Enhancing Reproduction in Female Redclaw Crayfish (*Cherax quadricarinatus*) for Commercial Juvenile Production

Thesis Submitted by

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In fulfilment of the requirements for the degree of

Doctor of Philosophy (Agriculture, Environmental and Related Studies)

College of Public Health, Medical and Veterinary Sciences

James Cook University, Townsville, Australia

June 2024

Acknowledgements

I am deeply indebted to Almighty creator, the benevolent and sovereign, whose blessings and glory enlightened my thoughts and thrived my ambitions to complete this research and thesis for the degree of Doctor of Philosophy in the College of Public Health, Medical and Veterinary Sciences at James Cook University, Australia. Pursuing this PhD has been one of the most transformative experiences of my life, filled with challenges, curiosity, critical thinking, exploring possibilities, immense growth and fulfilment. This journey has been enriched by the incredible support and guidance of my mentors, peers, and loved ones.

I would like to convey my heartfelt gratitude, profound indebtedness and sincere appreciation to my research supervisor Prof. John Cavalieri for his continuous support, scholastic guidance, innovative suggestions, constructive criticism, and untiring inspiration during the entire period of the research work. He provided kind cooperation from the beginning to the end of my research work and writing up of the thesis within the stipulated period.

I would also like to give my sincere thanks to A/Prof. Chaoshu Zeng, Dr. Lisa Elliott and A/Prof. Leo Nankervis to help me at laboratory work and their continuous valuable suggestions, intellectual instructions, and encouragement. My sincere thanks also to Professor Estelle Venter for her kind cooperation and mentoring during the period of study. I am also thankful to A/Prof. Damien Paris for his early introduction to this project. I would also like to thank Dr Melissa Crowe and the DTHM Cohort Doctoral Studies Program for providing additional support in creating networking opportunities through programs such as research education workshops and writing retreats.

A special thanks to my fellow lab members and colleagues for the inspiring discussions, constructive comments and for all the fun we have had in the last few years. In addition, I wish to extend my gratitude to laboratory and technical staffs of Australian Crayfish, Veterinary college and Marine and Aquaculture Research Facilities for their assistance in quick setting of recirculating aquaculture broodstock rearing systems.

Finally, my deepest gratitude towards my loving family including my baby girl Ayzal Mahiz Maryam, caring husband Dr. Sheik Md Moniruzzaman, affectionate parents and siblings, empathetic parents-in-law for their unwavering support, encouragement and understanding during the countless hours I spent on my research.

May Almighty bless all these people with long, happy and peaceful lives.

June 2024

NUR UN NESA

Publication Records

Peer-reviewed publications from this thesis:

1. Chapter 3: **Nesa N.U.**, Elliott L., Zeng C., Jones R., Cavalieri J. 2023. Redclaw, *Cherax quadricarinatus* sex-separated rearing strategy enhances reproduction in females. Aquaculture 573, 739592. https://doi.org/10.1016/j.aquaculture.2023.739592.

Conference presentations from this thesis:

1. **Nesa N.U.**, Elliott L., Paris D.B.B.P., Jones R., Zeng C., Nankervis L., Cavalieri J. 2023. Parenteral hormone administration induces spawning in redclaw, *Cherax quadricarinatus*. 11th Indo-Pacific Fish Conference (IPFC) and Annual Conference of the Australian Society for Fish Biology, Auckland, 20-24 November 2023.
2. **Nesa N.U.**, Elliott L., Zeng C., Cavalieri J. Use of dissociated aquaculture to enhance reproduction in redclaw crayfish. Australian Society for Fish Biology 2022 Conference, Surfers Paradise, 6-10 November 2022.
3. **Nesa N.U.**, Elliott L., Zeng C., Paris D. Developing biomarkers for fertility in female redclaw crayfish to boost commercial production. World Fisheries Congress 2021, Adelaide, South Australia, 20-24 September 2021.
4. **Nesa N.U.**, Elliott L., Zeng C., Paris D. Egg and embryo technologies to improve commercial juvenile production in redclaw crayfish. Students 4 Students International Conference 2021, James Cook University, Townsville, Australia, March 31, 2021.

Manuscripts under review from this thesis:

1. Chapter 5: **Nesa N.U.**, Nankervis L., Elliott L., Jones R., Zeng C., Cavalieri J. 2023. Dietary supplementation of astaxanthin improves gonad development and reproduction rates of female redclaw crayfish, *Cherax quadricarinatus*. Under review in Aquaculture.

Statement of the Contribution of Others

The table below outlines the co-contributors' contributions to this thesis.

Chapter	Title	Nature of contribution of co-contributors
	Abstract	**Nur Un Nesa**: conceptualisation, writing-original draft **John Cavalieri**: supervision, writing-review and editing
1	General introduction	**Nur Un Nesa**: conceptualisation, writing-original draft **John Cavalieri**: supervision, writing-review and editing
2	Literature review - Intensive breeding potential of female redclaw crayfish, *Cherax quadricarinatus*: induction of ovarian maturation and improvements of egg and embryo quality to enhance hatchery productivity	**Nur Un Nesa**: conceptualisation, writing-original draft **John Cavalieri**: supervision, writing-review and editing **Lisa Elliott**: supervision, writing-review and editing **Chaoshu Zeng**: supervision, writing-review and editing **Damien B.B.P. Paris**: conceptualisation, supervision, writing-review and editing
3	Development of sex-separated rearing strategy to induce ovarian maturation and spawning in redclaw, *Cherax quadricarinatus*	**Nur Un Nesa**: conceptualisation, formal analysis, methodology, visualisation, writing-original draft **Lisa Elliott**: conceptualisation, resources, supervision, writing-review and editing **Chaoshu Zeng**: supervision, writing-review and editing **Rhondda Jones**: data curation, formal analysis **John Cavalieri**: conceptualisation, formal analysis, methodology, supervision, writing-review and editing
4	Development of hormone-based method to induce ovarian maturation and spawning in redclaw, *Cherax quadricarinatus*	**Nur Un Nesa**: conceptualisation, formal analysis, methodology, visualisation, writing-original draft **Lisa Elliott**: conceptualisation, resources, supervision, writing-review and editing **Damien B.B.P. Paris**: conceptualisation, writing-

		review and editing **Rhondda Jones**: data curation, formal analysis **Chaoshu Zeng**: supervision, writing-review and editing **Leo Nankervis**: supervision, writing-review and editing **John Cavalieri**: conceptualisation, formal analysis, methodology, supervision, writing-review and editing
5	Formulation of practical diet to induce spawning condition and improve egg and embryo quality in redclaw, *Cherax quadricarinatus*	**Nur Un Nesa**: conceptualisation, formal analysis, methodology, visualisation, writing-original draft **Leo Nankervis**: conceptualisation, methodology, supervision, writing-review and editing **Lisa Elliott**: conceptualisation, resources, supervision, writing-review and editing **Rhondda Jones**: data curation, formal analysis **Chaoshu Zeng**: supervision, writing-review and editing **John Cavalieri**: conceptualisation, formal analysis, methodology, supervision, writing-review and editing
6	General discussion and conclusion	**Nur Un Nesa**: conceptualisation, writing-original draft **John Cavalieri**: supervision, writing-review and editing

The following table shows the people and organisations who have contributed to this thesis.

Nature of assistance	**Contribution**	**Name and Affiliation of co-contributor**
Administrative support	Mentoring and for assistance with the research and recommendations	Prof. Estelle Venter, College of Public Health, Medical and Veterinary Sciences, James Cook University
Financial support	Stipend	James Cook University Postgraduate Research Scholarship (JCUPRS): James Cook University PhD Top-up Scholarship: CRC for Developing Northern Australia (CRCNA)
	Research funding and conference support	Barry Jonassen Freshwater Award: Australian Society for Fish Biology
Industry partner	Supply of animals, feeds, breeding facilities, and hatching incubators for carrying out this study	Australian Crayfish Hatchery (ACH)
Facilities support	Providing breeding facilities for carrying out diet study	Marine and Aquaculture Research Facility (MARFU), James Cook University

Statement of the Use of Generative AI

During the preparation of this thesis, I acknowledge the use of ChatGPT (https://chatgpt.com/) for initial concepts or explanations and QuillBot (https://quillbot.com/paraphrasing-tool) to paraphrase. The prompts used include Message ChatGPT and Paraphraser. The output from these prompts was used to rewrite to standard or fluent English.

Abstract

Redclaw *Cherax quadricarinatus*, are indigenous to Australia and inhabit a variety of freshwater habitats in northern Queensland and southern Papua New Guinea. Due to its morphological, ecological, and economic traits, redclaw are regarded as an outstanding and sustainable alternative for tropical aquaculture when compared to other aquaculture species. However, several factors limit the productivity of commercial aquaculture of redclaw, and intensive breeding techniques are likely required to ensure year-round productivity of juveniles with uniform sizes. Improvement of reproductive efficacy in intensively reared systems will also require optimisation of broodstock nutrition, rearing and spawning conditions and the use of assisted reproductive technologies to improve the quality and quantity of eggs. The general aim of the studies included in this thesis was to improve the production of juveniles by enhancing the production of eggs and larvae from females. Through a series of studies this was accomplished by developing a sex-separated rearing approach, using hormonal treatments to stimulate female ovarian maturation and spawning and dietary interventions to induce spawning and improve egg and embryo quality in female redclaw.

In Chapter 2, the importance and scale of production of the redclaw aquaculture industry, the reproductive biology of redclaw, and challenges currently faced in aquaculture were reviewed, focusing particularly on female reproduction as a critical limiting factor in crustacean aquacultural systems. Key constraints identified included asynchronous hatching, malformed hatchlings, and juvenile mortality. Various diagnostic techniques utilised to assess the fertility of female mammals were considered, and similar techniques could enhance the reproductive success of broodstock in decapod crustaceans. The evaluation of egg and embryo quality in decapod crustaceans using both conventional and novel diagnostic tools were reviewed. Additionally, the development of several assisted reproductive techniques such as natural and artificial induction of gonadal maturation and spawning through manipulation of rearing strategies, hormone administration, and optimisation of broodstock nutrition coupled with the establishment of protocols for artificial fertilisation of decapod crustaceans were examined. These technologies have the potential to enhance the efficiency of intensive aquaculture production systems aiming to produce high quality juveniles by managing female reproduction and ensuring consistent egg and embryo quality.

In Chapter 3, research investigated whether a sex separated system with (associated) or without (dissociated) pre-exposure of female redclaw to males could stimulate spawning and increase reproductive efficiency. The study was conducted in two phases: a dissociated phase lasting 111 days and an associated phase lasting 34 days. Redclaw were held in vertical recirculating aquaculture systems. During the dissociated phase, females were either kept alone (0M, n = 36) or exposed to 1 (1M, n = 36) or 2 (2M, n = 36) males suspended in the uppermost row of the

system. In the associated phase, females (n = 108) were maintained at a sex ratio of 1M:1F. Results indicated that during the dissociated phase, the spawning rate was less (p = 0.026) but the moulting rate was greater (p = 0.009) in the control group compared to the male exposed groups. In contrast, during the associated phase, there were no significant differences in spawning rate, mean days to spawning, moulting rate and mean days to moulting in the control and male exposed groups (p>0.05). However, the mean total number of eggs, fecundity, hatching rate and total number of juveniles produced during the associated phase were greater in the male exposed groups compared to the control group (p>0.05). It was concluded that, in a dissociated recirculation system, pre-exposure of redclaw females to males enhances spawning rate during the dissociated phase and subsequently increases egg and juvenile production during the associated phase. This breeding technique shows potential for enhancing hatchery productivity.

In Chapter 4, an investigation into increasing productivity of female redclaw focused on the effects of exogenous, intramuscular (IM) administration of methyl farnesoate (MF), serotonin (5-HT), or naloxone on the maturation of the ovaries and egg production. On Days 0, 5, 10, 15, and 20 of the study, control (100 µL IM crayfish saline) and MF (8.3×10^{-2} µg/g BW IM) treatments were administered while on Days 10, 15, and 20, 5-HT (1.3 µg/g BW IM) and naloxone (6.7×10^{-1} µg/g BW IM) were administered (n = 42/treatment). On Day 25, 8 females from each treatment were sacrificed for histological examination and the remaining females were paired with males. Comparisons among treatments revealed that crayfish treated with 5-HT and naloxone exhibited greater mean GSI and oocyte diameter compared to the control and MF treated groups ($p < 0.001$). Moreover, a higher percentage of crayfish treated with that 5-HT and naloxone spawned ($p < 0.05$) and the interval to spawning was less (p<0.05) compared to the control and MF treated groups. Additionally, the mean number of eggs/female, fecundity and hatching rate were significantly greater (p<0.001) in the 5-HT and naloxone treated crayfish compared to those administered the control and MF treatments. While treatment with MF increased moulting frequency, it also increased mortality rates without significantly improving maturation or spawning. In contrast, parenteral administration of 5-HT and naloxone increased egg production and hatching rate in an indoor hatchery setting outside the reproductive season.

In Chapter 5, research focused on whether nutritional supplementation for 75 days (n = 34/treatment) with either a base diet, astaxanthin (AX; 100 mg/kg), cholesterol (CHOL; 1 g/kg) or both AX (100 mg/kg) and CHOL (1 g/kg) could enhance gonadal development, spawning rate, egg yield and hatchability, and the total number of juveniles. After 4 weeks, 8 females from each treatment were sacrificed to measure GSI, and histological examination of the ovary and males were introduced with remaining females at 1M:3F. Results indicated that both AX and AX + CHOL supplementation significantly increased GSI and shortened the interval to

spawning compared to control and CHOL supplementation ($p<0.001$). Moreover, supplementation with AX alone resulted in an elevated spawning rate ($p = 0.005$) and larger oocytes, greater fecundity ($p<0.001$), the number of eggs/female ($p<0.001$), hatching rate ($p = 0.003$) and the number of hatched juveniles ($p<0.001$) compared to crayfish fed the control, CHOL, and AX + CHOL diets. Female crayfish with a body weight of ≥60 g compared to crayfish weighing < 60 g also produced more eggs ($p < 0.001$) and juveniles ($p<0.001$). In contrast, redclaw supplemented with CHOL had greater moulting rates ($p = 0.003$) compared to crayfish fed the other treatment diets. The results implied that astaxanthin, an antioxidant, positively enhanced the reproductive attributes of female redclaw crayfish as evidenced by greater GSI, oocyte diameter, spawning rate, eggs/females, hatching rate and number of juveniles produced.

