have greater potential to improve on-farm production yields.

Throughout this thesis, a number of experiments were undertaken to examine aspects of broodstock performance in relation to bioactive nutritional intervention, repeat spawning and nutrition-gene interaction. The over-arching theme was to provide a rigorous scaffold for the development of improved broodstock nutritional and husbandry strategies. As a secondary goal, these works aimed to increase the availability of high-quality functional genomic resources in shrimp, particularly in relation to reproductively significant tissues such as ovary. The following discussion summarizes the results, limitations and opportunities related to these experiments in an attempt to facilitate the development and accessibility of elite domesticated-selected *P. monodon* lines, both within Australian and throughout the world.

6.1. Nutritional Intervention Using Biofloc-Derived Bioactives

Incorporation of microbial bioflocs within conditioning diets regimes has improved egg production, maturation and spawning rates in a number of *Penaeid* shrimp species (Emerenciano et al., 2013b,

Emerenciano et al., 2013a, Emerenciano et al., 2012). Despite the above improvements to reproduction, current use of microbial bioflocs in broodstock conditioning is largely relegated to broodstock grow-out only. This is primarily due to the turbidity of biofloc systems presenting a number of mechanical and logistical challenges within maturation facilities. Research contained within chapters 2 and 3 attempted to circumvent this major limitation by incorporating a dried biofloc ingredient within broodstock pelleted diets, allowing the reproduction-enhancing constituents within biofloc to be made available across all stages of the production cycle. The primary aim was to determine whether the reduced reproductive performance of domesticated broodstock could be improved via nutritional intervention, namely by incorporating the biofloc-derived bioactive Novacq™ within pelleted diets.

Preliminary farm-based reproductive trails (chapter 2) indicated that Novacq™ improved maturation, egg and nauplii production when fed to broodstock (20% inclusion, 2.4% of total diet) over an extended preconditioning period (11 weeks). However, such improvements could not be reliably substantiated in a subsequent comprehensive trial conducted under controlled experimental conditions. When broodstock were fed a pelleted conditioning diet containing Novacq™ (30% inclusion, 5.5% of total diet), a significant decrease in egg hatching was observed relative to the commercial control diet (chapter 3).

Variation in reproductive performance across the two experiments likely reflects the different pelleted diet formulation used, as well as the increased inclusion rate of Novacq™ outlined in chapter 3. Decreased reproductive performance in broodstock was primarily attributed to the increased Novacq™ inclusion rate compromising basal formulation quality, particularly by reducing diet protein content. Emerenciano et al. (2013b) demonstrated that the broad nutritional diet fed to broodstock influences the degree to which biofloc improved reproductive performance in *L. vannamei*. Therefore, it is reasonable to hypothesize that where the diet regime is otherwise lacking, the effect of biofloc on reproductive performance may be negated or minimized.

Interestingly, when Novacq™ was fed to juvenile *P. monodon,* the resulting growth increases were attributed, in part, to increased appetite (Glencross et al., 2013). A logical extension for this study is that reproductive performance in farm-based trials may have been improved purely as a function of increased appetite, and therefore accumulation of resources for spawning. Variation in broodstock performance seen under the different conditions described in chapters 2 and 3 may therefore simply reflect the quality of the diets. The pelleted diet used in farm-based experiments was a high-performance experimental diet containing a number of high-performance ingredients such as krill

meal, ARASCO® and Carophyll Pink™. Such ingredients are expensive and are not commonly found in high concentrations with commercial formulations, to which the diet used in chapter 3 was designed. If indeed Novacq™ does increase the appetite of adult broodstock, its inclusion within pelleted diets may promote accumulation of nutrient from the broader diet, a plausible conclusion in chapter 2. Conversely, increased appetite may further accentuate nutritional inadequacies present within broodstock diets, such as those fed in chapter 3.

Still, the mechanisms by which biofloc improves reproduction in *Penaeid* shrimp remain unknown. The potential role of Novacq™, and more generally bioflocs, as a stimulator of appetite (and therefore nutrition accumulation) in mature broodstock requires further investigation. Similarly, investigation of changes in bioactivity between live and dried biofloc substituents is required to determine suitable inclusion rates. However, unless broad improvements are made to basal diet formulations, particularly commercial-grade diets, determining the reproductive effect of biofloc and its substituents may be elusive.

6.2. Broodstock Nutrition In Relation To Repeated Spawning

The nutritional factors that underlie maturation and spawning in *P. monodon* remain poorly understood. Current maturation diet formulations are primarily designed to replicate the nutritional profile of wild broodstock ovaries. Such formulation strategies are overly simplistic, as they do not account for nutrient utilization and interaction, compositional variation in reproductively significant tissues (i.e. hepatopancreas), or the impact of repeated spawning. The aim of research described in chapter 4 was to identify nutritional variables with significant influence on reproductive performance, as well as provide a robust profile of nutrient utilization in relation to maturation and spawning. Using a multidisciplinary approach variation in reproductive performance and tissue biochemistry (ovary and hepatopancreas) were analyzed within a homogenous population across consecutive spawning events.

The outcome of this study outlined a number of interesting trends in relation to current high-performance diets, particularly in relation to fatty acid composition and broodstock utilization. Firstly, broad macronutrient categories such as total protein and lipid showed no significant correlation with reproductive parameters suggesting micronutrient composition elicits greater influence on performance. When investigated independently a number of fatty acids were significantly depleted in the hepatopancreas of second order spawners including C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:4n-6(ARA), C20:5n-3 (EPA), C22:6n-3 (DHA). Interestingly, significant reductions in hepatopancreas fatty acid content were not reflected in ovarian tissue, with

the exception of C20:4n-6 (ARA). Fatty acids C15:0, C17:0, C18:1n-9, C18:2n-6, C20:3n-6, C20:5n-3 (EPA), C22:6n-3 (DHA) did not appear to be limiting during the initial two spawning cycles, based on ovary profiles. However, given its role in nutrient storage (Marsden et al., 2007), reductions in specific hepatopancreas fatty acids may identify those that are likely to become limiting in subsequent spawning cycles.

As noted above, both hepatopancreas and ovary ARA content significantly decreased in second order spawners, suggesting that both the requirement for and the utilization of ARA across repeated spawning is high. Chapter 4 largely suggests that current maturation diets do not contain sufficient quantities of ARA to sustain the requirement of spawning *P. monodon* and therefore limiting ARA may be contributing to decreased reproductive performance in domesticated broodstock. Furthermore, experiments outlined in chapter 5 demonstrated that ovarian ARA content varied significantly at an individual level, even within a seemingly homogenous population of broodstock. This has further implications on the regulation of reproductively significant hormones such as prostaglandin (discussed in the next section). These works further support research by Coman et al. (2011), which suggest an increase in the availability of ARA within *P. monodon* broodstock diets is both beneficial and warranted. However, any increases should be done with the careful consideration of the effects of ARA overdose (Furuita et al., 2003, Glencross and Smith, 2001, Tveiten et al., 2004). To date, the requirement for ARA has been determined for juvenile *P. monodon* only (Glencross and Smith, 2001). In light of the results of Chapter 4, a systematic review of ARA requirement in adult broodstock is required to optimize dietary inclusion levels.

A significant limitation to the experimental design of chapter 4 was the inability to measure reproductive performance and biochemical attributes from the same animals. The destructive sample methods required for biochemical analyses limit the capacity to obtain reliable repeat spawn data. In addition, following spawning ovarian tissue is rapidly regressed and therefore not representative of the pre-spawning condition. Future research efforts should aim to utilize non-destructive methods of biochemical analysis. Technologies such as near-infrared spectroscopy (NIRS) have the potential for the reliable and non-destructive quantification of broodstock tissue composition across multiple spawning events for a single individual. Such technology would significantly increase our power to detect and make causative associations between nutrition and variable reproductive performance. Therefore, optimization and validation of such technologies for *P. monodon* represents a significant research priority.

6.3. Functional Genomic Resources And Nutrient-Gene Interactions In *P. Monodon*

Currently, functional links between broodstock nutrition and endocrine regulation (i.e. prostaglandins) are poorly characterized for *P. monodon*. In a bid to improve our understanding of this significant area of broodstock biology, I investigated key interactions between nutrition and gene regulation (Chapter 5). Specifically, the chapter aimed to investigate the effect of variable ovarian ARA content on the regulation of both prostaglandin biosynthesis genes and global expression. As a secondary aim, chapter 5 sought to increase the availability of comprehensive functional genomic tools in *P. monodon* via the construction of a high quality ovarian transcriptome and subsequent RNA-seq analysis.