In summary, studies reported in this thesis, describe a range of techniques for improving productivity in female redclaw. This includes the first report that sex-separated aquaculture of *C. quadricarinatus* can induce gonadal maturation and egg release and could be used as a technique to increase the numbers of eggs produced per female. In addition, unfertilised eggs produced using a dissociated and sex-separated technique could, in the future, be cryopreserved and utilised for artificial fertilisation. Additionally, administration of 5-HT and naloxone, at dosages used in this study, could synchronise egg production, accelerate spawning, and improve juvenile production. Furthermore, dietary supplementation with astaxanthin can improve the fertility of females which could result in a notable improvement in the productivity of redclaw hatcheries and the quality of eggs and embryos produced. Finally, the limitations of the applied methodology within the context of each study were outlined and prospects of further research were proposed for additional optimisation that could help the redclaw industry successfully implement these proven female fertility techniques for the long-term sustainable aquaculture production of this promising species.

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Chapter 1. General Introduction

1.1 Redclaw Crayfish

Redclaw *Cherax quadricarinatus*, are endemic to northern Queensland (Piper, 2000; Saoud et al., 2013), and southern Papua New Guinea (Jones, 1990). This species inhabits a variety of freshwater domains including stagnant ponds, small creeks, isolated rock pools, lakes, lagoons and fast-flowing rivers. Redclaw are non-burrowing species and prefer rocky territories for scavenging, foraging and also as shelter during moulting (Souty-Grosset et al., 2006). In comparison to other aquaculture species, redclaw are considered an outstanding and sustainable candidate for tropical aquaculture due to several physical, biological and economic characteristics. These include hardiness and tolerance to a wide range of environmental conditions: lower oxygen level (>1 ppm), varied hardness and alkalinity (20 to 300 ppm), a wide range of pH (6.5 to 9; Masser and Rouse, 1997; Ruscoe, 2002), and salinities (up to 12 ppt; Ruscoe, 2002). Additionally, redclaw are eurythermal and mesohaline (Meade et al., 2002), because they can maximize energy efficiency, gain weight and survive in broader range of temperatures typically with a temperature range of 26-29 °C and 21-22 °C during summer and winter, respectively (Karplus et al., 2003b; Riek, 1972). Redclaw can survive at temperatures as low as 10 °C, but growth rate is significantly impaired under such low temperatures (Karplus et al., 1998; Masser and Rouse, 1997).

Redclaw attain sexual maturity within one year and spawn multiple times annually (Karplus et al., 2003a; Masser and Rouse, 1997). Fecundity ranges from 300-1000 eggs/female (Jones, 1990). Moreover, direct development of juveniles from eggs without independent planktonic larval stages (Jones, 1990) and a non-aggressive nature (Jones, 1990; Masser and Rouse, 1997) facilitate simple breeding technology (Masser and Rouse, 1997). Commercially, redclaw aquaculture is profitable because animals can reach market size within 7 months (Luchini and Panné-Huidobro, 2008; Meade et al., 2002) to one year (Jones, 1990), even with the simple dietary supplementation (Ruscoe, 2002). In contrast to other crustaceans such as giant freshwater prawn (that require brine shrimp as food through all larval stages), juvenile redclaw can be fed the same diet as adults, which is beneficial from an economic, operational and management perspective (Curran et al., 2015). Redclaw also have a higher market acceptance due to their intense flavour, vivid colouration, and good flesh recovery (40% live weight; (Jones, 1990). In regards to their eating quality they make an excellent substitute for marron, yabby, lobster and tiger shrimp (Medley et al., 1994). Moreover, the availability of different strains with minimal genetic variation is regarded as a unique feature, suitable for the selective breeding program (Jones, 1990). Broodstock can be selected from existing pond stocks for advantageous traits such as a rapid growth and high fecundity. Genetic selection and domestication of redclaw can increase growth rates and production by 10% per generation

(Ruscoe, 2002). All these features have made redclaw an ideal species for aquaculture in tropical Australia.

The life cycle of redclaw involves several stages and typically begins with mating with moulted female as her exoskeleton is soft and more receptive. After mating the male deposits a spermatophore between the third and fifth pair of a female's walking legs (Masser and Rouse, 1997). The female will then release their eggs 12 to 24 h later which are then fertilised externally and attached to the females swimmerets (Yeh and Rouse, 1994). The female ensures offspring survival by careful incubation that lasts about 6 weeks, although the duration can vary with water temperature and environmental conditions (García-Guerrero et al., 2003b). Once the juveniles are capable of independent movement and feeding, they detach from the mother, moult regularly to increase in size and weight (García-Guerrero et al., 2003b).

1.2 Global Crayfish Market Value and Aquaculture Production

According to FAO-FIGIS (2020), global production of crayfish increased in Asia, Americas, Africa and Europe throughout the last two decades (2000-2017). The largest production came from red swamp crawfish, danube, marron, redclaw, yabby, and noble crayfish. Asia was the leading producer of crayfish in 2017, producing 1.13 million tonnes (t) with an estimated value of USD 10 billion (FAO-FIGIS, 2020), with China being the biggest contributor (1,129,708 t; USD 9.81 billion), followed by Malaysia (173 t). In the same year, the USA produced 63,626 t (USD 0.19 billion), Islamic Republic of Iran produced 36 t, and Armenia produced 20 t. However over the last one to two decades, countries in South America, Africa, Europe and Oceania saw considerable declines in crayfish production (FAO-FIGIS, 2020). The primary producers of redclaw are Australia, Argentina, Uruguay, Ecuador, and Mexico. In addition to these countries, redclaw cultivation is also practiced in Indonesia, Belize, China, Panama, Israel, Morocco, Spain and the USA (FAO, 2024). Between 2000 to 2015, the average yearly production of redclaw in Australia, Ecuador, and Mexico was 88, 75, and 30 tonnes, respectively, with all of each countries product being traded domestically due to the relatively small scale of production (Núñez-Amao et al., 2019). Based on the FAO-FIGIS (2020), the total production of redclaw in the continents of America, Asia and Oceania was 239 tonnes with an aquaculture value of 2,406,000 USD.

Australia's crayfish production has decreased from 409 to 149 t (USD 3.5 million) over the last two decades. Similarly, as the leading producer of farmed redclaw in Australia, Queensland's redclaw production dropped by 30.9%, from 31.2 tonnes in 2021-22 to 21.6 tonnes in 2022-23 (Table 1.1; DAF, 2024). The value of the redclaw industry in Australia decreased from USD 0.59 million in 2021-22 to $0.46 million in 2022-23. However, despite this decline, the average market price of redclaw rose from USD 17.99/kg in 2021-22 to USD 21.03/kg in 2022-23

(DAF, 2024). The failure of the industry to keep increasing its level of production in the face of global demand may suggest that there are factors limiting production. For instance, recurrent poor hatch rates with no determined cause have been observed which has likely contributed to declines in productivity (Valverde et al., 2020), necessitating further research to elucidate potential causes and to develop and optimise management strategies to boost productivity.

Table 1.1: Annual aquaculture production of redclaw (*Cherax quadricarinatus*) in Queensland, Australia (DAF, 2024).

Variable	**Year**									
	2014	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**	**2022**	**2023**
Production (t)	35.2	45.0	51.3	64.8	48.8	44.9	61.6	32.5	31.2	21.6
Gross value (USD million)	$0.5	$0.7	$0.9	$1.1	$0.9	$0.8	$1.2	$0.6	$0.6	$0.5

1.3 Redclaw Juvenile Production

In the 1990s, Queensland Department of Primary Industries recommended a simple method for cost-efficient production of redclaw, prioritising the need for optimum stocking density, water condition, dietary nutrition and shelter provision (Ruscoe, 2002). Traditionally, redclaw production is performed by separating juveniles into earthen ponds for grow-out to market size, without a controlled hatchery phase (Figure 1.1; Jones and Ruscoe, 1996; Masser and Rouse, 1997). However, the simple approach is limited to asynchronous breeding and significant size variation, which is not favourable to produce consistent size of redclaw juveniles (Rigg, 2021). Moreover, feeding trials of broodstock with commercial diets showed poor (average 32 to 35%) and highly variable (16.3 to 53.9%) juvenile survival (Valverde et al., 2020). These limitations necessitated an intervention in traditional juvenile production methods, with an emphasis on improving broodstock husbandry conditions and dietary supplementation.

Figure 1.1. Conventional production pattern of redclaw, *Cherax quadricarinatus* (FAO, 2024).

1.4 Limitations and Future Research Priorities

The redclaw industry is facing several production issues currently impeding its expansion. Traditional methods of redclaw production in earthen ponds are laborious (QCFA, 2013) and require meticulous maintenance of husbandry practices over a large area including feeding, disease management, predator control and harvesting (Jones, 1998). Production is seasonal and often impossible out of the spawning season with an unforeseeable harvest yield (QCFA, 2013). Mature redclaw broodstock are currently stocked by size without genetic records which may lead to inbreeding that eventually decreases genetic fitness (QCFA, 2013; Stevenson et al., 2013). In the absence of age records a proportion of stocked juveniles could be propagated that have markedly reduced growth rates compared to the average animal within a given group of juveniles of a similar age. These often have physical deformities such as a lesser head to tail ratio (Stevenson et al., 2013), which can contribute to variable sizes of individuals at the time of harvesting (QCFA, 2013) leading to low market acceptability. Moreover, juvenile growth and survival are very low (5 to 10%) due to improper husbandry, inadequate provision of protein-rich diets, and cannibalism which is prevalent among early juveniles (Jones, 1995b; Masser and Rouse, 1997). The ability to find solutions to overcome limitations in production is also hampered by insufficient capital for supporting research to address these issues (Bitomsky, 2008; Stevenson et al., 2013).

To overcome these problems, intensive breeding techniques that include hatchery and nursery phases are necessary to ensure increased productivity of uniform-sized juveniles (Jones, 1990; Saoud et al., 2013). Selective breeding of several commercially important crustaceans has shown promising results (Argue et al., 2002; Jerry et al., 2005). For example, selective breeding of the freshwater yabby, *Cherax destructor*, resulted in 32.7% heavier females by the third generation compared to unselected controls (Jerry et al., 2005), representing a 15.5% average genetic gain per generation. Similarly, a 21.2% increase in weight was observed in selectively bred Pacific white shrimp *Litopenaeus vannamei* (Argue et al., 2002). Unlike several other aquaculture industries, the redclaw industry still requires further development of an intensification system and selective breeding program to improve fertility, maturation, quality of eggs and ultimately productivity. While hatcheries are able to produce comparatively high-quality juveniles using the above-mentioned *in vitro* embryo incubation system, the numbers of fertilised eggs required for incubation are insufficient to meet the rising national and international demand (Lisa Elliott, personal communication). Even when pond farmers are stocking relatively high-quality hatchery produced juveniles, they often encounter significant variability in survival rates and unpredictable yields after the 6 to 9 months grow-out period (Jones and Valverde 2020). Moreover, at the hatchery level, juvenile production suffers from several bottlenecks including poor broodstock condition, asynchronous hatching, production of deformed larvae, parasitic infestation and huge juvenile mortality that are regarded as the major constraints to industry growth and export potential. When survival rates are low and yields are poor, the exact reasons are often unclear. These primarily could be due to the suboptimal methods currently used for producing fertilised eggs. To address this, it's essential to understand the underlying causes of subfertility and use techniques that enhance reproduction by improving egg quality, thereby contributing to overall juvenile production in aquaculture systems.

Improvement of reproductive efficiency (fecundity) is crucial for increasing juvenile production and relies on several important factors, including optimising husbandry practices, refining rearing and spawning conditions (both natural and hormonal), and ensuring proper nutrition for the animals. Methods that have been used and investigated to attempt to improve performance in decapod crustaceans such as crayfish, prawns, crabs have included techniques which aim to modify the normal physiological processes that affect reproduction. Some examples are eyestalk ablation to prevent the release of gonad and moult inhibiting hormones, administration of exogenous hormones such as methyl farnesoate, serotonin and naloxone to trigger the secretion of vitellogenin or gonad stimulating hormones, and supplementation of important nutrients such as proteins, vitamins, minerals, fatty acids and carotenoids to induce ovarian maturation and spawning in hatchery animals.

1.5 Research Aims

The aims of the review and studies presented in this thesis is to identify methods that can enhance juvenile production in female *C. quadricarinatus* by inducing gonadal maturation and spawning and improve egg and embryo quality. This was accomplished through testing the following hypotheses:

i) Inclusion of 1 or 2 males within the top level of a recirculating culture system will reduce the time interval to spawning and improve the reproductive performance of redclaw females.

ii) Intramuscular injection of methyl farnesoate, 5-HT and naloxone in females will increase the mean GSI, oocyte diameter, spawning rate, total number of eggs and decrease the mean interval to spawning compared to saline-treated Controls.

iii) Dietary supplementation with astaxanthin, and cholesterol alone or in combination in female redclaw will increase the mean GSI, spawning rate, total number of eggs, and juveniles produced and decrease the mean interval to spawning during the breeding period compared to Control group with no supplementation of astaxanthin and cholesterol.

This thesis is structured into the following chapters:

Chapter 1: General Introduction

Chapter 2: Literature Review: The review identified the viable methods for the intensive breeding of decapod crustaceans with the goal of enhancing reproduction and juvenile production in redclaw. The methods reviewed are non-invasive natural manipulations, hormonal applications, nutritional supplements, tools for evaluating the quality of eggs and embryos, and artificial fertilisation.

Chapter 3: The aim of this study was to develop a non-invasive technique that utilised maintaining females in close proximity to males to induce gonadal maturation and spawning in redclaw, *C. quadricarinatus* into a vertical recirculating aquaculture system. The study analysed the important reproductive parameters such as spawning rate, number of eggs produced, fecundity, hatching rate and number of juveniles.

Chapter 4: The main objective of this study was to investigate the effects of exogenously administered hormones on inducing ovarian maturation and spawning in female redclaw, *C. quadricarinatus.* In this study the effects of intramuscular injection of MF, 5-HT and naloxone was investigated on similar reproductive parameters that were recorded in the study described in Chapter 3.

Chapter 5: The aim of this study was to investigate whether dietary supplementation with astaxanthin and/or cholesterol could enhance reproductive development and the number of viable eggs in female redclaw. Similar to other studies effects on the gonadosomatic index,

oocyte diameter, spawning, egg production, hatching rate and number of juveniles produced were recorded.

Chapter 6: In this chapter, a general discussion, implications of the results, limitations of the studies and conclusion of the main thesis outcomes are presented.

Chapter 2. Literature Review - Intensive Breeding Potential of Female Redclaw Crayfish, *Cherax quadricarinatus*: Induction of Ovarian Maturation and Improvements of Egg and Embryo Quality to Enhance Hatchery Productivity

2.1 Abstract

Reproductive performance can limit production within aquaculture systems including crustacean aquaculture. To date, most studies of female fertility have focused on vertebrates, but invertebrate fertility has been remarkably under-explored. Factors such as asynchronous hatching, deformed hatchlings, and mortality among juveniles are major contributors to reduced reproductive performance in decapod crustaceans. This review discussed the development of several assisted reproductive techniques such as natural and artificial induction of gonadal maturation and spawning by novel rearing technique, hormone administration, improvement of egg quality by nutritional supplementation. These technologies have the potential to improve the efficiency of intensive aquaculture production systems by controlling female reproduction and improving the consistency of egg and embryo quality. Different diagnostic approaches are used to evaluate female fertility in mammals which could be applied to improve broodstock reproductive success in decapod crustaceans including redclaw crayfish. This review also evaluated the application of novel and more traditional diagnostic tools to decapod crustaceans for assessing egg and embryo quality coupled with the establishment of protocols for artificial fertilisation of decapod crustaceans which could be applicable in redclaw. Importantly, this will permit reproductive performance to be benchmarked and will allow infertile versus highly fecund female broodstock to be identified for selective breeding to increase the supply of juveniles and intensify production.

2.2 Introduction

Globally, aquaculture is one of the most rapidly expanding food-production industries, yielding over 82 million tonnes of seafood worth US$250 billion in 2019 (FAO, 2021). Crustaceans make a significant contribution (9.4 million tonnes, US$69.3 billion) to this sector and provide a valuable source of protein (FAO, 2021). Over the past decade, production of marine and freshwater decapod crustaceans, particularly shrimp, crab, lobster and crayfish, has grown steadily in various parts of the world from 5.48 million tonnes in 2010 to 10.48 million tonnes in 2019 (FAO-FIGIS, 2021). A crayfish is a freshwater crustacean that resembles a lobster, which possesses a hard exoskeleton, a segmented body, and jointed limbs, including large chelae on the front pair of legs. The abdomen also features five pairs of tiny appendages, which are largely employed for swimming and circulating water for breathing (Crandal et al., 2000; Crandall and Buhay, 2008). Redclaw has ranked as the second most important crayfish species after red swamp crayfish, *Procambarus clarkii* in terms of economic importance globally (Haubrock et al., 2021) Such recent growth has stimulated increased demand for juveniles to stock aquaculture production systems. This requires optimal and efficient breeding to meet growing demand for juveniles.

Reproductive performance significantly affects the spawning rate, fecundity, hatching success, and juvenile production of both wild-caught and hatchery-produced broodstock in decapod crustaceans (Keys and Crocos, 2006). Research indicates that several key factors influence broodstock reproductive performance in decapods, including broodstock husbandry condition, maturation practices including natural and hormonal manipulation and nutrient supplementation (Feng et al., 2023). Additionally, developing a suite of advanced fertility tools to identify and select highly fecund females for broodstock is necessary to maximise reproductive output Thus, implementing these management strategies will enhance the reproductive performance and overall fertility of female broodstock leading to improved production of juveniles (Harlıoğlu and Farhadi, 2017).

Control and acceleration of gonadal maturation is considered one of the priorities for commercial production of crustacean juveniles (Liu et al., 2014b). Several studies in shrimp and crayfish have used exogenous hormone treatment as a substitute for eyestalk ablation to trigger ovarian maturation and yield greater numbers of high-quality juveniles (Alfaro-Montoya et al., 2019; Kulkarni et al., 1992; Meeratana et al., 2006; Nagaraju et al., 2002; Ngernsoungnern et al., 2008; Ngernsoungnern et al., 2009; Tinikul et al., 2009; Treerattrakool et al., 2013). In the spiny lobster *Panulirus interruptus*, use of steroid hormone treatment reduced the time to reach sexual maturity in females (Nan et al., 2015). This review will focus on the most crucial reproductive hormones that influence gonadal maturation in female decapod crustaceans and therefore have the greatest potential for application within redclaw aquaculture systems. Female

decapods often fail to undergo synchronous ovarian development, oocyte maturation and ovulation, which ultimately may result in a failure of spawning at the required time within a production facility (Hewitt, 1992; Rahman and Ohtomi, 2020; Sun et al., 2018). This dysfunction might be caused by a lack of environmental cues (e.g., suboptimal temperature, light or appropriate nutrients) in captive females, resulting in a failure of endocrine signalling pathways to release hormones required to trigger oocyte maturation and release. Although, hormone-induced oocyte release is a relatively reliable method in practise over the past nine decades in the finfish industry (Hu et al., 2020a; Zohar and Mylonas, 2001), this method to induce spawning is largely unexplored in commercially important decapod crustaceans. Therefore, this review explored putative mechanisms and candidate hormones that might promote ovulation and oviposition in decapods particularly redclaw extrapolated from our knowledge in mammals and some invertebrates.

Due to the energy demanding nature of ovarian maturation and reproduction, dietary deficiencies may cause a decrease in the number of larvae produced, which may have an impact on the hatchability and survival of the larvae (Hernández-Abad et al., 2018; Thien and Yong, 2017). The reproductive capabilities of crustaceans are influenced by different dietary components (Díaz-Jiménez et al., 2019; Harlioğlu et al., 2012; Thien and Yong, 2017). Crustacean reproductive performance is influenced by several dietary components (Díaz-Jiménez et al., 2019; Harlioğlu et al., 2012; Thien and Yong, 2017). In particular, it has been demonstrated that feeding decapod crustaceans supplements containing protein, carotenoids, lipids and fatty acids enhances ovarian maturation, spawning rates, and fecundity (Barim-Öz and Şahin, 2016; Niu et al., 2014). This chapter delineated important nutrients that can influence the reproductive efficiency of decapod crustaceans, transferrable to other commercially viable species such as redclaw.

Egg quality or oocyte competence refers to the ability of an egg to generate a viable embryo (Bobe and Labbé, 2010; Bonnet et al., 2007; Brooks et al., 1997; Kjørsvik et al., 1990). In this review, the term oocyte refers to an unfertilised female gamete that has not yet been ovulated and oviposited. While egg is the newly oviposited oocyte that has/has not yet been fertilised nor completed syngamy and embryo is the fertilised egg that has completed syngamy (fusion of male and female pronuclei) to form a 1-cell zygote, followed thereafter by cell division. Production of superior quality oocytes is crucial for improved embryo survival and optimal reproductive performance of broodstock (Migaud et al., 2013), thus developing methods to assess egg and embryo quality is critical (Migaud et al., 2013; Swetha et al., 2011). However, the heavy yolk content and various maternally derived compounds in egg cytoplasm, make it more difficult to visually evaluate egg and early embryo quality when compared with, for example, sperm (Bobe, 2015). Current assessment of egg and embryo quality is predominantly

based on traditional morphological parameters such as ovarian index (OI) or the number, diameter, dry weight, and colour of eggs (Churchill, 2003; Coleman et al., 2019; Habashy et al., 2012). In mammals and finfish, however, advanced tools that evaluate factors such as cell viability, mitochondrial function, and DNA damage have been employed as prospective markers of egg and embryo quality in addition to more traditional techniques for assessing morphological development (Browman et al., 2003; Cerdà et al., 2008; Hoelker et al., 2006; Sturmey et al., 2008; Van Blerkom, 2008). To our understanding, effective markers for assessing egg and embryo quality have not yet been established in commercially important decapod crustaceans including redclaw. Several potential markers could include fertilisation and hatching rates, embryo heart rate, as well as molecular markers employed in other species such as cell viability, mitochondrial function, and DNA damage (Agnello et al., 2017; Arcos et al., 2003; Bukowska et al., 2012; Khosravi-Farsani et al., 2010; Yűce and Sadler, 2001).

The development of artificial fertilisation (AF) techniques is the ultimate component toward accomplishing a successful intensive breeding programme. The high economic value of redclaw and their huge demand in the global market have prompted the need for AF technique to be established. AF offers several potential advantages that include supply of sperm anytime and anywhere through the use of cryopreserved gametes; which avoids the movement of whole animals and thus controls disease transmission by eliminating the introduction of potential pathogens into the breeding system (Colenbrander et al., 1993; Parkinson and Morrell, 2019). AF also saves space by reducing the number of males needed in the rearing system, and promotes trade in valuable genetics via global shipment of sperm as in the cattle industry, which can further accelerate selective breeding (Parkinson and Morrell, 2019). This novel source of genetic exchange could be critical in overcoming the continued dependence of industry on wild-caught individuals to supplement hatchery broodstock, which combined with high rates of disease prevalence put the sustainability of the decapod industry at significant risk (Browdy, 1998; De Grave et al., 2015). Moreover, in traditional aquaculture systems, spawning is typically limited to an animal's seasonal cycle of reproduction. AF coupled with hormone-induced ovarian maturation/ovulation could facilitate the production of fertilised eggs at any time of the year, ensuring year-round production and expansion of the redclaw industry.

The present review assesses the induction of ovarian maturation and spawning following natural manipulation, hormone treatment, RNA interference and monoclonal antibody technology, dietary intervention for improving egg quality, development of fertility tools for evaluating egg and embryo quality, as well as AF techniques in female decapod crustaceans. Optimisation and validation of female fertility tools and intensive breeding strategies could maximise the number and viability of early embryos, which is critical to ensure consistent year-round production of juveniles to supply the commercial redclaw industry.

2.3 Taxonomy, Habitat, and Distribution of Redclaw Crayfish

Freshwater crayfish belong to the order Decapoda, which also includes crabs, prawns, lobsters, and Moreton Bay bugs (Courtney, 2002; Crandall and Buhay, 2008; Schweitzer and Feldmann, 2014; Wolfe et al., 2019). Crayfish are believed to exist since the Triassic period 185-225 million years ago (Crandal et al., 2000; Crandall and Buhay, 2008).

Although lobsters and crayfish have a similar external skeleton and segmented body, they do not belong to the same family. Lobsters are exclusively marine creatures and include several species where Homarus (large-clawed), Nephrops (small-clawed), and palinurid (spiny or rock, without claws) lobsters dominate the fisheries (Penn et al., 2001). The American (*Homarus americanus*) and European (*H. gammarus*) lobsters are two major commercial species that inhabit different oceans of the world (Jørstad et al., 2004; Plagányi et al., 2018). Moreton Bay bugs (flathead or slipper; *Thenus spp*,) live abundantly on the seabed of tropical regions along the Western Australian and the Queensland coast (Courtney, 2002; Holthuis, 1991). In contrast, crayfish live in freshwater habitats including rivers, lakes, streams, ponds, burrows and caves (Crandall and Buhay, 2008). All freshwater crayfish species belong to one of three families: Astacidae, Cambaridae or Parastacidae, with their native distribution across different continents. For example, the natural distribution of Astacidae and Cambaridae was exclusively in the northern hemisphere, with Cambaridae confined to the American continent and Astacidae predominantly found in Europe and some part of western USA (Crandall and Buhay, 2008).On the other hand, Parastacidae is distributed across Australia, New Zealand, New Guinea, Madagascar and certain regions of America (Austin, 1996; Crandall and Buhay, 2008).

Parastacidae consists of 13 genera including the most widespread and distinctive genus (*Cherax*) in the southern hemisphere (Austin, 1996). Ten species exist within the *Cherax* genus, three of which are commercially farmed i.e., yabby (*C. destructor*), marron (*C. tenuimanus*) and redclaw crayfish (*C. quadricarinatus*; Piper, 2000). Although a late arrival to the aquaculture sector (Saoud et al., 2013), compared to yabby and marron, it has been suggested that redclaw has greater production potential (Jones, 1990).

2.4 Basic Biology of Redclaw Crayfish

2.4.1 Anatomy

Anatomically the body of redclaw is divided into the abdomen (tail) and the cephalothorax (head; Figure 2.1; Jones, 1990). The cephalothorax is covered by the carapace, which shields the internal organ, and is toughened by a rostrum at the forefront. Despite possessing large eyes, eyesight of redclaw is proportionately poor, with the primary sensory organs being the large antennae and antennules. Antennae are only used as touch sensors while the antennules are

transducers for both touch and taste and used for locating food and recognising water parameters such as temperature and salinity (Jones, 1990).

Figure 2.1. General anatomy (dorsal view) of redclaw crayfish, *Cherax quadricarinatus* (Jones, 1990).

Redclaw have two large claws (chelipeds), four pairs of walking legs (pereiopods), and swimming legs (pleopods) situated between segments 2 to 5 of a six-segmented abdomen. Female crayfish use fine hairs on their pleopods to clutch their eggs. The pleopods of the sixth abdominal segment become extended to form the tail fan, which helps quickly propel the animal backward to evade predators. The abdomen of redclaw is also used to protect eggs during incubation by folding to form a temporary brood chamber (Jones, 1990).

Sexually dimorphic structures are clearly distinguishable among adult redclaw. The male cheliped is larger and exhibits a soft, vivid red membrane on the external surface of the propodous (Curtis et al., 2007; Karplus et al., 2003b). Additionally, redclaw has two other anatomical features: the statocyst, used to maintain body balance; and gastrolith, needed to harden the newly moulted shell (Jones, 1990).