The results of chapter 5 outlined a number of links between ARA content and prostanoid synthesis gene regulation. Significant positive correlations were observed between ovarian ARA content and expression levels of *PmcPLA2* and *PmCOX*, suggesting that reduced ARA may limit the synthesis PGE2 and PGF2α by reducing PGH2 availability. This notion was complemented by the improved reproductive performance observed in broodstock fed high levels of dietary ARA (Coman et al., 2011), although other biochemical or gene expression effects were not investigated in that study. Combined, these results suggest that dietary and ovarian ARA levels have a direct effect on production of hormones linked to maturation and egg viability (two key areas for improvement in domesticated *P. monodon*). When differential expression was investigated between LOW and HIGH ARA content groups, significant up-regulation of *PmPGFS* occurred in individuals with greater ovarian ARA content, suggesting a greater capacity for continued development of oocytes. The regulation of the three *PmPGES* isoforms was not consistent, and requires further investigation, particularly with respects to their impact on total ovary PGE2 content. Unfortunately, attempts to quantify ovarian prostaglandin content using LC-MS were unsuccessful, but represent an area that would provide valuable functional confirmation of the results seen in this study. Alternative detection methods such as ELISA should be pursued in future research, and the investigation of prostaglandin synthesis and regulation remains a significant research priority for *P. monodon*. Notably, there is preliminary evidence to suggest that ovarian prostaglandin concentration may vary in relation to broodstock origin, i.e. wild vs. domesticated (Wimuttisuk et al., 2013). Given the results of chapters 4 and 5, the limited availability of ARA in current maturation diets may underlie the some of the reproductive performance differences between wild and domesticated broodstock.

Future experimental works should therefore strive to assess prostaglandin regulation in both wild and domesticated broodstock.

Assessment of global gene expression in relation to ovarian ARA content was complicated by the limited functional annotation available for marine invertebrate species. DGE analyses using RNA-seq identified 757 unique genes with greater than 2-fold log expression difference. However, whilst 754 putative *P. monodon* could be assigned homologs, the significant majority of these homologs were 'predicted' genes with no further functional annotation. As a result, traditional methods of assigning gene ontology such as Blast2GO could not be utilised, and only 19 of the 754 genes could be assigned ontology using manual methods. With such limited power to identify and assign functionally to gene sets, attributing higher-level function to genomic studies is difficult.

The transcriptome developed as part of chapter 5 represents significant progress towards the development of comprehensive genomic resources in *P. monodon*, and has putatively identified a list of genes involved in ARA regulation. Further development of *P. monodon* specific functional resources is needed. Several of the differentially expressed genes identified in this study require experimental confirmation using qPCR. Some key targets from within the current data set of differentially expressed genes are listed in Table 16. Future efforts should be made to expand UniProt annotation beyond the current 40 most differentially expressed genes, in a bid to identify a greater diversity of genes with putative links to reproduction. The assemblies presented in chapter 5 were derived from stage IV ovary tissues, and future functional characterisation should aim to develop transcriptome assemblies across multiple ovarian or life history stages. Development of functional gene analysis technologies to specifically down-regulate or investigate differentially expressed genes identified in this study, such as CRISPR, RNAi, *in situ* hybridization are needed. These may provide further evidence of their involvement in ovarian development, the link with nutritional requirements, and the functional mechanisms underlying reproductive performance in

Table 16. Biological process ontology of genes with predicted putative link to reproductive processes. Genes were extracted from UniProt annotations of the 40 most differentially expressed genes in *P. monodon* ovarian tissue between LOW and HIGH groups.

Log FC	Significance	Pmon Contig #	GO ID	GO Name	Aspect
5.429196	<0.001	TR12891-c0_g1_i1	GO:0008406	gonad development	Biological Process
-3.01176	0.001	TR8190-c0_g3_i1	GO:0000281	mitotic cytokinesis	Biological Process
-3.01176	0.001	TR8190-c0_g3_i1	GO:0048477	oogenesis	Biological Process
-3.01176	0.001	TR8190-c0_g3_i1	GO:0051297	centrosome organization	Biological Process
-3.01176	0.001	TR8190-c0_g3_i1	GO:0051301	cell division	Biological Process
-3.01176	0.001	TR8190-c0_g3_i1	GO:0051321	meiotic cell cycle	Biological Process

Penaeid shrimp. A major challenge may lie in the fact that the functional regulatory mechanisms underlying reproductive production traits in shrimp may be highly specialised, and require dedicated species-specific studies.

6.4. Conclusion

In this research project, a range of approaches were undertaken to determine factors underlying the reduced reproductive performance of domesticate *P. monodon* broodstock. Studies aimed at improving reproductive performance using microbial bioactives highlight the need for supplements to be fed within the context of a highly optimized broodstock diet. Notably, current high-performance maturation diets do not appear to be optimized for repeated maturation and spawning, as a number of potentially limiting fatty acids were identified. In particular, the low dietary availability of ARA may be contributing to the reduced maturation, egg production and hatching rates of domesticated broodstock either directly or through the disruption on the downstream prostanoid synthesis pathway. A number of technical constraints were identified in relation to RNA-seq analysis, and therefore ongoing research of nutrient-gene interactions in domesticated broodstock is required. Similarly, the continued development and optimization of broodstock nutrition and husbandry practices are essential to improve reproductive outcomes in domesticated *P. monodon.*

References

ALAVA, V. R., KANAZAWA, A., TESHIMA, S. & KOSHIO, S. 1993. Effect of dietary phospholipids and n-3 highly unsaturated fatty-acids on ovarian development of kuruma prawn. *Nippon Suisan Gakkaishi,* 59**,** 345-351.

ANAND, P. S. S., KOHLI, M. P. S., KUMAR, S., SUNDARAY, J. K., ROY, S. D., VENKATESHWARLU, G., SINHA, A. & PAILAN, G. H. 2014. Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*. *Aquaculture,* 418**,** 108-115.

ANDERSON, J. & VALDERRAMA, D. 2013. Shrimp Production Review. *Global Outlook on Aquaculture Leadership 2013.* Paris, France.

ARCOS, F. G., PALACIOS, E., IBARRA, A. M. & RACOTTA, I. S. 2005. Larval quality in relation to consecutive spawnings in white shrimp Litopenaeus vannamei Boone. *Aquaculture Research,* 36**,** 890-897.

ARNOLD, S. J. & COMAN, G. J. 2012. Parental influence on embryo development in *Penaeus monodon*. *Aquaculture,* 360**,** 41-44.

ARNOLD, S. J., COMAN, G. J., BURRIDGE, C. & RAO, M. 2012. A novel approach to evaluate the relationship between measures of male fertility and egg fertilization in *Penaeus monodon*. *Aquaculture,* 338**,** 181-189.

ARNOLD, S. J., COMAN, G. J. & EMERENCIANO, M. 2013. Constraints on seedstock production in eighth generation domesticated *Penaeus monodon* broodstock. *Aquaculture,* 410**,** 95-100.

BAUER, W., PRENTICE-HERNANDEZ, C., TESSER, M. B., WASIELESKY, W. & POERSCH, L. H. S. 2012. Substitution of fishmeal with microbial floc meal and soy protein concentrate in diets for the pacific white shrimp Litopenaeus vannamei. *Aquaculture,* 342**,** 112-116.

BEARD, T. W. & WICKINS, J. F. 1980. BREEDING OF PENAEUS-MONODON FABRICIUS IN LABORATORY RECIRCULATION SYSTEMS. *Aquaculture,* 20**,** 79-89.

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*.

BRAGA, A., LOPES, D. L. A., MAGALHÃES, V., POERSCH, L. H. & WASIELESKY, W. 2015. Use of biofloc technology during the pre-maturation period of Litopenaeus vannamei males: effect of feeds with different protein levels on the spermatophore and sperm quality. *Aquaculture Research,* 46**,** 1965-1973.

BRAY, W. A. & LAWRENCE, A. L. 1992. Reproduction of *Penaeus* species in captivity. *Marine shrimp culture: principles and practices.*: Elsevier, Netherlands.

BRAY, W. A., LAWRENCE, A. L. & LESTER, L. J. 1990. Reproduction of Eyestalk-Ablated *Penaeus stylirostris* Fed Various Levels of Total Dietary Lipid. *Journal of the World Aquaculture Society,* 21**,** 41-52.

BROWDY, C. 1992. *A review of the reproductive-biology of penaeus species - perspectives on controlled shrimp maturation systems for high-quality nauplii production,* Baton Rouge, World Aquaculture Soc.

BROWDY, C. L. 1998. Recent developments in penaeid broodstock and seed production technologies: improving the outlook for superior captive stocks. *Aquaculture,* 164**,** 3-21.

BROWDY, C. L. & SAMOCHA, T. M. 1985. The effect of eyestalk ablation on spawning, molting and mating of Penaeus semisulcatus de Haan. *Aquaculture,* 49**,** 19-29.

BURFORD, M. A., THOMPSON, P. J., MCINTOSH, R. P., BAUMAN, R. H. & PEARSON, D. C. 2004. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system. *Aquaculture,* 232**,** 525-537.

CAHU, C., GUILLAUME, J. C., STEPHAN, G. & CHIM, L. 1994. Influence of phospholipid and highly unsaturated fatty-acids on spawning rate and egg and tissue composition in *Penaeus vannamei* fed semi-purified diets. *Aquaculture,* 126**,** 159-170.

CAHU, C. L., CUZON, G. & QUAZUGUEL, P. 1995a. Effect of highly unsaturated fatty acids, alpha-tocopherol and ascorbic acid in broodstock diet on egg composition and development of Penaeus indicus. *Comparative Biochemistry and Physiology a-Physiology,* 112**,** 417-424.

CAHU, C. L., CUZON, G. & QUAZUGUEL, P. 1995b. Effect of highly unsaturated fatty acids, α-tocopherol and ascorbic acid in broodstock diet on egg composition and development of *Penaeus indicus*. *Comparative Biochemistry and Physiology -- Part A: Physiology,* 112**,** 417-424.

CASTILLE, F. L. & LAWRENCE, A. L. 1989. Relationship between maturation and biochemical-composition of the gonads and digestive glands of the shrimps *Penaeus aztecus ives* and *Penaeus setiferus (l). Journal of Crustacean Biology,* 9**,** 202-211.

CHIMSUNG, N. 2014. Maturation diets for black tiger shrimp (*Penaeus monodon*) broodstock: A review. *Songklanakarin Journal of Science and Technology,* 36**,** 265-273.

CHRISTIE, W. 2003a. *Lipid analysis: isolation, separation, identification and structural analysis of lipids,* Bridgwater, England : Oily Press.

CHRISTIE, W. W. 2003b. *Lipid analysis, isolation, separation, identification and structural analysis of lipids.,* Bridgewater, UK, PJ Barnes and Associates.

CLARKE, A., BROWN, J. H. & HOLMES, L. J. 1990. The biochemical composition of eggs from *Macrobrachium rosenbergii* in relation to embryonic development. *Comparative Biochemistry and Physiology - Biochemistry and Molecular Biology,* 96**,** 505-511.

COLBOURNE, J. K., PFRENDER, M. E., GILBERT, D., THOMAS, W. K., TUCKER, A., OAKLEY, T. H., TOKISHITA, S., AERTS, A., ARNOLD, G. J. & BASU, M. K. 2011. The ecoresponsive genome of *Daphnia pulex*. *Science,* 331.

COMAN, G., WOOD, A., GOODALL J., GLENCROSS, B., MURPHY, B., SELLARS, M. 2014. Effect of Novacq™ supplementation of a broodstock maturation diet on reproductive performance of *Penaeus monodon*.

COMAN, G. J., ARNOLD, S. J., BARCLAY, M. & SMITH, D. M. 2011. Effect of arachidonic acid supplementation on reproductive performance of tank-domesticated *Penaeus monodon*. *Aquaculture Nutrition,* 17**,** 141-151.

COMAN, G. J., ARNOLD, S. J., CALLAGHAN, T. R. & PRESTON, N. P. 2007a. Effect of two maturation diet combinations on reproductive performance of domesticated *Penaeus monodon*. *Aquaculture,* 263**,** 75-83.

COMAN, G. J., ARNOLD, S. J., JONES, M. J. & PRESTON, N. P. 2007b. Effect of rearing density on growth, survival and reproductive performance of domesticated *Penaeus monodon*. *Aquaculture,* 264**,** 175-183.

COMAN, G. J., ARNOLD, S. J., PEIXOTO, S., CROCOS, P. J., COMAN, F. E. & PRESTON, N. P. 2006. Reproductive performance of reciprocally crossed wild-caught and tank-reared *Penaeus monodon* broodstock. *Aquaculture,* 252**,** 372-384.

COMAN, G. J., ARNOLD, S. J., WOOD, A. T. & PRESTON, N. P. 2013. Evaluation of egg and nauplii production parameters of a single stock of domesticated *Penaeus monodon* (Giant Tiger Shrimp) across generations. *Aquaculture,* 400–401**,** 125-128.

COMAN, G. J., CROCOS, P. J., ARNOLD, S. J., KEYS, S. J., PRESTON, N. P. & MURPHY, B. 2005. Growth, survival and reproductive performance of domesticated Australian stocks of the Giant Tiger Prawn, *Penaeus monodon*, reared in tanks and raceways. *Journal of the World Aquaculture Society,* 36**,** 464-479.

COMAN, G. J., CROCOS, P. J., PRESTON, N. P. & FIELDER, D. 2004. The effects of density on the growth and survival of different families of juvenile *Penaeus japonicus Bate*. *Aquaculture,* 229**,** 215-223.

CRAB, R., CHIELENS, B., WILLE, M., BOSSIER, P. & VERSTRAETE, W. 2010. The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. *Aquaculture Research,* 41**,** 559-567.

CROCOS, P. J. 1997. *Factors affecting the reproductive performance of captive and wild broodstock prawns / P.J. Crocos, D.M. Smith and G. Marsden,* Melbourne, CSIRO, Division of Fisheries.

CROCOS, P. J. & COMAN, G. J. 1997. Seasonal and age variability in the reproductive performance of *Penaeus semisulcatus* broodstock: optimising broodstock selection. *Aquaculture,* 155**,** 55-67.

CUZON, G. & GUILLAUME, J. 1997. Energy and protein: energy ratio. In: LR Abramo (Editor) ; OF Conklin (Editor) ; Akiyama DM (Editor). *In:* World Aquaculture Society, B. R. E.-U. (ed.) *Crustacean nutrition.* Baton Rouge, USA: World Aquaculture Society: Advances in World Aquaculture, 6.

D'SOUZA, F. M. L. & KELLY, G. J. 2000. Effects of a diet of a nitrogen-limited alga (*Tetraselmis suecica*) on growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae. *Aquaculture,* 181**,** 311-329.

DAVIDSON, N. M. & OSHLACK, A. 2014. Corset: enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biology,* 15.

EMERENCIANO, M., CUZON, G., ARÉVALO, M. & GAXIOLA, G. 2013a. Biofloc technology in intensive broodstock farming of the pink shrimp *Farfantepenaeus duorarum*: Spawning performance, biochemical composition and fatty acid profile of eggs. *Aquaculture Research*.

EMERENCIANO, M., CUZON, G., AREVALO, M., MIQUELAJAUREGUI, M. M. & GAXIOLA, G. 2013b. Effect of short-term fresh food supplementation on reproductive performance, biochemical composition, and fatty acid profile of *Litopenaeus vannamei* (*Boone*) reared under biofloc conditions. *Aquaculture International,* 21**,** 987-1007.

EMERENCIANO, M., CUZON, G., GOGUENHEIM, J., GAXIOLA, G. & AQUACOP 2012. Floc contribution on spawning performance of blue shrimp *Litopenaeus stylirostris*. *Aquaculture Research,* 44**,** 75-85.

EMERENCIANO, M., CUZON, G., PAREDES, A. & GAXIOLA, G. 2013c. Evaluation of biofloc technology in pink shrimp *Farfantepenaeus duorarum* culture: growth performance, water quality, microorganisms profile and proximate analysis of biofloc. *Aquaculture International,* 21**,** 1381-1394.

EMMERSON, W. D. 1980. Induced maturation of prawn Penaeus-indicus. *Marine Ecology Progress Series,* 2**,** 121-131.

FAO 2010. The State of World Fisheries and Aquaculture 2010. Rome: FAO.

FAO 2016a. FAO Yearbook: Fisheries and Aquaculture Statistics (2014). Food and Agriculture Organisation of the United Nations

FAO 2016b. The State of World Fisheries and Aquaculture 2014.

FOLCH, J. A. S.-S., G. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry,* 226**,** 497-509.

FUNK, C. D. 2001. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science,* 294**,** 1871-1875.