2.4.2 Reproduction

Although in redclaw, several types of intersex individuals can arise as a form of non-functional hermaphroditism, they are considered gonochoristic (Ghanawi and Saoud, 2012; Parnes et al., 2003; Sagi et al., 1996a). Depending on their sex, testes or ovaries are in the thorax on top of the hepatopancreas. Gonad size varies according to maturity status of individual crayfish (McLay and van den Brink, 2016). In redclaw, the presence of prominent cement glands on the female's abdomen is evidence of sexual maturity when carapace length reaches 42 mm (McLay and van den Brink, 2016). Usually, redclaw attain sexual maturity after 6-12 months at a body weight of

100-120 g (Jones, 1990). During the breeding season, female ovaries expand in size and contain many yellow-brown eggs (Vogt, 2002).

The northern equatorial redclaw populations are multiple spawners, could spawn more than four times a year (Jones, 1990; Jones, 1995a; Masser and Rouse, 1997; Sammy, 1988). Barki et al. (1997) identified three spawning events in redclaw under laboratory condition at 26-28 °C temperature and 14L:10D photoperiod. On average female spawned three times mostly during spring and summer season of a year similar to three spawning events during rainy summer (26-29 °C) and twice during same summer season when it starts to cool and dryer (21–22 °C), observed by Sammy (1988). Unlike some other decapod crustaceans, moulting does not have to take place before copulation (Ghanawi and Saoud, 2012). The common successive spawning and moulting pattern in redclaw is spawn–spawn–moult or spawn–moult–spawn (Sagi et al., 1997). As breeding season approaches, in response to external environmental stimulation, female redclaw starts to prepare for spawning by conditioning the ovary and cleaning the pleopods (Jones, 1990). During conditioning, the ovary becomes responsive to external stimulation which ripens them, resulting in the release of ova through the oviduct towards the gonopore to maximise oviposition and fertilisation success during spawning (Jones, 1995a).

Reproduction in crayfish is generally initiated by female who releases urine in water that triggers the onset of courtship behaviour (Berry and Breithaupt, 2010). Redclaw reproductive behaviour includes fighting and mating (Barki et al., 2003), with three stages of mating behaviour: pre-copulation, copulation and post-copulation (Barki and Karplus, 1999). Pre-copulatory behaviour comences with the female or male searching for a mate in its shelter (Barki and Karplus, 1999). Thereafter male and female face each other with chelipeds upheld. The female uses her chelae to make constant contact with the male chelae and cephala. Sometimes this face-to-face position may lead to aggression and fighting. Copulation demonstrates the cooperation between males and females. The female pushes the male downward while he curls his abdomen to insert himself under the female where he finally unfolds in a supine position. With their ventral surfaces facing each other, the male uses his telson to continually force the female’s sternum upward and bring her abdomen closest to him. Thereafter, the male pereiopods embrace the female's carapace and the pair remain in a frozen male-beneath-female posture. The male then successfully deposits a spermatophore between the third and fifth pair of walking legs (Masser and Rouse, 1997) of female which rapidly solidifies (Figure 2.2; López Greco and Lo Nostro, 2008). Female terminates copulation with a rapid tail-flick that propels her backward and upward, thereby detaching her from the male.

Figure 2.2. Spermatophores (sp) attached to the sternum of a freshly berried female redclaw, *Cherax quadricarinatus* (Photos by Laura López Greco reproduced from McLay and van den Brink, 2016).

During post-copulation, the male assumes a dominant posture by taking an upright position lifting his body and chelipeds, trying to make contact with females via his antenna. By contrast, the female avoids the male by lowering her body while rubbing her sternum with her walking legs to break open the deposited spermatophore and fertilise her eggs (Barki and Karplus, 1999).

2.4.3 Egg Developmental Stages

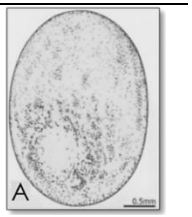
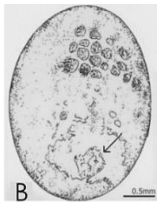
Jones (1990) characterised developmental stages of fertilised eggs based on morphological changes during *in vitro* incubation. At stage 1, immediately after release, eggs are round, dull olive-green and about 2 mm long and 1 mm wide. At stage 2, eggs appear chocolate-brown and become more rounded by increasing in size. This stage is very short and does not appear in every egg. At stage 3, eggs appear orange and opaque, with no clear embryo development visible. At stage 4, eggs appear red and become partially transparent, with yolk of variable consistency. At stage 5, dark eye spots become partially visible through red eggs and continue to develop. At stage 6, appendages appear beneath the eyes as the red yolk sac of the egg case. At stage 7, red yolk is lost, and the larvae are released completely from the egg capsule. This last stage is described as 'hatched and attached' since the larval crayfish remain attached to the female's pleopods for some time before becoming independent. The duration of each stage is shown in Figure 2.3.

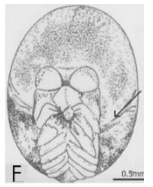
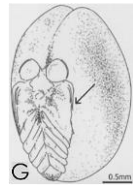
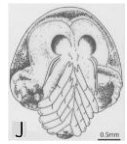
Figure 2.3. Average duration of each of 7 stages of redclaw (*Cherax quadricarinatus*) fertilised egg development during external incubation at 24.5-27.6 °C and 14L:10D (Jones, 1990).

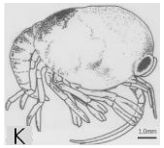
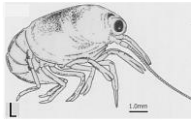
Similar egg developmental stages with a clear indication of days of development has been reported by (Yeh and Rouse, 1994). However, the time between spawning and hatching was shorter as eggs were incubated at a higher water temperature (28 ± 1 °C) and increased photoperiod (14L:10D). As such eye-stage embryos were observed at Day 22 and larvae were released at Day 35-40 (Yeh and Rouse, 1994) compared to Day 35 and Day 56-71 respectively in the study by Jones (1990). Thereafter, hatched and fully independent juveniles grow rapidly by 50 - 100 g to become adult within one year, and will survive as adults for 4-5 years (Jones, 1990).

In another study, thirteen stages of embryo development was described for redclaw (Table 2.1; García-Guerrero et al., 2003b).

Table 2.1: Embryonic development of redclaw, *Cherax quadricarinatus* (García-Guerrero et al., 2003b).

Stage	Development (%)	Duration (days)	Characteristics	Figure
1	10	1-3	Newly spawned fertilised eggs are oval, yolky with no evidence of cell division by stereomicroscope, even after eggshell puncture. Yolk droplets and some tissue formation is visible at Day 3. Progressive cell division gives rise to the germinal disk on the ventral surface of the egg after three days.	
2	10 to 20	4-6	Complete division of yolk into tiny droplets, a patch of white cells (arrow) in the lower part that spreads forming a depression of gastrulation. Blastopore appears in the ventral part of gastrula and caudal papilla starts to develop.	
3	20 to 30	7-9	Appearance of primordial eyes, three pairs of antennules, antennae, and mandibles, at the back of eyes, Post-naupliar somites grow (arrow) in between caudal papilla.	
4	30 to 40	10-12	Undifferentiated but pronounced eye lobes with thicker and darker edges. More defined and larger antennules, antennae, and mandibles. Cephalic appendages like maxillules and maxillae start growing. Caudal papilla forms as a horseshoe shape (arrow).	

Stage	Development (%)	Duration (days)	Characteristics	Figure
5	40 to 50	13-15	Formation of all abdominal somites. Bulging eyes (arrow), antennules, antennae, and larger, folded caudal papilla. The heart (dorsal) beats regularly.	
6	50 to 60	16-18	Larger spherical eyes and short rostrum between eyes. Continuous egg and embryo contractions and transverse medial groove (arrow) in the yolk. Mandibles and antennae longer, folded backwards of the head. Abdominal segments and the presence of rudimentary pereiopods with chelae.	
7	60 to 70	19-21	Larger embryo with differentiated and pigmented eyes, folded antennae towards chelipeds (arrow). Three pairs of chelipeds in the frontal part of the body. Differentiated larger mouthparts. Carapace deepens laterally forming cavities of branchial chambers.	
8	70 to 80	22-24	Deeper yolk grooves, fully pigmented eyes with cornea on the dark zone of eyes. Appendages differentiated and chelipeds cover mouthparts; pereiopods enlarge until the edge of the posterior labrum. Embryo is capable of movement.	
9	80 to 90	25-27	Larger eyes extending beyond cephalothorax with two separated eye lobes. Rostrum is thicker and larger. Thoracic appendages and chelipeds completely develop. Embryo entirely takes a ventral position.	
10	hatching	28-31	Embryo hatches but attached to the loose strand of the chorionic membrane. Pereiopods extend. Pereiopods moving, appendages are functional and like an	

Stage	Development (%)	Duration (days)	Characteristics	Figure
			adult. Absence of hair setae.	
11	Post-embryo I	32-36	Chorion is lost and first hatched stage. Convex cephalothorax, transparent body, sessile eyes. Released antennules and antennae curved backwards. Curled abdomen with the complete formation of pleopods on each somite. All adult appendages develop except uropods. No independent feeding and locomotion.	
12	Post-embryo II	37-41	Cephalothorax acquires final shape. Pigmentation on the upper side of carapace and setae hair appear on pereiopods. Eyestalk develops and rostrum emerges between eyes. Abdomen straightens and telson and uropods are evident.	
13	Juvenile	42	First stage juvenile capable of independent locomotion. Extended abdomen, continuous movement of pleopods and pereiopods. Gastroliths visible inside cephalothorax. Separated uropods and telson bearing many setae. All pereiopods, antennae and rostrum are hairy. Carapace translucent, but pigmentation begins with the appearance of red spots on the entire body.	

2.5 Reproductive Biology of Female Freshwater Crayfish

2.5.1 Female Reproductive Biology and Ovarian Maturation

Crustacean reproductive systems are highly diverse (Nagaraju, 2011) and those of commercially or ecologically important crustaceans have been studied extensively (Ando and Makioka, 1998; Vallina et al., 2014; Vazquez et al., 2008; Wortham-Neal, 2002). The reproductive biology of crustaceans spans several areas including the structural anatomy of the gonads (testis and ovary), size and appearance of the gonad at sexual maturity, gametogenesis, ovulation and fertilisation, and embryo development that shapes the hatching of early juveniles (Subramoniam, 2017a). Understanding these processes, particularly for female crayfish, is often essential for sustainable management and prudent exploitation of crayfish resources. The crayfish industry is rapidly developing to meet the demand in local and international markets. Since berried females are limited in their natural habitat, the production of berried females in captive aquaculture systems is becoming essential to maximise egg and offspring production.

In redclaw, different components of the female reproductive system have been reported focussing on ovarian physiology, egg (oocyte) development including yolk formation, vitellogenin (Vg) synthesis, and reproductive endocrinology (Abdu et al., 2000; Sagi et al., 1996b; Soroka et al., 2000).

2.5.1.1 Structure of the Reproductive Tract and Ovaries.

The redclaw female reproductive tract consists of paired ovaries, short and narrow oviducts, and genital apertures situated at the base of the third pereiopodal segment (Abdu et al., 2000; Vazquez et al., 2008). The ovary resembles a sac that consists of a pair of anterior ovarian lobes and a single posterior ovarian lobe (Figure 2.4A). Anterior ovarian lobes are connected posteriorly via paired connectors. The oviducts are short, straight, spherical, and translucent pipe-like structures that extend from the posterior ovarian lobes to the gonopores located on the base of each of the third pereiopods (Ando and Makioka, 1998; Vazquez and López Greco, 2007; Vazquez et al., 2008). The proliferative zone, positioned in the medial gonad on the surface of the ovarian lumen, contains copious oogonia, which are encircled by oocytes in a tangential position (Vazquez and López Greco, 2007; Vazquez et al., 2008).

The ovarian lumen persists through all ovarian developmental stages but is most pronounced during fully mature stages. It is lined by a single-layered ovarian cuboidal epithelium. The ovarian epithelium swells and folds repeatedly generating a form of ‘oogenic pouch’, as well as surrounding the follicle cells of secondary oocytes that are present in the interstitium (Ando and Makioka, 1998; Vazquez and López Greco, 2007; Vazquez et al., 2008). The ovary is also lined by an ovarian sheath made up of two layers. The inner muscular tunica layer consists of multilayered (usually three to four layers) smooth muscle cells. The outer layer of the sheath

consists of a thick single layer of connective cells. The proliferative zone is centrally positioned on the ovarian epithelium while the oocytes are located between the ovarian epithelium and the connective external tunica (Figure 2.4B; Vazquez, et al., 2008).

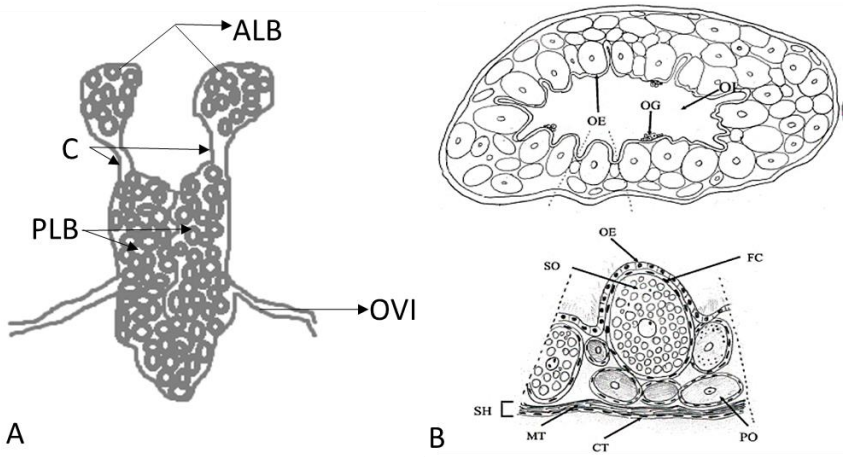


Figure 2.4. Redclaw crayfish, *Cherax quadricarinatus*, ovary. (Reproduced from Vazquez et al. (2008) with permission from Invertebrate Biology, John Wiley and Sons). (A) Sac-like ovary containing anterior and posterior lobes. (B) Cross-section of the ovary surrounded by a sheath. Follicle cells encircle all types of oocytes while the ovarian epithelium predominately envelops secondary oocytes. Oogonia reside in the epithelium adjacent to a central lumen. ALB, anterior ovarian lobes; C, connectors; PLB, posterior ovarian lobes; OVI, oviduct; CT, connective tissue; SH, ovarian sheath; MT, muscular tunica; PO, primary oocyte; SO, secondary oocyte; FC, follicular cells; OE, ovarian epithelium; OL, ovary lumen; OG, oogonium

2.5.1.2 Developmental Stages and Maturation of the Ovaries.

The immature ovary undergoes three structural changes during maturation, appearing as an ovary with: (i) disconnected parallel strands, (ii) a modified H-shape, and (iii) a post-spawning Y-shape (Figure 2.5A, 2.5B and 2.8A). In decapod crustaceans, histology is widely used as a method for assessing ovarian maturation, as it allows for the identification of different stages of oocyte maturation, from early oogonia (immature reproductive cells) to fully mature oocytes (Arcos et al., 2011; Palacios et al., 1999). In redclaw, four ovarian developmental stages can be identified according to ovary colour, histological appearance and the presence of different oocyte types (Vazquez et al., 2008).