FURUITA, H., YAMAMOTO, T., SHIMA, T., SUZUKI, N. & TAKEUCHI, T. 2003. Effect of arachidonic acid levels in broodstock diet on larval and egg quality of Japanese flounder Paralichthys olivaceus. *Aquaculture,* 220**,** 725-735.

GARCÍA-GUERRERO, M., RACOTTA, I. S. & VILLARREAL, H. 2003. Variation in lipid, protein, and carbohydrate content during the embryonic development of the crayfish *Cherax quadricarinatus* (decapoda: parastacidae). *Journal of Crustacean Biology,* 23**,** 1-6.

GARCIA, S. M. & ROSENBERG, A. A. 2010. Food security and marine capture fisheries: characteristics, trends, drivers and future perspectives. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 365**,** 2869-2880.

GINJUPALLI, G. K., GERARD, P. D. & BALDWIN, W. S. 2015. Arachidonic acid enhances reproduction in *Daphnia magna* and mitigates changes in sex ratios induced by pyriproxyfen. *Environmental Toxicology and Chemistry,* 34**,** 527-535.

GLENCROSS, B. 2009. Exploring the nutritional demand for essential fatty acids by aquaculture species. *Reviews in Aquaculture,* 1**,** 71-124.

GLENCROSS, B., ARNOLD, S. & IRVIN, S. 2015. Bioactive factors in microbial biomass have the capacity to offset reductions in the level of protein in the diet of Black Tiger Shrimp, *Penaeus monodon*. *Aquaculture,* 446**,** 74-79.

GLENCROSS, B., IRVIN, S., ARNOLD, S., BLYTH, D., BOURNE, N. & PRESTON, N. 2014. Effective use of microbial biomass products to facilitate the complete replacement of fishery resources in diets for the Black Tiger Shrimp, *Penaeus monodon*. *Aquaculture,* 431**,** 12-19.

GLENCROSS, B., TABRETT, S., IRVIN, S., WADE, N., ANDERSON, M., BLYTH, D., SMITH, D., COMAN, G. & PRESTON, N. 2013. An analysis of the effect of diet and genotype on protein and energy utilization by the Black Tiger Shrimp, *Penaeus monodon* - why do genetically selected shrimp grow faster? *Aquaculture Nutrition,* 19**,** 128-138.

GLENCROSS, B. D. & SMITH, D. M. 2001. A study of the arachidonic acid requirements of the giant tiger prawn, *Penaues monodon*. *Aquaculture Nutrition,* 7**,** 59-69.

GOODALL, J. D., WADE, N. M., MERRITT, D. J., SELLARS, M. J., SALEE, K. & COMAN, G. J. 2016. The effects of adding microbial biomass to grow-out and maturation feeds on the reproductive performance of black tiger shrimp, *Penaeus monodon*. *Aquaculture,* 450**,** 206-212.

HAAS, B. J., PAPANICOLAOU, A., YASSOUR, M., GRABHERR, M., BLOOD, P. D. & BOWDEN, J. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protocols,* 8.

HALL, M. R., MASTRO, R., YOUNG, N., FRASER, C., STRUGNELL, J., KENWAY, M. 2003. High Quality Eggs and Nauplii for the Australian Prawn Industry. *FRDC 1995/166.* . Fisheries Research and Development Corporation.

HAMPSON, A. J., ROWLEY, A. F., BARROW, S. E. & STEADMAN, R. 1992. Biosynthesis of eicosanoids by blood-cells of the crab, carcinus maenas. *Biochimica Et Biophysica Acta,* 1124**,** 143-150.

HANSFORD, S. W. & MARSDEN, G. E. 1995. Temporal Variation in Egg and Larval Productivity of Eyestalk Ablated Spawners of the Prawn *Penaeus monodon* from Cook Bay, Australia. *Journal of the World Aquaculture Society,* 26**,** 396-405.

HARRISON, K. E. 1990. The role of nutrition in maturation reproduction and embryonic development of decapod crustaceans a review. *Journal of Shellfish Research,* 9**,** 1-28.

HOA, N. D., WOUTERS, R., WILLE, M., THANH, V., DONG, T. K., HAO, N. V. & SORGELOOS, P. 2009. A fresh-food maturation diet with an adequate HUFA composition for broodstock nutrition studies in Black Tiger Shrimp *Penaeus monodon* (*Fabricius*, 1798). *Aquaculture,* 297**,** 116-121.

HUANG, J. H., JIANG, S. G., LIN, H. Z., ZHOU, F. L. & YE, L. 2008. Effects of dietary highly unsaturated fatty acids and astaxanthin on the fecundity and lipid content of pond-reared *Penaeus monodon* (*Fabricius*) broodstock. *Aquaculture Research,* 39**,** 240-251.

IZQUIERDO, M., FORSTER, I., DIVAKARAN, S., CONQUEST, L., DECAMP, O. & TACON, A. 2006. Effect of green and clear water and lipid source on survival, growth and biochemical composition of Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture Nutrition,* 12**,** 192-202.

JACKSON, C. I. & WANG, Y. G. 1998. Modelling growth rate of *Penaeus monodon fabricius* in intensively managed ponds: effects of temperature, pond age and stocking density. *Aquaculture Research,* 29**,** 27-36.

JIANG, S. G., HUANG, J. H., ZHOU, F. L., CHEN, X., YANG, Q. B., WEN, W. G. & MA, Z. M. 2009. Observations of reproductive development and maturation of male *Penaeus monodon* reared in tidal and earthen ponds. *Aquaculture,* 292**,** 121-128.

JU, Z. Y., FORSTER, I., CONQUEST, L., DOMINY, W., KUO, W. C. & HORGEN, F. D. 2008. Determination of microbial community structures of shrimp floc cultures by biomarkers and analysis of floc amino acid profiles. *Aquaculture Research,* 39**,** 118-133.

KLINBUNGA, S., SITTIKANKAEW, K., YUVANATEMIYA, V., PREECHAPHOL, R., PRASERTLUX, S., YAMANO, K. & MENASVETA, P. 2009. Molecular Cloning and Expression Analysis of Ovary-Specific Transcript 1 (Pm-OST1) of the Giant Tiger Shrimp, *Penaeus monodon*. *Zoological Science,* 26**,** 783-790.

KNIGHT, J., ROWLEY, A. F., YAMAZAKI, M. & CLARE, A. S. 2000. Eicosanoids are modulators of larval settlement in the barnacle, *Balanus amphitrite*. *Journal of the Marine Biological Association of the United Kingdom,* 80**,** 113-117.

KUHN, D. D., BOARDMAN, G. D., CRAIG, S. R., FLICK, G. J. & MCLEAN, E. 2008. Use of microbial flocs generated from Tilapia effluent as a nutritional supplement for shrimp, *Litopenaeus vannamei*, in recirculating aquaculture systems. *Journal of the World Aquaculture Society,* 39**,** 72-82.

KUHN, D. D., BOARDMAN, G. D., LAWRENCE, A. L., MARSH, L. & FLICK, G. J. 2009. Microbial floc meal as a replacement ingredient for fish meal and soybean protein in shrimp feed. *Aquaculture,* 296**,** 51-57.

KUHN, D. D., LAWRENCE, A. L., BOARDMAN, G. D., PATNAIK, S., MARSH, L. & FLICK JR, G. J. 2010. Evaluation of two types of bioflocs derived from biological treatment of fish effluent as feed ingredients for Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture,* 303**,** 28-33.

LANGMEAD, B. & SALZBERG, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods,* 9.

LANGMEAD, B., TRAPNELL, C., POP, M. & SALZBERG, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology,* 10**,** 1-10.

LEELATANAWIT, R., UAWISETWATHANA, U., KLINBUNGA, S. & KAROONUTHAISIRI, N. 2011. A cDNA microarray, UniShrimpChip, for identification of genes relevant to testicular development in the black tiger shrimp (Penaeus monodon). *Bmc Molecular Biology,* 12.

LUMARE, F. 1979. Reproduction of penaeus-kerathurus using eyestalk ablation. *Aquaculture,* 18**,** 203-214.

LYTLE, J. S., LYTLE, T. F. & OGLE, J. T. 1990. Polyunsaturated fatty-acid profiles as a comparative tool in assessing maturation diets of *penaeus vannamei*. *Aquaculture,* 89**,** 287-299.

MACBETH, M., KENWAY, M., SALMON, M., BENZIE, J., KNIBB, W. & WILSON, K. 2007. Heritability of reproductive traits and genetic correlations with growth in the black tiger prawn *Penaeus monodon* reared in tanks. *Aquaculture,* 270**,** 51-56.

MACMANES, M. D. 2016. Establishing evidenced-based best practice for the de novo assembly and evaluation of transcriptomes from non-model organisms. *bioRxiv*.

MAKINOUCHI, S. & HIRATA, H. 1995. Studies on maturation and reproduction of pond-reared *penaeus monodon* for developing a closed life-cycle culture system. *Israeli Journal of Aquaculture-Bamidgeh,* 47**,** 68-77.

MARSDEN, G., MATHER, P. & RICHARDSON, N. 2007. Captivity, ablation and starvation of the prawn *Penaeus monodon* affects protein and lipid content in ovary and hepatopancreas tissues. *Aquaculture,* 271**,** 507-515.

MARSDEN, G., MCGUREN, J. M., SARAC, H. Z., NEILL, A. R., BROCK, I. J. & PAIMER, C. L. 1991. Nutritional composition of some natural marine feeds used in prawn maturation.

MARSDEN, G. E., MCGUREN, J. J., HANSFORD, S. W. & BURKE, M. J. 1997. A moist artificial diet for prawn broodstock: Its effect on the variable reproductive performance of wild caught *Penaeus monodon*. *Aquaculture,* 149**,** 145-156.

MAZORRA, C., BRUCE, M., BELL, J. G., DAVIE, A., ALOREND, E., JORDAN, N., REES, J., PAPANIKOS, N., PORTER, M. & BROMAGE, N. 2003. Dietary lipid enhancement of broodstock reproductive performance and egg and larval quality in Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture,* 227**,** 21-33.

MCCULLAGH, P. & NELDER, J. A. 1989. An Outline of Generalized Linear Models: Generalized Linear Models. 2nd ed. London: Chapman & Hall.

MENASVETA, P., PIYATIRATITIVORAKUL, S., RUNGSUPA, S., MOREE, N. & FAST, A. W. 1993. Gonadal maturation and reproductive-performance of Giant Tiger Prawn (*Penaeus monodon fabricius*) from the andaman sea and pond-reared sources in Thailand. *Aquaculture,* 116**,** 191-198.

MENDOZA, R. 1997. Nauplii Production from Wild, Cultivated, and Mixed Populations of Blue Shrimp, *Penaeus stylirostris*. *Journal of Applied Aquaculture,* 7**,** 41-50.

MEUNPOL, O., DUANGJAI, E., YOONPUN, R. & PIYATIRATITIVORAKUL, S. 2010. Detection of prostaglandin E-2 in polychaete *Perinereis sp* and its effect on *Penaeus monodon* oocyte development in vitro. *Fisheries Science,* 76**,** 281-286.

MEUNPOL, O., MEEJING, P. & PIYATIRATITIVORAKUL, S. 2005. Maturation diet based on fatty acid content for male *Penaeus monodon* (*Fabricius*) broodstock. *Aquaculture Research,* 36**,** 1216-1225.

MILLAMENA, O. M. 1989. Effect of fatty acid composition of broodstock diet on tissue fatty acid patterns and egg fertilization and hatching in pond-reared *Penaeus monodon*. *Asian Fisheries Science,* 2**,** 127-134.

MILLAMENA, O. M., PUDADERA, R. A. & CATACUTAN, M. R. 1993. Tissue lipid-content and fatty-acid composition during ovarian maturation of ablated *Penaeus monodon*. *Israeli Journal of Aquaculture-Bamidgeh,* 45**,** 120-125.

MOTOH, H. 1985. Biology and ecology of *Penaeus monodon*

In: TAKI, Y., PRIMAVERA, J. H. & LLOBRERA, J. A. (eds.) *Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps.* Iloilo City, Philippines: Iloilo City, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.

NAESSENS, E., LAVENS, P., GOMEZ, L., BROWDY, C. L., MCGOVERNHOPKINS, K., SPENCER, A. W., KAWAHIGASHI, D. & SORGELOOS, P. 1997. Maturation performance of *Penaeus vannamei* co-fed *Artemia* biomass preparations. *Aquaculture,* 155**,** 87-101.

OGATA, H. Y., EMATA, A. C., GARIBAY, E. S. & FURUITA, H. 2004. Fatty acid composition of five candidate aquaculture species in Central Philippines. *Aquaculture,* 236**,** 361-375.

PALACIOS, E., RACOTTA, I. S., HERAS, H., MARTY, Y., MOAL, J. & SAMAIN, J. F. 2001. Relation between lipid and fatty acid composition of eggs and larval survival in white pacific shrimp (*Penaeus vannamei, Boone,* 1931). *Aquaculture International,* 9**,** 531-543.

PARNES, S., RAVIV, S., AZULAY, D. & SAGI, A. 2007. Dynamics of reproduction in a captive shrimp broodstock: Unequal contribution of the female shrimp and a hidden shortage in competent males. *Invertebrate Reproduction and Development,* 50**,** 21-29.

PEIXOTO, S., COMAN, G., ARNOLD, S., CROCOS, P. & PRESTON, N. 2005. Histological examination of final oocyte maturation and atresia in wild and domesticated *Penaeus monodon* (*Fabricius*) broodstock. *Aquaculture Research,* 36**,** 666-673.

PONGTIPPATEE, P., VANICHVIRIYAKIT, R., CHAVADEJ, J., PLODPAI, P., PRATOOMCHART, B., SOBHON, P. & WITHYACHUMNARNKUL, B. 2007. Acrosome reaction in the sperm of the black tiger shrimp *Penaeus monodon* (Decapoda, Penaeidae). *Aquaculture Research,* 38**,** 1635-1644.

PRASAIN, J. K., HOANG, H. D., EDMONDS, J. W. & MILLER, M. A. 2013. Prostaglandin Extraction and Analysis in Caenorhabditis elegans. e50447.

PRATOOMCHAT, B., PIYATIRATITIVORAKUL, S. & MENASVETA, P. 1993. Sperm quality of pond-reared and wild-caught *Penaeus monodon* in Thailand. *Journal of the World Aquaculture Society,* 24**,** 530-540.

PRESTON, N., COMAN, G., SELLARS, M., COWLEY, J., DIXON, T., LI, Y. & MURPHY, B. Advances in *Penaeus monodon* breeding and genetics. 2009 Baton Rouge Louisiana, USA. The World Aquaculture Society.

PRIMAVERA, J. H. & CABALLERO, R. M. V. 1992. Light color and ovarian maturation in unablated and ablated giant tiger prawn *penaeus monodon* (*fabricius*). *Aquaculture,* 108**,** 247-256.

PRIMAVERA, J. H. & POSADAS, R. A. 1981. Studies on the egg quality of *penaeus-monodon fabricius,* based on morphology and hatching rates. *Aquaculture,* 22**,** 269-277.

RACOTTA, I. S., PALACIOS, E. & IBARRA, A. M. 2003. Shrimp larval quality in relation to broodstock condition. *Aquaculture,* 227**,** 107-130.

ROBINSON, M. D., MCCARTHY, D. J. & SMYTH, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics,* 26**,** 139-140.

RODRIGUEZ-GONZALEZ, H., GARCIA-ULLOA, M., HERNANDEZ-LLAMAS, A. & VILLARREAL, H. 2006. Effect of dietary protein level on spawning and egg quality of redclaw crayfish *Cherax quadricarinatus*. *Aquaculture,* 257**,** 412-419.

RODRIGUEZ-GONZALEZ, H., HERNANDEZ-LLAMAS, A., GARCIA-ULLOA, M. & VILLARREAL, H. 2013. Effect of dietary protein and lipid levels on gonadal development of female redclaw crayfish *Cherax quadricarinatus*. *Aquaculture Research,* 45**,** 80-86.

ROWLEY, A. F., VOGAN, C. L., TAYLOR, G. W. & CLARE, A. S. 2005. Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. *Journal of Experimental Biology,* 208**,** 3-14.

SALZE, G., TOCHER, D. R., ROY, W. J. & ROBERTSON, D. A. 2005. Egg quality determinants in cod (*Gadus morhua L.*): egg performance and lipids in eggs from farmed and wild broodstock. *Aquaculture Research,* 36**,** 1488-1499.

SAOUD, I. P., DE YTA, A. G. & GHANAWI, J. 2012. A review of nutritional biology and dietary requirements of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). *Aquaculture Nutrition,* 18**,** 349-368.

SAS INSTITUTE SOFTWARE 1999. SAS/STAT software, Version 8.2. Cary, NC, USA

SAS Institute Inc.