The stage I ovary marks the beginning of ovarian maturation, resembling transparent tandem parallel strings that later fuse to form the H-shaped ovary (Figure 2.5A; Vazquez, et al., 2008). At this stage, acidophilic primary oocytes (122-125 μm) with homogenous cytoplasm and small oogonial nests of 5-10 cells (11 μm) are visible. Both primary oocytes and oogonia are surrounded by basophilic follicular cells (Figure 2.5; Vazquez, et al., 2008).

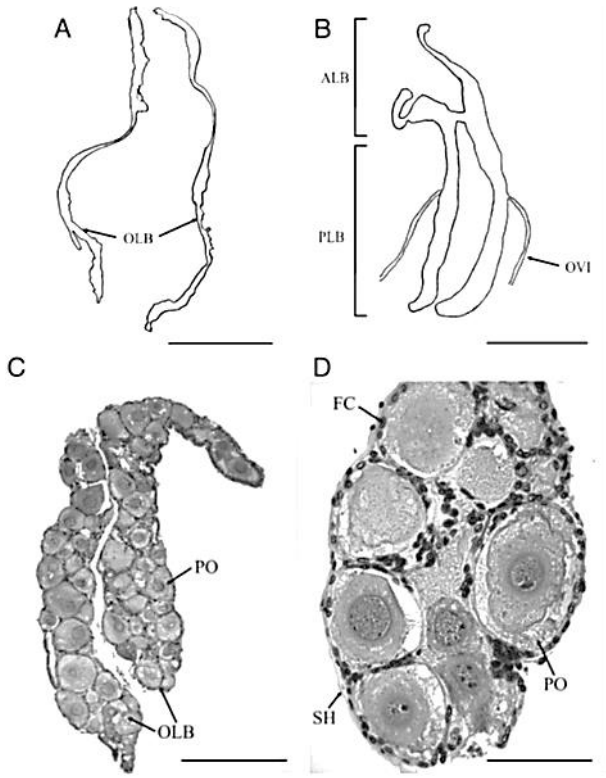


Figure 2.5. Redclaw crayfish, *Cherax quadricarinatus*, stage I ovary. (Reproduced from Vazquez et al. (2008) with permission from Invertebrate Biology, John Wiley and Sons). (A) Parallel ovarian strands, (B) H-shaped ovary, (C) Histology of longitudinal and (D) transverse ovarian sections stained with haematoxylin-eosin and Masson-Trichrome. (Scales: 130, 510, 195 and 78 mm respectively). OLB, ovarian lobes; ALB, anterior ovarian lobes; PLB, posterior ovarian lobes; OVI, oviduct; PO, primary oocytes; FC, follicular cells; SH, ovarian sheath

The H-shaped ovary at stage II increases in size and changes to a light orange colour. Connectors and the ovarian lumen appear that facilitate the evacuation of full-grown oocytes from the ovary (Vazquez and López Greco, 2007). Though primary oocytes are the most common types of cells in this ovary, intermediate oocytes of similar size appear for the first time and are identified by the presence of tiny yolk platelets at their periphery. Both primary and intermediate oocytes have regions of homogeneous and heterogeneous (due to mucopolysaccharides) cytoplasm. Oogonia and follicular cells have a similar appearance and form as at stage I. (Figure 2.6; Vazquez, et al., 2008).

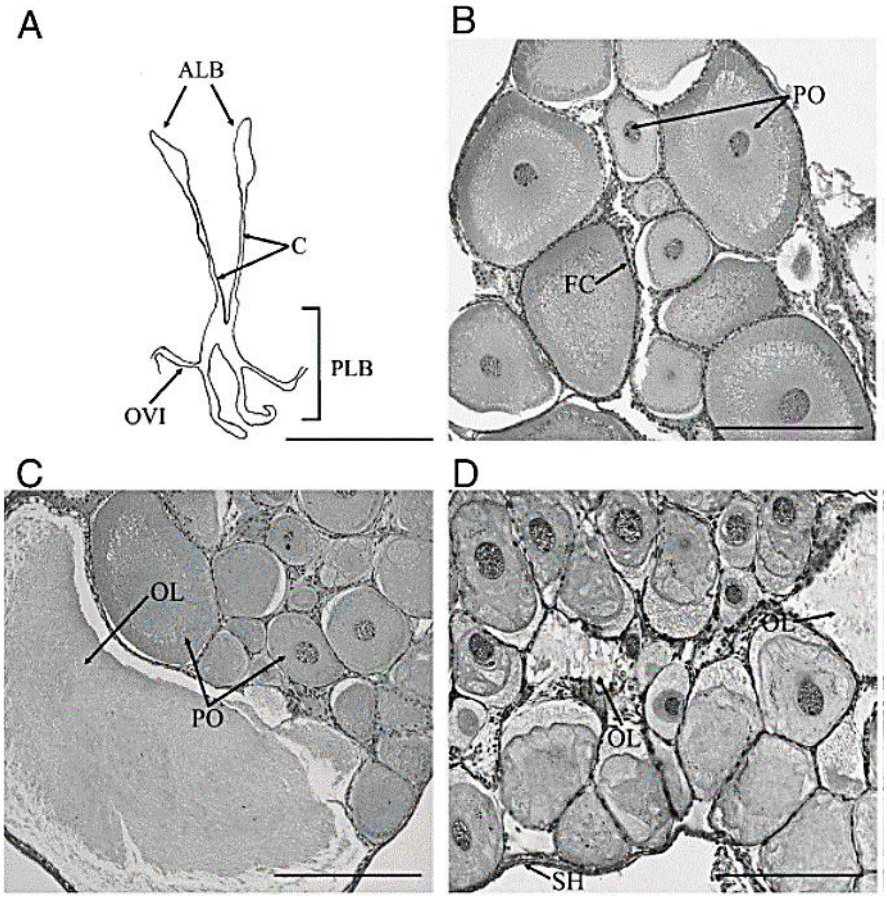


Figure 2.6. Redclaw crayfish, *Cherax quadricarinatus*, stage II ovary. (Reproduced from Vazquez et al. (2008) with permission from Invertebrate Biology, John Wiley and Sons) (A) Differentiation of connectors in H-shaped ovary, (B, C, and D) Histology of different haematoxylin-eosin stained ovarian cross-sections. (Scales: 1200, 140, 220 and 210 mm, respectively). ALB, anterior ovarian lobes; OVI, oviduct; C, connectors; PLB, posterior ovarian lobes; FC, follicular cells; PO, primary oocytes; OL, ovarian lumen; SH, ovarian sheath

Stage III is characterised by the presence of olive-green primary, intermediate and a few secondary oocytes (Figure 2.7). The ovary is now larger than stage II, with the first appearance of large secondary oocytes (460-480 µm in diameter) signifying the onset of sexual maturity in redclaw. Secondary oocytes have eosinophilic cytoplasm that contains yolk droplets and globules and are surrounded by pycnotic follicular cells (Figure 2.8; Vazquez, et al., 2008).

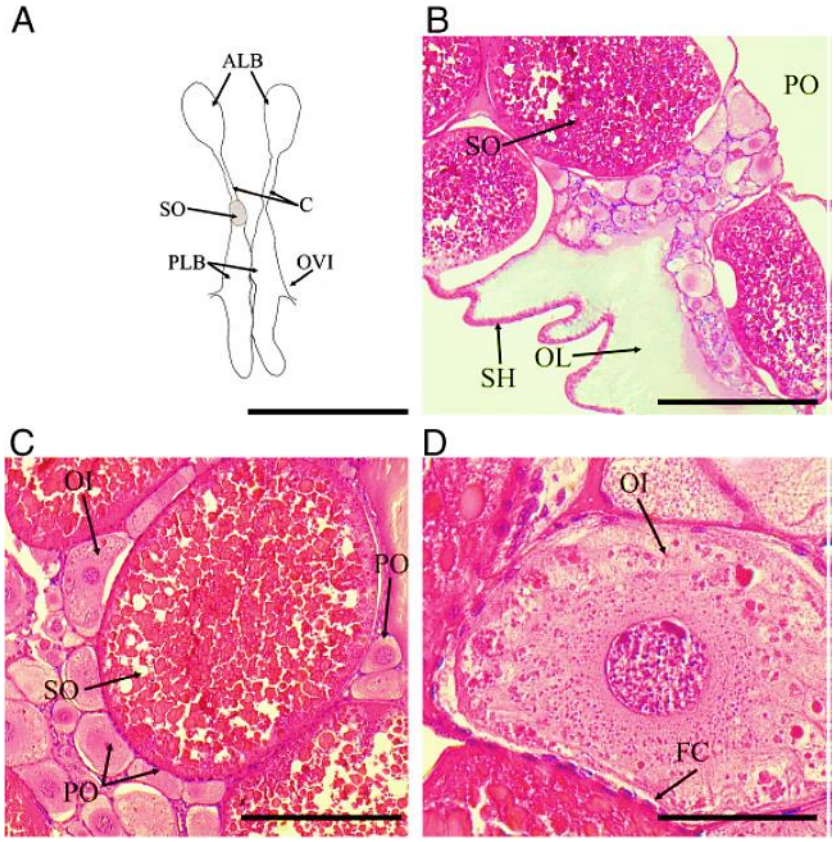


Figure 2.7. Redclaw crayfish, *Cherax quadricarinatus*, stage III ovary. (Reproduced from Vazquez et al. (2008) with permission from Invertebrate Biology, John Wiley and Sons). (A) Differentiation of posterior lobes and oviduct, (B, C, and D) Histology of different haematoxylin-eosin-stained ovarian cross-sections. (Scales: 4.3, 142, 100 and 40 mm, respectively). ALB, anterior ovarian lobes; OVI, oviduct; C, connectors; PLB, posterior ovarian lobes; FC, follicular cells; OI, intermediate oocytes; PO, primary oocytes; SO, secondary oocytes; OL, ovarian lumen; SH, ovarian sheath

Stage IV, the last completely mature ovarian stage, has an olive-green Y-shaped appearance that extends towards the abdomen because of the coalescence and expansion of the anterior lobes into the pleon. All types of primary, intermediate and secondary oocytes are present with secondary oocytes being most prevalent reaching a diameter of 1.06-1.08 mm. The thin muscular tunica of the ovary is involved in ovulation at this stage (Figure 2.8; Ando and Makioka, 1998; Vazquez, et al., 2008).

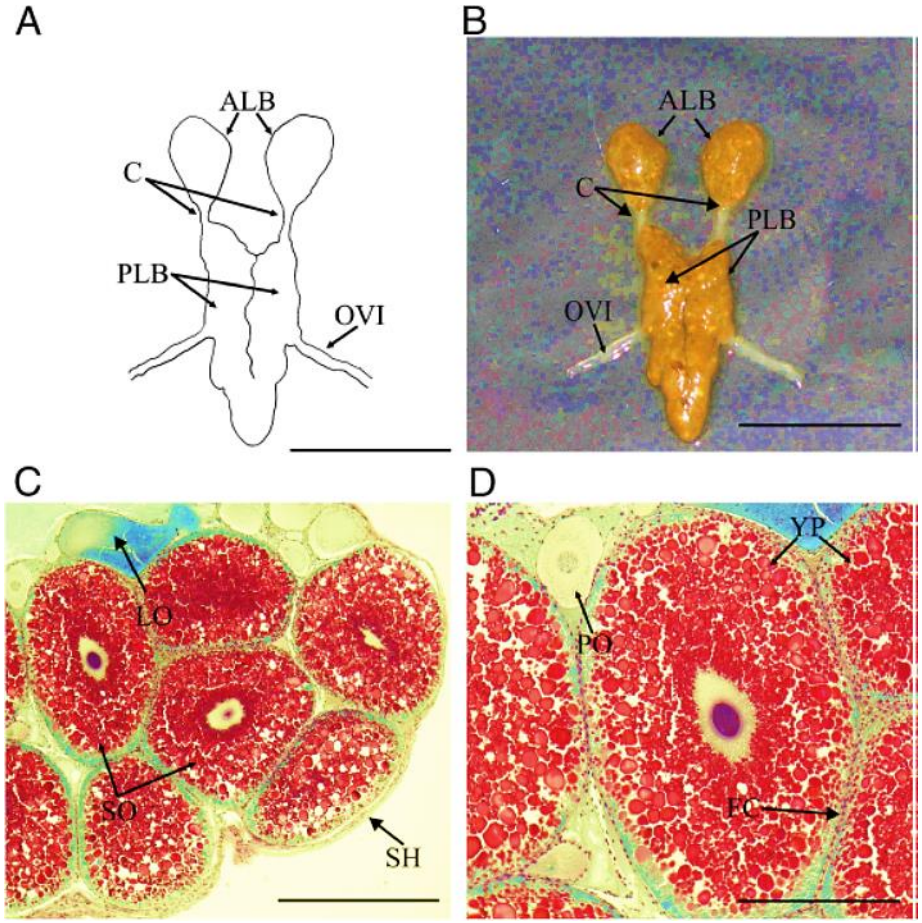


Figure 2.8. Redclaw crayfish, *Cherax quadricarinatus*, stage IV ovary. (Reproduced from Vazquez et al. (2008) with permission from Invertebrate Biology, John Wiley and Sons). (A, B) Mature olive-green Y-shaped ovary, (C and D) Histology of different Masson-Trichrome stained ovarian cross-sections. (Scales: 16.2, 16.2, 489 and 217 mm, respectively). ALB, anterior ovarian lobes; OVI, oviduct; C, connectors; PLB, posterior ovarian lobes; FC, follicular cells; PO, primary oocytes; SO, secondary oocytes: LO, ovary lumen; YP, yolk platelets; SH, ovary sheath

The post-spawning redclaw ovary remains Y-shaped but transforms into a swollen, pale orange structure with a distinctly prominant ovarian lumen (Vazquez et al., 2008). Presence of empty oogenic pouches (analogous to post-ovulatory follicles in mammals) are the predominant feature of this stage, with a flower-like appearance delineated by ovarian epithelium (Figure 2.9; Ando and Makioka, 1998; Vazquez et al., 2008).