SELLARS, M., RAO, M., POLYMERIS, N., IRVIN, S., COWLEY, J., PRESTON, N. & GLENCROSS, B. 2015a. Feed containing Novacq improves resilience of black tiger shrimp to gill-associated virus-induced mortality. *Journal of the World Aquaculture Society,* 46.

SELLARS, M. J., RAO, M., POLYMERIS, N., IRVIN, S. J., COWLEY, J. A., PRESTON, N. P. & GLENCROSS, B. D. 2015b. Feed Containing Novacq Improves Resilience of Black Tiger Shrimp, *Penaeus Monodon*, to Gill-associated Virus-induced Mortality. *Journal of the World Aquaculture Society,* 46**,** 328-336.

SIEGEL, S. 1956. *Non-parametric statistics for the behavioral sciences,* New York, McGraw-Hill.

SIMÃO, F. A., WATERHOUSE, R. M., IOANNIDIS, P., KRIVENTSEVA, E. V. & ZDOBNOV, E. M. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*.

SMITH-UNNA, R. D., BOURSNELL, C., PATRO, R., HIBBERD, J. M. & KELLY, S. 2015. TransRate: reference free quality assessment of de-novo transcriptome assemblies. *bioRxiv*.

SOKAL, R. R. & ROHLF, F. J. 1981. Nonparametric Tests for Association. *Biometry.* New York: W. H. Freeman and Company.

SONG, L. & FLOREA, L. 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. *GigaScience,* 4**,** 1-8.

SPAZIANI, E. P., HINSCH, G. W. & EDWARDS, S. C. 1993. Changes in prostaglandin E2 and F2 α during vitellogenesis in the florida crayfish *Procambarus paeninsulanus*. *Journal of Comparative Physiology B,* 163**,** 541-545.

SPAZIANI, E. P., HINSCH, G. W. & EDWARDS, S. C. 1995. The effect of prostaglandin E(2) and prostaglandin F-2 alpha on ovarian tissue in the Florida crayfish *Procambarus paeninsulanus*. *Prostaglandins,* 50**,** 189-200.

SREENIVASULA REDDY, P., RAMACHANDRA REDDY, P. & PURNA CHANDRA NAGARAJU, G. 2004. The synthesis and effects of prostaglandins on the ovary of the crab *Oziotelphusa senex senex*. *General and Comparative Endocrinology,* 135**,** 35-41.

STANLEY, D. 2006. Prostaglandins and other eicosanoids in insects: Biological significance. *Annual Review of Entomology.* Palo Alto: Annual Reviews.

STANLEY, D. W. & HOWARD, R. W. 1998. The biology of prostaglandins and related eicosanoids in invertebrates: Cellular, organismal and ecological actions. *American Zoologist,* 38**,** 369-381.

TAHARA, D. & YANO, I. 2004. Maturation-related variations in prostaglandin and fatty acid content of ovary in the kuruma prawn (*Marsupenaeus japonicus*). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* 137**,** 631-637.

TANFERMIN, J. D. & PUDADERA, R. A. 1989. Ovarian maturation stages of the wild giant tiger prawn, *Penaeus-monodon fabricius*. *Aquaculture,* 77**,** 229-242.

TEAM, R Studio. 2015. R Studio: Integrated Development for R. RStudio, Inc., Boston, MA

TVEITEN, H., JOBLING, M. & ANDREASSEN, I. 2004. Influence of egg lipids and fatty acids on egg viability, and their utilization during embryonic development of spotted wolf-fish, *Anarhichas minor Olafsen*. *Aquaculture Research,* 35**,** 152-161.

WASIELESKY, W., ATWOOD, H., STOKES, A. & BROWDY, C. L. 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. *Aquaculture,* 258**,** 396-403.

WEN, W., YANG, Q., MA, Z., JIANG, S., QIU, L., HUANG, J., ZHOU, F. & QIN, J. G. 2015. Comparison of ovarian maturation and spawning after unilateral eyestalk ablation of wild-caught and pond-reared *Penaeus monodon*. *Spanish Journal of Agricultural Research,* 13.

WILKS, S. S. 1946. Sample criteria for testing equality of means, equality of variances and equality of covariances in a normal multivariate distribution. *Ann Math Statist,* 17**,** 257-281.

WIMUTTISUK, W., TOBWOR, P., DEENARN, P., DANWISETKANJANA, K., PINKAEW, D., KIRTIKARA, K. & VICHAI, V. 2013. Insights into the Prostanoid Pathway in the Ovary Development of the Penaeid Shrimp *Penaeus monodon*. *PLoS ONE,* 8.

WOUTERS, R., GÓMEZ, L., LAVENS, P. & CALDERÓN, J. 1999. Feeding enriched *Artemia* biomass to *Penaeus vannamei* broodstock: Its effect on reproductive performance and larval quality. *Journal of Shellfish Research,* 18**,** 651-656.

WOUTERS, R., LAVENS, P., NIETO, J. & SORGELOOS, P. 2001a. Penaeid shrimp broodstock nutrition: An updated review on research and development. *Aquaculture,* 202**,** 1-21.

WOUTERS, R., PIGUAVE, X., BASTIDAS, L., CALDERÓN, J. & SORGELOOS, P. 2001b. Ovarian maturation and haemolymphatic vitellogenin concentration of Pacific white shrimp *Litopenaeus vannamei* (*Boone*) fed increasing levels of total dietary lipids and HUFA. *Aquaculture Research,* 32**,** 573-582.

XU, X. L., JI, W. J., CASTELL, J. D. & ODOR, R. K. 1994a. Essential fatty-acid requirement of the chinese prawn, *penaeus-chinensis*. *Aquaculture,* 127**,** 29-40.

XU, X. L., JI, W. J., CASTELL, J. D. & ODOR, R. K. 1994b. Influence of dietary-lipid sources on fecundity, egg hatchability and fatty-acid composition of chinese prawn (*Penaeus-chinensis*) broodstock. *Aquaculture,* 119**,** 359-370.

ZAR, J. H. 1984a. One-sample hypotheses: Biostatistical Analysis. 2nd ed. Englewood Cliffs, NJ Prentice-Hall, Inc.

ZAR, J. H. 1984b. Two-factor analysis of variance: Biostatistical Analysis. 2nd ed. Englewood Cliffs, NJ: Prentice-Hall, Inc.

ZENG, V., VILLANUEVA, K. E., EWEN-CAMPEN, B. S., ALWES, F., BROWNE, W. E. & EXTAVOUR, C. G. 2011. De novo assembly and characterization of a maternal and developmental transcriptome for the emerging model crustacean *Parhyale hawaiensis*. *BMC Genomics,* 12**,** 581.

Appendices

Supplementary Table 1. tblastx analysis of known prostaglandin-associated genes against the final assembly

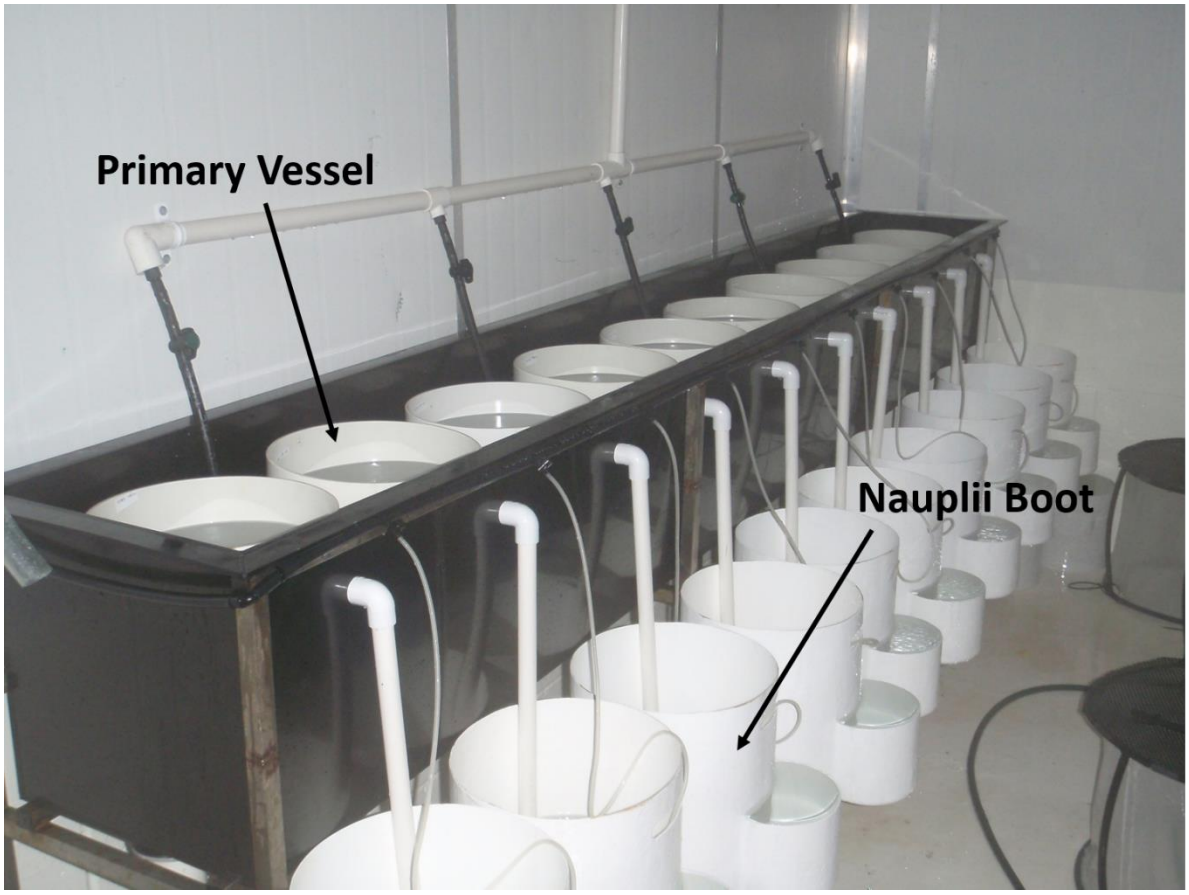
Gene	Contig #	Contig Size (bp)	Matched Accession	Reference Species	Sequence Identity (%)	E-value
cPLA2	TR16503-c0_g1_i1	1118	JN003878	*Penaeus monodon*	99.46	0
COX	TR25076-c0_g1_i1	516	KF501342	*Penaeus monodon*	99.42	3.21E-116
gPGDS	TR18226-c0_g1_i1	638	JN003880	*Penaeus monodon*	100	1.60E-104
PGFS	TR29149-c0_g1_i1	1407	JN003884	*Penaeus monodon*	72.01	2.99E-166
PGES1	TR17519-c0_g1_i1	588	JN003882	*Penaeus monodon*	97.33	1.50E-101
PGES2	TR20851-c0_g1_i1	1823	JN003883	*Penaeus monodon*	99.79	0
PGES3	TR28136-c0_g1_i1	1003	JN003881	*Penaeus monodon*	99.56	0
PTGR1	TR2673-c0_g1_i1	1771	JF834156	*Penaeus monodon*	98.12	0

Supplementary Table 2. FastQC summary statistics for LOW, HIGH and MEDIAN samples following trimming and read correction of Illumina 2500, 100 base pair, paired-end read sequencing.

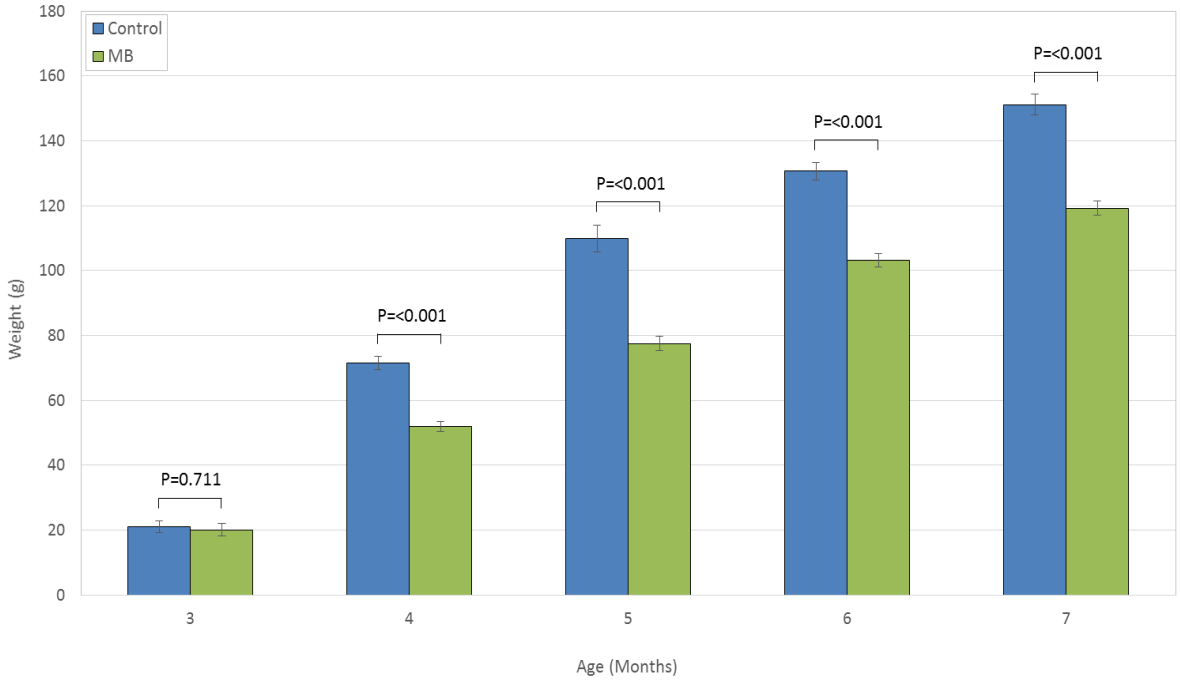
Sample Rep #	Sequencing Group	Total Sequences	Flagged as Poor Quality	Sequence Length	%GC Content	Used in d*e novo* Assembly
1	LOW	11517375	0	50-100	48	-
2	LOW	10556680	0	50-100	47	-
3	LOW	12030854	0	50-100	46	Yes
4	LOW	10907147	0	50-100	47	-
10	MEDIAN	10691389	0	50-100	46	Yes
16	HIGH	10744830	0	50-100	46	-
17	HIGH	10190109	0	50-100	46	-
18	HIGH	10427726	0	50-100	47	-
19	HIGH	11226801	0	50-100	46	Yes

Supplementary Table 3. BUSCO and Transrate statistics for both preliminary (prelim.) and final *Penaeus monodon* ovarian transcriptome assemblies.