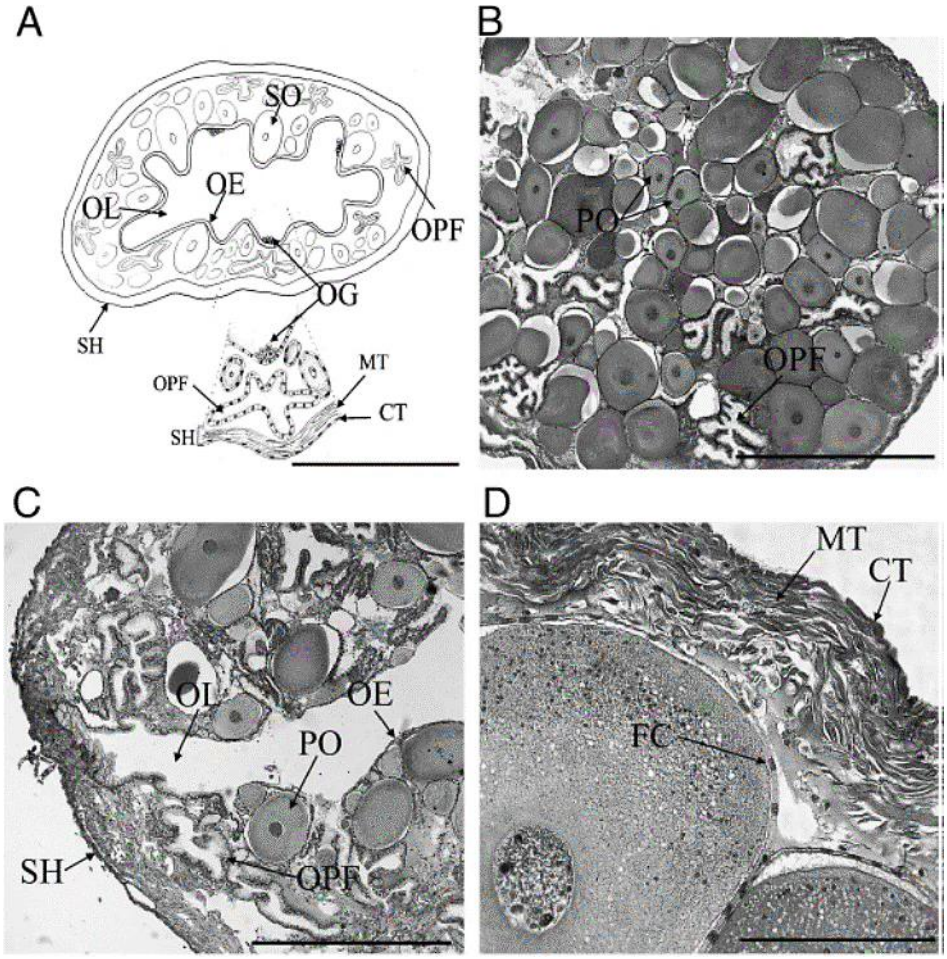


Figure 2.9. Redclaw crayfish, *Cherax quadricarinatus*, post-spawning ovary (Reproduced from Vazquez et al. (2008) with permission from Invertebrate Biology, John Wiley and Sons). (A) Diagram of ovarian cross-section with a close-up of an oogenic pouch. Histology of different Masson-Trichrome stained (B and C) ovarian cross-sections and (D) primary oocyte in close-up. (Scales: 30, 392, 223 and 43 mm, respectively). SO, secondary oocytes; OL, ovarian lumen; FC, follicular cells; PO, primary oocytes; OG, oogonium; OPF; oogenic pouch; OE, ovarian epithelium, MT, muscular tunica; CT, connective tissue; SH, ovarian sheath

2.5.1.3 Oogenesis.

Oogenesis is an energy-demanding process that can be divided into several phases (Tsukimura, 2015). Initial stages of oocyte development are generally quite slow, while the last stages of oogenesis are accelerated (Subramoniam, 2017b). These later stages are characterized by the rapid accumulation of yolk protein in the maturing oocytes, a process referred to as vitellogenesis leading to a significant increase in oocyte diameter (Tsukimura, 2015). During ovarian development in redclaw, oogenesis comprises both primary and secondary vitellogenesis (Abdu et al., 2000). Primary vitellogenesis involves two distinct oocytes groups: the first being 0.1 mm uniform milky oocytes at chromatin, chromatin-nucleolus, early peri-nuclear and late peri-nuclear stages (Figure 2.10, I-IV). Chromatin stage oocytes appear in

groups with a diameter of 12.3 ± 0.5 µm. Their oocyte membrane is not well-developed but the centrally located basophilic nucleus (9.7 ± 0.1 µm) has a well-defined membrane. At chromatin-nucleolus stage, nucleus and oocyte diameters are 16.0 ± 0.3 µm and 30.2 ± 3.5 µm respectively and have a well-defined plasma membrane and centrally located nucleus containing 2-10 small round basophilic nucleoli at its periphery. During the early-perinuclear stage, the nucleus and oocyte diameters increase to 31 ± 1.24 µm and 81.0 ± 10 µm respectively. The nucleus contains 4-5 large round intensely basophilic nucleoli at the periphery, surrounded by a periodic acid-Schiff (PAS) positive thin peri-nuclear zone appears. 1-2 µm thick basophilic follicle cells appear in limited numbers around the oocyte plasma membrane. The diameter of the nucleus and oocyte at the late-perinuclear stage is 52.2 ± 1.37 µm and 209.5 ± 55 µm respectively, with the periphery of the nucleus containing 4-5 large round strongly basophilic nucleoli surrounded by a much thicker perinuclear zone. The oocyte plasma membrane is now enveloped by a 35 µm thick complete monolayer of basophilic follicle cells (Abdu, et al., 2000).

The second to undergo primary vitellogenesis are 0.4 mm oocytes (Figure 2.10, V) that include both small milky white and larger yellow to orange oocytes. The larger yellow lipid-stage oocytes have a nucleus and oocyte diameter of 71 ± 1.9 µm and 349.7 ± 60 µm respectively, and a nuclear appearance similar to late-perinuclear stage oocytes. Numerous PAS-positive 4.0 ± 0.05 µm oil droplets appear in the cytoplasm, with a 6.0-8.0 µm thick single follicle cell layer surrounding the plasma membrane. The appearance of oil droplets in the cytoplasm at this stage marks the end of primary vitellogenesis.

Secondary vitellogenesis spans the yolk, prematuration and maturation stages of oocyte development (Figure 2.10, VI-VIII) in which oocytes develop synchronously through these stages and change colour from deep orange to olive green or creamy brown. Yolk is deposited in the cytoplasm during this time and the oocyte reaches a maximum diameter of 2 mm. More specifically, the nucleus and oocyte diameters of yolk stage oocytes are 75.62 ± 2.3 µm and 498.7 ± 80 µm respectively, with few to no nucleoli present in the centrally located nucleus. The cytoplasm contains both evenly distributed lipid globules (5.2 ± 0.05 µm) and peripherally distributed yolk globules (3 to 10 µm). The thickness of the single follicle cell layer increases up to 12 µm. Prematuration stage oocytes have nucleus and oocyte diameters of 63.7 ± 2.0 µm and 828.9 ± 126 µm respectively. The centrally located nucleus is surrounded by an exclusive perinuclear zone and is smaller and strongly basophilic than the previous stage, containing 2-3 peripherally distributed basophilic nucleoli. Yolk globules are larger (3 to 20 µm) together with lipid globules are homogeneously distributed throughout the cytoplasm except at the perinuclear area, and a 8.0-14.0 µm thick single follicle cell layer surrounds the plasma membrane. Final maturation-stage oocytes have a diameter of 1800.3 ± 181 µm and a small irregular nucleus

with indistinct membrane. Follicle cells around the oocyte shrink (0.5-1 μm), and a clear layer is evident between the follicular cells and oocyte membrane.

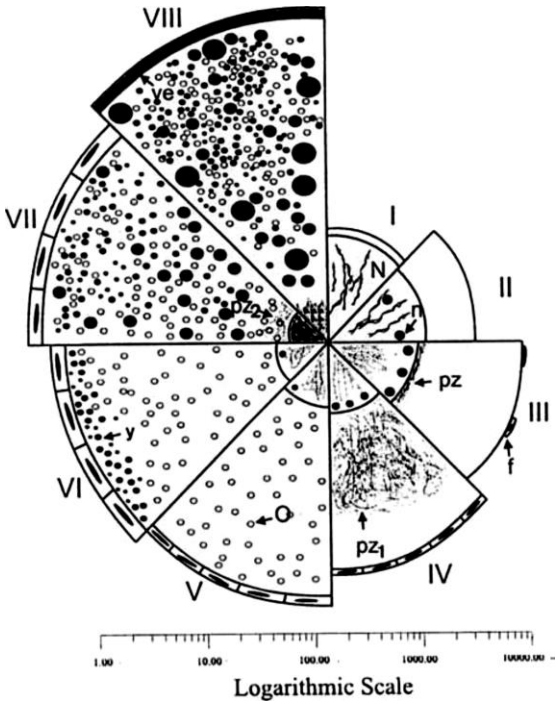


Figure 2.10. Stages of oocyte development in redclaw, *Cherax quadricarinatus*.(Reproduced from Oocyte development and polypeptide dynamics during ovarian maturation in the red-claw crayfish Cherax quadricarinatus, Uri Abdu, Galit Yehezkel and Amir Sagi, Invertebrate Reproduction and Development, and © copyright # 2000, reprinted by permission of Informa UK Limited, trading as Taylor and Francis Group, http://www.tandfonline.com). I, Chromatin stage; II, Chromatin-nucleolus stage; III, Early perinuclear stage; IV, Late perinuclear stage; V, Lipid stage; VI, Yolk stage; VII, Prematuration stage; VIII, Maturation stage. N, nucleus; n, nucleolus; f, follicle cell; pz, perinuclear zone in the early-perinuclear stage; pz1, perinuclear zone in the late-perinuclear stage; o, oil globules; y, yolk globules; pz2, perinuclear zone in the prematuration stage; ve, vitellin envelope. The scale represents the oocyte diameter

2.5.1.4 Ovulation and Fertilisation.

During sexual differentiation and the onset of sexual maturity in redclaw, there is a significant increase in the size of oocytes (reaching 2.2 mm long when fully mature), and an increase in the proportion of secondary relative to primary oocytes (Vazquez et al., 2008). The wall of the redclaw ovary is surrounded by a thin and stretched muscular layer similar to that observed in *P. clarkia*, that participates in ovulation (Vazquez et al., 2008) possibly mediated by $PGF_{2\alpha}$ (Spaziani et al., 1995). In addition, the tubular connectors of the ovary and the oviducts are lined with highly secretory epithelia that may further modify the oocyte envelop and could function to lubricate/facilitate ovulation and evacuation of mature oocytes from the anterior

lobes in an individual and coordinated manner (Vazquez et al., 2008). At ovulation, the secondary oocyte and surrounding follicle cells enter the ovarian lumen (leaving an ovarian epithelium surrounding an empty oogenic pouch). From there they pass through the connectors and into the oviduct before being oviposited through gonopores (Vazquez et al., 2008). Thus the presence of connectors in the ovary may assist the complete discharge of oocytes from the ovary (Vazquez et al., 2008).

Release of eggs in female freshwater crayfish is triggered by mating behaviour when spermatophore are deposited by the male onto the female (Skurdal and Taugbol, 2002). After mating, the female secretes a sticky glue from glair glands that breaks-down the male's spermatophore enabling her to smear spermatozoa towards her sternum prior to egg release (Niksirat et al., 2015; Vogt, 2016). Female redclaw usually release eggs from gonopores situated at the base of the third pair of walking legs within 24 hours of mating (Masser and Rouse, 1997). Eggs are released towards a brood chamber formed by the female curling its abdomen under its legs (Andrews, 1906; Niksirat et al., 2014). The highly viscous glair glue within the brood chamber ensures the attachment of eggs to the pleopods for fertilisation and hatching for 4-6 weeks (García-Guerrero et al., 2003b; Niksirat et al., 2015; Vogt, 2018; Yeh and Rouse, 1994).

Fertilisation is the union of sperm and egg to generate a diploid zygote (Siu et al., 2021). The process of fertilisation in decapod crustaceans varies between species but universally involves activation of the egg after fertilisation by spermatozoa, triggering physical and chemical changes (Goudeau, 1982; Goudeau and Jacqueline, 1982; Niksirat et al., 2015; Pongtippatee-Taweepreda et al., 2004). The process of fertilisation has not yet been described in redclaw, but has been documented in the narrow-clawed freshwater crayfish, *A. leptodactylus* (Niksirat et al., 2014). At the commencement of fertilisation, the spermatophore layers are dissolved by female glair gland secretions. The extracellular capsule of the spermatozoon, plasma membrane, and membranous lamellae are removed, and a mass of filaments are released from the anterior acrosome. The acrosome's innermost layer also loses electron-dense content, creating a filament/droplet arrangement at the anterior portion of the spermatozoon thought to facilitate the process of sperm-egg attachment in crayfish (Niksirat et al., 2014).

The growing demand for decapod crustaceans in aquaculture has necessitated developing more intensive production methods to boost yields and profits. However, in conventional aquaculture systems, there are no established methods to maximise synchronous gonadal maturation and spawning. Furthermore, insufficient production of fertilised eggs, variable fertilisation rates, asynchronous hatching and mass mortality of early juveniles are limiting the consistent and reliable production of juveniles (Valverde. et al., 2020). These production drawbacks suggest

that improvements in captive aquaculture systems should be prioritised to increase the number of berried females and maximise egg and juvenile production.

Manipulation of gonadal maturation in broodstock coupled with AF is a promising strategy that could be used to increase year-round production of fertilised eggs in decapods. Moreover, AF is a valuable tool that permits complete control of mating pairs and their reproduction in order to accelerate selective breeding of commercially important aquaculture species (Haldar, 2018). This can be achieved because AF uncouples the critical time window normally required for spermatozoa to meet oocytes. Disease-free gametes can be sourced and exchanged between broodstock across the industry to help maximise genetics, while maintaining biosecurity by limiting the introduction of pathogens through the movement of whole animals into production systems (Parkinson and Morrell, 2019). Sperm freezing coupled with AF could actually decrease the number of males that must be maintained for breeding purposes, since cryopreserved gametes could be transferred between facilities and over long distances, as is routinely practiced in terrestrial animal production systems (Nagata et al., 2019; Ugur et al., 2019). Lastly, through timed gonadal maturation and AF techniques, it should be possible to achieve synchronous hatching, accurately determine the age of progeny, and generate consistently sized juveniles at any time of the year, facilitating expansion of the decapod industry. The development of different AF techniques such as artificial spermatophore deposition or *in vitro* fertilisation (IVF) in decapod crustaceans, including crayfish, offers potential to improve productivity (Ikhwanuddin et al., 2015).

IVF involves the mixing of spermatozoa and oocytes, resulting in the fertilisation of oocytes in absence of whole animals (Beirão et al., 2019). IVF has been conducted on a limited scale in some decapod crustaceans, resulting in successful fertilisation with relatively high hatching rates (Ikhwanuddin et al., 2015; Sarker et al., 2009; Talbot et al., 1991). In lobster *H. americanus*, IVF was performed using large (1.6 mm diameter) pre-ovulatory oocytes dissected from ovaries and spermatophore obtained from either the dissected male proximal vas deferens or female seminal receptacles (Talbot et al., 1991). Initially, about 20 to 30 oocytes were pipetted into a glass Petri dish containing either 10 mL 2.5 mM Ca^{2+} in 20 mM HEPES-buffered lobster saline (2.5 LSH) or 10 mL artificial seawater (ASW) and spermatozoa added after disaggregating the spermatophore using forceps. Spermatozoa were able to fertilise 94% of oocytes in 2.5 LSH compared to 33% of oocytes in ASW. Fertilisation was verified by transmission electron microscopy to directly observe sperm binding to or penetration of the vitelline envelope of the oocyte during the acrosome reaction. The higher proportion of fertilised eggs in 2.5 LSH than in ASW suggests it is a more effective medium for IVF, possibly explained by the difference in pH (2.5 LSH = pH 7.5; ASW = pH 8.0) or calcium content (2.5 LSH = 2.5 mM; ASW = ~10 mM) (Talbot et al., 1991). Although IVF was successful in terms

of high fertilisation rate (94%), hatching did not occur (Talbot et al., 1991). Failure of fertilised eggs to undergo hatching could be attributed to poor *in vitro* culture conditions.

In banana shrimp *Fenneropenaeus merguiensis*, IVF was performed in natural seawater (NSW) as a control medium, ASW and Calcium-free saline (Ca-F saline), and compared with natural spawning (Ikhwanuddin et al., 2015). Approximately 2,000 eggs from pre-spawn maturation stage (IV) ovaries were collected and transferred into 3 glass Petri dishes containing 20,000 sperm/mL in NSW, ASW or Ca-F saline and mixed for 5 min to facilitate fertilisation (Ikhwanuddin et al., 2015). Fertilised eggs were then transferred to a large incubation chamber containing UV-sterilised NSW, and egg development monitored periodically by light microscopy until hatching. Although fertilisation was successful in all treatments (NSW: 19.7 ± 7.3% *vs.* ASW: 8.7 ± 4.0% *vs*. Ca-F saline: 4.3 ± 4.0% *vs.* natural spawning: 98.3 ± 1.2%), hatching did not occur in NSW or ASW treatments. By contrast, the Ca-F saline fertilisation when incubated over a 16 h period in NSW produced actively swimming nauplii and zoea larvae at the end of the experiment with 3.0 ± 2.6% hatching rate compared to 60.5 ± 45.6% after natural spawning. These studies of IVF in decapods provide us with valuable information critical to the development of improved IVF protocols for commercially important redclaw.

2.5.2 Husbandry Requirements for Female Broodstock

Owing to the lack of well-established husbandry protocols, redclaw production varies annually between producers (Jones and Ruscoe, 1996). Such variability can be overcome by implementing best husbandry protocols such as proper site selection and farm design, quality broodstock selection, efficient utilisation of water resources, water quality control, proper nutrition and abiotic supplementation, disease and stress reduction, careful harvesting and post-harvest handling, etc. (Jones and Ruscoe, 1996). Such practices can help to build a strong foundation for sustainable redclaw aquaculture.

The farm site should be located in a place where soil contains a high amount of clay (Jones, 2000) because it is viscous and has high nutrient and water retention capacity year-round, even during extreme drought. Sufficient water is needed from one of three sources: surface runoff, groundwater or dam irrigation. Good water quality is determined by parameters such as optimum temperature, transparency, pH, as well as absence of insecticides and heavy metals (Jones, 2000; Jones et al., 1998).

For successful aquaculture, maturation and spawning season is effectively controlled to facilitate continuous large-scale supply of quality seed stock. This can be achieved through provision of optimal nutrition and environmental conditions to broodstock (Migaud et al., 2013). For female broodstock management, several factors are important to enhance sexual maturation and sufficient quantities of good quality eggs, that can be harvested in a sustainable

fashion (Duncan et al., 2013). Among different environmental factors, temperature and photoperiod are considered the most important factors that trigger the onset of spawning in different aquatic species (Lawrence and Soame, 2004).

2.5.2.1 Temperature and Photoperiod.

The two major environmental cues affecting the growth and reproductive cycles of crayfish are temperature and photoperiod (Jones, 1990; Yeh and Rouse, 1995). In the natural environment, depending upon temperature (26-29 °C) and photoperiod (14L:10D) redclaw can spawn 3-5 times per year from spring to summer (Bugnot and López Greco, 2009; C. Jones, 1995; Jones, 1998) . Temperature and photoperiod can be manipulated in aquaculture systems in order to control redclaw reproduction and extend the brooding period in captivity (Yeh and Rouse, 1994; Yeh and Rouse, 1995). Under laboratory condition, early exposure of juvenile redclaw to high temperature (28 ± 1 °C) accelerated the onset of sexual maturity, inducing enlarged oocytes some of which reached vitellogenic stages (Yeh and Rouse, 1994). Moreover, long-term exposure of redclaw females to 30 °C accelerates ovarian development and first maturation (Tropea et al., 2010). Temperatures of 26-28 °C and photoperiod of 14L:10D increased female redclaw spawning by 60% in the first spawners (Karplus et al., 2003a). In another study, daily exposure of redclaw females to 24.5-27.6 °C and 14L:10D successfully induced spawning in 97% females achieving hatching (Jones, 1990; Jones, 1995). Furthermore, a higher spawning rate (~80%) was achieved when redclaw reared at 28 °C and 14L:10D (Yeh and Rouse, 1994).

The egg incubation period is also significantly influenced by water temperature. At 28 °C, the incubation period is shortened to 30-40 days, compared to taking more than 45-71 days at a temperatures below 25 °C (Masser and Rouse, 1997; Yeh and Rouse, 1994). Hatching can be synchronised and accelerated by 1 month by incubating fertilised eggs at 32 °C instead of 22 °C (King, 1993a). Conversely, redclaw eggs lose their regenerative capacity and exhibit retarded development when stored at or below 10 °C for more than 30 days (King, 1993a). For example, at temperatures below 18 and 20 °C, egg development arrests after the eyespot phase (King, 1993a). This is due to a 30-fold increase in metabolism that normally occurs at this stage, which cannot be supported at lower temperatures, thereby preventing full development of redclaw eggs (King, 1993). Additionally, temperature and photoperiod also influence the growth and survival of redclaw offspring. When redclaw juveniles (0.61 g to 1.27 g) were reared over 70 days at 20, 24, 28, 32 and 34 °C, 65% survived at 24, 28 and 32 °C, compared to less than 40% at 34 °C. Moreover, the best growth (5.03 g) occurred at 28 °C (Jones, 1990; C. Jones, 1995). In another study, 96.7% survival was achieved at 28 °C with a final average weight of 923 ± 92.5 g (Meade et al., 2002).

In the above studies, both temperature and photoperiod act synergistically to facilitate sexual maturation, reproduction, embryo development, as well as juvenile growth and survival in redclaw. In most of the cases, a temperature range of 27-29 °C and photoperiod of 14L:10D is suitable for modulating the growth and reproduction, facilitating year-round production of redclaw in captivity.

2.5.2.2 Stocking Density and Sex Ratio.

Redclaw are considered adaptive to higher stocking densities (Macreadie, 1990), an important attribute for aquaculture. Approximately 84% of females mated and produced eggs successfully at a gender ratio of 1M:5F (Jones, 1990). Spawning rate (40.2 *vs.* 45.6%) did not differ significantly when redclaw were reared for 90 days in tanks at 10 *vs.* 20 individuals/m^2 with a sex ratio of 1M:1F (Yeh and Rouse, 1995). In the same experiment, males/females sex ratios of 1:1, 1:3, and 1:5 were tested at 10 individuals/m^2. Though no significant spawning difference was observed between the sex ratios, a higher spawning rate (35.9%) was found at 1M:5F compared to 33.8 and 29.3% at sex ratios of 1:1 and 1:3, respectively (Yeh and Rouse, 1995). Similarly, stocking redclaw broodstock at 15-25 individuals/m^2 at a sex ratio of 1M:2-3F resulted in 7.5 eggs and 6.6 offspring/g of female redclaw (Austin, 1998). In another study, reproductive efficiency of redclaw was evaluated rearing at 20 and 60 individuals/m^2 at a sex ratio of 1M:5F (Barki and Karplus, 2000). Although the results indicated almost the same reproductive outputs; spawning rate (75.6%), egg clutch size (7.5 ± 2.7/g female), average number of pleopodal eggs in each tank (12,367 eggs/m^2), and female survival (75.6%) at both stocking densities (20 and 60/m^2), it is advisable to use the reduced stocking density (20/m^2) and sex ratio (1M:4F) to permit efficient reproduction (Barki and Karplus, 2000). Such a stocking plan for intensive aquaculture of redclaw, might reduce the production footprint and allow close screening and sorting of each individual into berried and non-berried female cohorts that ultimately facilitate consistent egg production and animal recycling, thereby increasing overall management efficiency (Barki and Karplus, 2000).

2.5.3 Female Broodstock Nutrition

Maternal body reserves are a determining factor in reproduction, therefore, broodstock must be fed a high-quality diet throughout the first few months of gonadal maturation to increase their ability to reproduce (Liñán-Cabello et al., 2002). Optimising broodstock nutrition is important for triggering gonadal maturation, improving fecundity and fertilisation, and subsequent embryo and larval development (Snyder and Zeigler, 2013; Wouters et al., 2001). Different nutrients play various important roles, but mainly the most important nutrients of which will be covered in this section. Through an increased understanding of broodstock nutrition, it may be possible to develop improved broodstock diets specific for redclaw.

2.5.3.1 Proteins.

Protein plays an important role in maturing gonads as well as egg and embryo quality (García-Guerrero et al., 2003b; Wouters et al., 2001). Broodstock diet for female crayfish with sufficient protein and amino acids can promote proper growth and Vg synthesis in eggs (Harlıoğlu and Farhadi, 2017). Redclaw broodstock fed four different protein-rich diets (22, 27, 32, and 37%) containing 20.2-21.6 kJ/g gross energy, exhibited improved reproduction (25% spawning rate, 82% survival, and 9.3 eggs/g female fecundity) when fed the 32% protein diet (Rodríguez-González et al., 2006a). Moreover, the 32% protein diet significantly enhanced egg quality, with an enlarged diameter 2.27 mm, surface area 3.90 mm^2, volume 39.3 mm^3 and weight 5.44 µg (Rodríguez-González et al., 2006a). In another study, a 33% crude protein diet containing 21.37 kJ/g gross energy, resulted in increased gonadosomatic and hepatosomatic index as well as increased numbers of secondary vitellogenic oocytes, both signs of proper ovarian maturation (Rodriguez-Gonalez et al., 2009a). Furthermore, dietary protein at a certain amount can improve embryo growth and resistance to detrimental environmental conditions. For example, a broodstock diet containing 26% protein increased the growth and quality of juvenile offspring subjected to conditions of low dissolved oxygen, high salinity and high ammonia (Rodríguez-González et al., 2014). From the above studies, it appears that 32-33% crude protein and 20.5-21.5 kJ/g gross energy is optimum for the preparation of female broodstock diet.