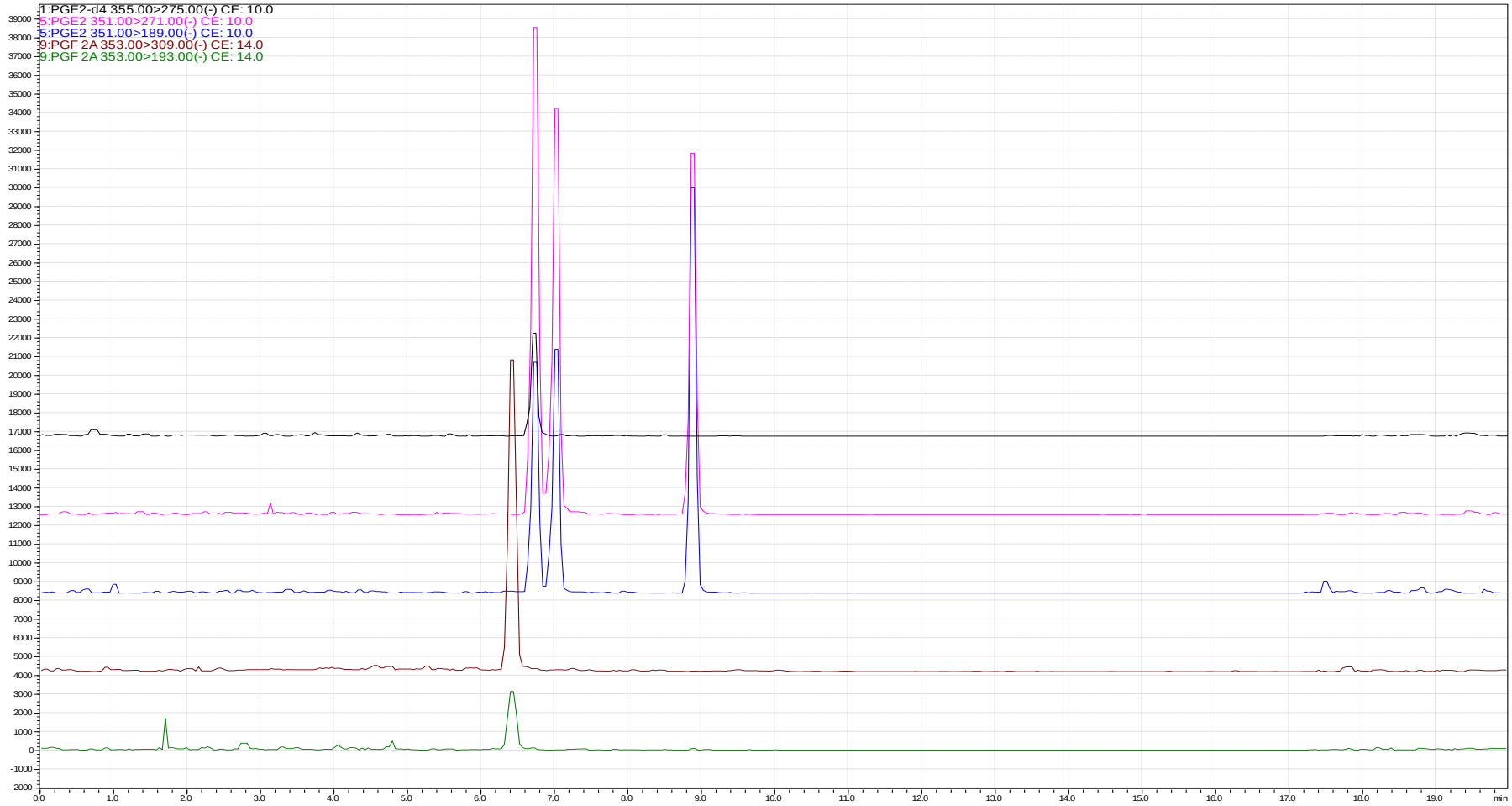
Parameter	Prelim. Assembly	Final Assembly
BUSCO		
Complete Single-Copy BUSCOs (%)	91	90
Complete Duplicated BUSCOs (%)	28	28
Fragmented BUSCOs (%)	5.2	5.1
Missing BUSCOs	3.6	4
Transrate		
contigs	44481	40171
smallest contig	224	224
largest contig	14125	14125
bases	47521243	43502525
mean contig length	1068	1083
contigs <200 bases	0	0
contigs > 1000 bases	13823	13028
contigs >10,000 bases	54	37
contigs with an open reading frame	14832	13955
mean % of contig covered by open reading frame	59.9	59.5
n90	390	405
n70	1071	1083
n50	2014	1968
n30	3206	3101
n10	5601	5423
GC content (%)	42.6	42.5
GC skew	-0.04	-0.05
AT skew	-0.03	-0.03
CpG ratio	1.5	1.5
bases that are N	0	0
linguistic complexity	0.18	0.18
read pairs provided	34323238	34322881
read pairs mapping	31277543	32145288
good mappings	27366314	30246112
bad mappings	3911229	1899176
potential bridges	11123	8989
bases uncovered	4742307	1815180
contigs containing at least one uncovered base	17567	13007
contigs with mean per-base read coverage of < 1	3085	2125
contigs with mean per-base read coverage of < 10	3402	32206
contigs with ≥ 50% estimated chance of being segmented	1790	1481
assembly score	0.39	0.61



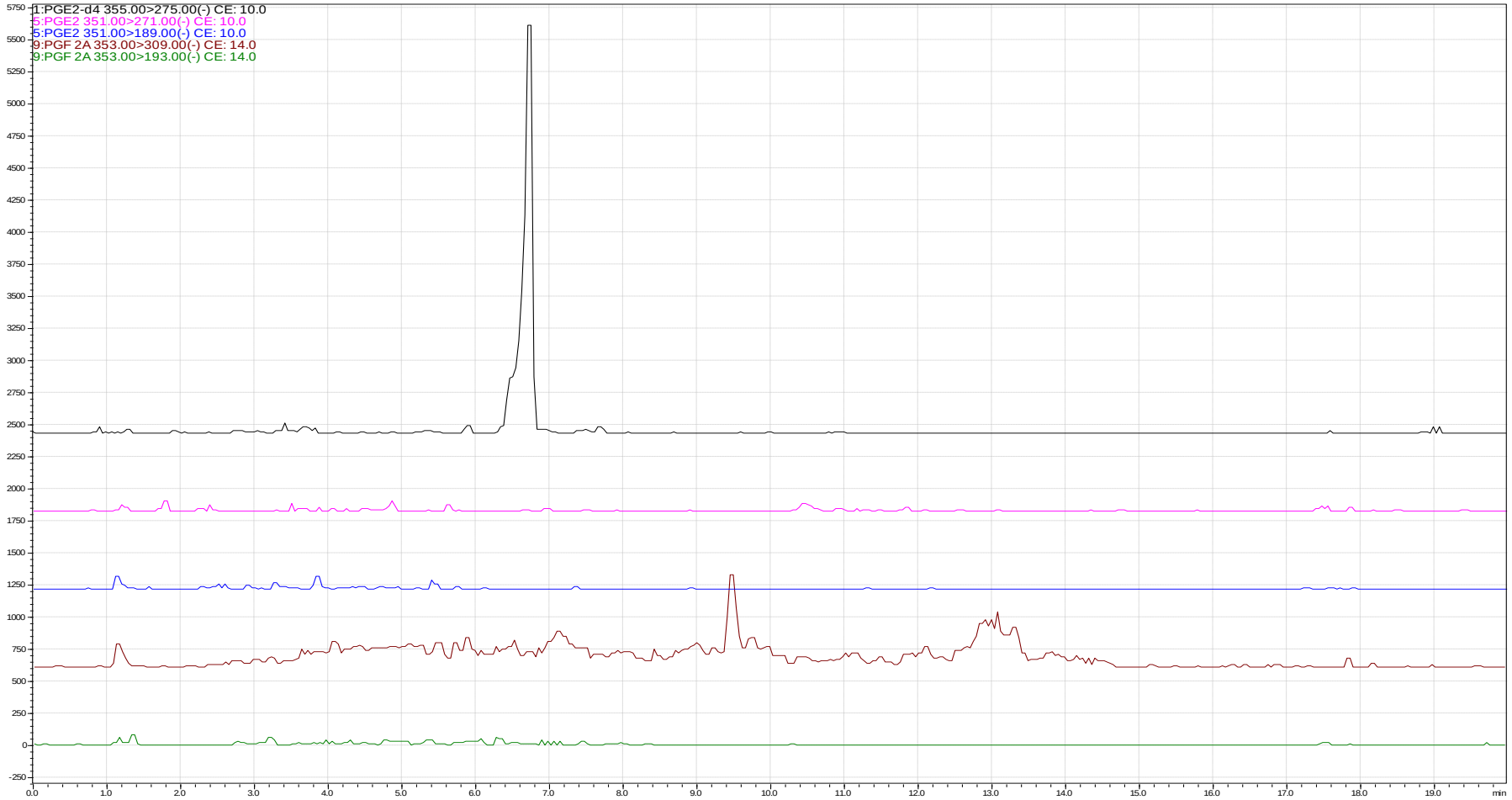
Supplementary Figure 1. Representation of the on-farm spawning facilities used in chapter 2. Both the primary vessel and nauplii boot are denoted by arrows



Supplementary Figure 2. Female broodstock weight in response to grow-out diet treatment. Female weight was sampled on a monthly basis, between three and seven months of age (i.e. the conclusion of the grow-out period). During the grow-out period female broodstock were reared on either a control diet (commercial pellet; blue) or a pelleted diet containing 10% microbial biomass (MB; green).



Supplementary Figure 3. Chromatograph showing detection peaks for all prostaglandin standards within the mixed standard. The single transition product of the deuterated prostaglandin E2 (PGE2-d4) is represented in black (355.0>275.0); the two transition products of prostaglandin E2 (PGE2,) represented in pink (351.0 >271.0) and blue (351>189.0) respectively; and the two transition products of prostaglandin F2α represented in red (353.0>309.0) and green (353.0>193.0) respectively.



Supplementary Figure 4. Chromatograph showing detection peaks for all prostaglandin present within 0.5g of extracted ovary tissue. The single transition product of the deuterated prostaglandin E2 (PGE2-d4) is represented in black (355.0>275.0); the two transition products of prostaglandin E2 (PGE2,) represented in pink (351.0 >271.0) and blue (351>189.0) respectively; and the two transition products of prostaglandin F2α represented in red (353.0>309.0) and green (353.0>193.0) respectively.