2.5.3.2 Lipids.

Gonadal maturation and reproduction in crustaceans is an energy and nutrient demanding process in which females transfer energy reserves to eggs in order to nourish the developing embryo (Hernández-Abad et al., 2018). Lipids and their constituent fatty acids are considered fundamental dietary elements for the successful reproduction and progeny survival of crustaceans (Izquierdo et al., 2001), since they are a major energy source for gonadal development and are indispensable components of cellular structures in the embryo (Hernández-Abad et al., 2018). Female redclaw generally demand higher lipid content in their diet than males (Ghanawi and Saoud, 2012) because of extra energy requirement for egg development (Rodriguez-Gonzalez et al., 2009; Rodríguez-González et al., 2006b). Hepatopancreas and adipose tissues act as energy reservoirs (Luo and Liu, 2016; Wang et al., 2014) from which stored lipids actively circulate to the gonad for development of gametes in several crustaceans (Mourente et al., 1994). The composition of lipid changes at the beginning of oocyte maturation in redclaw and a steady supply of lipid from hepatopancreas to the gonad continues to facilitate yolk deposition during oogenesis (Rodríguez-González et al., 2006b). As such, sufficient quantities of lipids are required in broodstock diets for proper oocyte maturation. In this regard, egg quality (based on egg area, volume and weight) of female redclaw was highest when fed a diet containing 8.7% lipids compared to 4 and 12% (Rodriguez-Gonzalez et al., 2009b).

As the female redclaw ovary matures, the proportion of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the ovary increase from 33.2% to 51.9% and 18.6% to 27.3% respectively, during stage IV vitellogenesis (Li et al., 2010b). However, the amount of saturated fatty acids (SFA) in the ovary decreases markedly as it matures (Li et al., 2010a, 2010b). High levels of MUFA during stage IV vitellogenesis indicate it is a chief energy source in developing oocytes, while PUFA appears to have important functions in fertilisation and hatching (Li et al., 2010b). In the quest for an economic source of dietary fatty acids for broodstock diets, it is thought that plant-derived PUFA such as soybean oil (with lower n-3 series fatty acids) may be a cheap substitute for more expensive fish oil, rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA; (Li et al., 2010b). Female redclaw fed higher quantities of PUFA (42.4%) had increased Vg mRNA expression in the hepatopancreas, and attained ovarian maturation earlier (Li et al., 2010b). Linoleic acid (C18:2n6; high in soybean oil) was identified as the primary PUFA responsible for ovarian maturation in diets low in EPA and DHA (Li et al., 2011).

Additionally, phospholipids are regarded as another essential component of broodstock diets that influence reproductive efficiency in crustaceans (Sui et al., 2009; Wouters et al., 2001; Wu et al., 2007). Soybean lecithin (SL), one source of the dietary phospholipid, can induce ovarian maturation when applied at optimum levels in broodstock diet (Wang et al., 2013b). Redclaw broodstock diet containing >2% SL significantly increased survival rate (87.5%), gonadosomatic index (GSI) (2.2) and hepatopancreatic Vg mRNA expression in females (Wang et al., 2013b). Vg is the precursor of yolk protein produced in the hepatopancreas during vitellogenesis, which is transported to the ovary via haemolymph, then converted to vitellin (Vn) and accumulated in growing oocytes (Wang et al., 2013b). Vn can also be synthesised directly in the hepatopancreas of redclaw (Serrano-Pinto et al., 2004). Once deposited in eggs, Vn serves as a nutritional source for the developing embryo (Abdu et al., 2002; Wang et al., 2013b). Thus, based on the above studies, 2% SL in broodstock diets appears to improve ovarian growth and maturation.

Cholesterol a primary sterol found in all cells and the haemolymph of crustaceans, can exist alone or in combination with fatty acids (Kumar et al., 2018b). Cholesterol contributes to the structural integrity of eggs and embryos, playing a crucial role in their development and survival. In the copepod *Acartia hudsonica*, a diet high in cholesterol increased egg production and hatching rates without affecting the cholesterol level of the plasma membranes (Crockett and Hassett, 2005). However, despite the comparatively high sterol content of crustacean tissue, like arthopods they are incapable of synthesising sterols on their own and must obtain these vital nutrients from their diet to grow, develop, and survive (Wacker and Martin-Creuzburg, 2007). Although dietary supplementation of cholesterol improved growth and survival of juvenile

redclaw, there is no study demonstrating the impact of cholesterol supplementation on the reproduction of redclaw broodstock (Hernández et al., 2004).

2.5.3.3 Carbohydrate.

Carbohydrate supplementation appears to be of little concern for redclaw growth and nutrition (Jones and Ruscoe, 1996), with a scarcity of studies investigating the dietary requirement of carbohydrates for redclaw broodstock (Ghanawi and Saoud, 2012; Harlıoğlu and Farhadi, 2017). Carbohydrate is important only during the initial stage of vitellogenesis in the maturing redclaw ovary and decreases with increasing GSI and dependence on protein and lipid reserves (Rodríguez-González, Hernández-Llamas, et al., 2006). Moreover, carbohydrate is not utilised as a major energy source during embryo development, since very little carbohydrate is deposited in freshly spawned eggs (García-Guerrero, Racotta, et al., 2003).Thus, compared to other nutritional components, carbohydrates likely to have relatively low importance to gonadal maturation of redclaw (Rodríguez-González, Hernández-Llamas, et al., 2006). Nonetheless, as a low-cost source of energy, carbohydrates will always be a base element of artificial diets for redclaw (Saoud et al., 2012).

2.5.3.4 Vitamins, Carotenoids and Minerals.

Vitamins are considered essential micronutrients for crayfish (Saoud et al., 2012) and in shrimp broodstock, vitamin A, D, E, and C have been shown to play important roles (Harlıoğlu and Farhadi, 2017). Broodstock diet supplemented with 80 mg/kg of vitamin E increased the number of eggs attached to the pleopods of freshwater crayfish, *Astacus leptodactylus* (Harlioglu et al., 2002). However, the importance of dietary vitamins and carotenoids to female redclaw broodstock is poorly understood (Harlıoğlu and Farhadi, 2017). Supplementation of 0.0192% vitamin E in redclaw broodstock diet can significantly improve female reproduction with increased gonadosomatic index (6.55), weight gain (30.42%), spawning rate (84.0%), total weight of eggs per female (2.86 g) and hatching rate (45.53%; Luo, et al., 2004b). Individual eggs had an average weight of 5.08 mg, and overall lipid content and essential amino acids in fertilised eggs increased to 31.69% and 37.23% respectively (Luo et al., 2004b). Optimal levels of vitamin E can also prevent oxidation of *n*-3 series fatty acids and so increase the accumulation of substantial amino acids and fatty acids in fertilised eggs (Luo et al., 2004b).

Vitamin A (as either retinol and its synthetic analogues, or to certain carotenoids such as provitamin A) is also important for broodstock nutrition (Harlıoğlu and Farhadi, 2017; Liñán-Cabello et al., 2004). Carotenoids function as antioxidants and can trigger the vitellogenesis in developing oocytes during gonadal maturation (Liñán-Cabello and Paniagua-Michel, 2004). Injection of carotenoid (18.3 µg/g BW) and retinol palmitate (RP; 100 µl per 50 g body weight) increased the pronounced nuclear zone which are characteristics of late perinuclear stage

redclaw oocytes and induced the greatest number of oil globules with an average of 13.25 and 13.23 per oocyte (Liñán-Cabello et al., 2004). Moreover, carotenoid injection significantly increased average oocyte diameter by 283 µm and RP increased the number of follicle cells to 37 per follicle (Liñán-Cabello et al., 2004). During RP treatment, several isomers of vitamin A take part in gene activation that leads to ontogenic development, producing follicles and fat-globules in oocytes (Liñán-Cabello et al., 2004; Liñán-Cabello and Paniagua-Michel, 2004), suggesting a lack of vitamin A and carotenoids could result in reduced follicle numbers. In crustaceans, astaxanthin has been identified as the most common by product of carotenoid metabolism (Meyers and Latscha, 1997). Astaxanthin rich diet are important for promoting gonadal maturation, spawning, improved egg quality, and enhanced embryo survival in crustaceans (Barım-Öz and Şahin, 2016; Paibulkichakul et al., 2008). Astaxanthin enhances the development of follicles and oocytes by enhancing the antioxidant capacity of follicles and oocytes and reducing the oxidative stress during follicular development and oocyte maturation (Li et al., 2022). In pond-reared *P. monodon* diet with 8% fish oil and 280 mg/kg astaxanthin improved the maturation and spawning rate (Paibulkichakul et al., 2008).

In conclusion, carotenoids and retinoids are important dietary nutrients that help triggering the onset of redclaw ovary and oocyte maturation, which could be a suitable alternative to eyestalk ablation (Liñán-Cabello and Paniagua-Michel, 2004). Further studies are necessary to determine combined effect of carotenoids and vitamin A on redclaw egg and offspring quality. To date, there are no studies reporting the mineral requirements for redclaw broodstock (Harlıoğlu and Farhadi, 2017; Saoud et al., 2012).

2.5.4 Role of Stress in Limiting Broodstock Fertility

Animals in the natural environment may encounter physiological disruptions that can jeopardise their survival and another important life status such as reproduction. Every individual has a unique strategy to respond to the stressors (Moore and Jessop, 2003). In response to physiological stressors, the vertebrate hypothalamic-pituitary-adrenocortical (HPA) axis is increasingly found as a dynamic physiological mechanism capable of mediating broad variations (Sapolsky, 1992). HPA mediated physiological responses include energy accumulation, gluconeogenesis, and acceleration or suppression of growth and reproduction (Wingfield et al., 2015). The HPA axis can perceive different stressors and can control the adrenocortical modulation (Wingfield and Romero, 2001). Different features such as body size, disease, age, sex type, dominant-subordinate interaction, and brooding condition can cause a change in the hormonal cascade of vertebrates that expedite the production of cortisol and corticosterone level during reproduction (Dunlap and Schall, 1995; Elwood et al., 2009; Fraipont et al., 2000; Grassman and Hess, 1992; Young et al., 2006). The increased level of stress hormone (cortisol/ corticosterone) reduces estradiol production by affecting the functions

of follicular granulosa cells, that may result deterioration in oocyte quality (Prasad et al., 2016). In the breeding mammal meerkat (*Suricata suricatta*), the dominant aggression was found as a stress related suppressor that affected the reproductive physiology and glucocorticoid level of the subordinates (Young et al., 2006). Subordinate females showed chronic elevation of their glucocorticoid adrenal levels, reduced pituitary sensitivity to gonadotrophin-releasing hormone (reproductive down-regulation), reduced conception rates, and increased abortion rates (Young et al., 2006). In rain bow trout, repeated exposure to acute stress (aerial emersion for 3 min) during reproductive development, delayed ovulation, significantly reduced egg production and progeny survival in stressed fish compared to control (Campbell et al., 1992). Plasma cortisol level was significantly higher (26 ± 3 ng/ml) in stressed fish than the unstressed control fish (13.5 ± 4.7 ng/ml).

Additionally, repeated injections, extensive handling, crowding, anaesthesia, and surgery also exhibited stress on sexual behaviours of amphibians (Moore and Miller, 1984). Male amphibian (*Taricha granulosa*) confining in a small box (20 L water) for 60 min, stressful condition was created and a significant drop in the incidence of sexual behaviour was observed. The corticosterone level was also higher in stressed animal and only 10% of them mated compared to 90% of unstressed animals (Moore and Miller, 1984). Rearing animals under laboratory condition out of their natural environment can be stressful (Romero and Wingfield, 1999). Captivity induces physiological stress to white-crowned sparrows and alters the adrenal and pituitary function (Pagé and Cooper, 2004; Romero and Wingfield, 1999). Collectively, the above results suggest that in vertebrates the cortisol level increases in response to different husbandry stressors which adversely impact their reproductive efficiency. But in crustacean the mechanism by which stress impacts on reproduction is not clearly understood.

Tank size and stocking density were found to impact on reproductive performance in two eyestalk ablated groups of *L. vannamei* (Kumlu et al., 2011). One group was stocked in a 2 m round tank following a stocking density of 9.44 shrimps/m^2 (1:2, male/female), while another group was stocked in a 3 m tank at a density of 5.67 shrimps/m^2 (1:1, female/male). The experiment was continued in a recirculation system for 2 months until maturation. At the end of the experiment, the spawning, fecundity and hatching rates were significantly lower in the smaller-sized tank group. Adult female shrimp *P. californiensis* rearing in captivity showed a reduced fecundity (Moore Jr. et al., 1974). Again, Indian white shrimp, *Penaeus indicus* rearing in captivity showed reduced reproductive performances in comparison to the wild species (Shyne Anand et al., 2019). The eggs produced from wild species were 8,126 ± 3,502 eggs per gram BW while the captive species produced 1,481 ± 863 eggs per gram BW. The egg hatching rate from wild spawners was 80% whereas captive spawners showed 50-70% hatching rate (Shyne Anand et al., 2019*)*. In another study, however, there was no difference between

hatchability and metamorphosis to protozoa I for spawns of wild-caught female species and those of spawns of the same females restocked and cultured under laboratory condition (Browdy et al., 1986; Browdy et al., 1987). Although stress level was not measured in these crustaceans (*L. vannamei*, *P. californiensis*, and *P. indicus),* these findings indicate husbandry may impact reproductive fitness of animals and suggested that broodstock needs to be well managed with proper diet, health status and conditions to improve the quality of spawns from captive animals (Browdy, 1998).

Female crayfish face similar conditions of captivity, handling, transportations and diseases (AquaVerde, 2020) that might result to physiological stress for them. Crayfish also have several well-characterised and well-established habits that can be used to analyse the consequences of the physiological stress response on reproduction. A heat stress protein (HSP86) has been quantified from the muscle of thermally stressed male redclaw. Since the physiological stress level of egg and embryo can be determined by the expression study of different HSPs (Hallare et al., 2004), HSP86 may provide a sound basis for studying the role of HSPs in reproduction and gamete quality of female redclaw. Therefore, by utilising the knowledge from other vertebrate studies it would be possible to begin research on how redclaw female will respond to several physiological stressors to compensate or balance their reproduction.

2.5.5 Current Hatchery Practices

Despite predominantly used earthen ponds, there has been regular attempts to use indoor tank systems to produce juveniles (FAO, 2024). In temperate regions, earthen pond-based outdoor culture of redclaw is not feasible until the water temperature reaches 25 °C, narrowing the production season to 3-5 months (Webster et al., 2004). Indoor hatcheries can expand the growing cycle, increase the quantity and quality of juveniles available for stocking, and promote larger juveniles for differential stocking (Masser and Rouse, 1997).

For indoor hatcheries, broodstock are treated in salt (1000-2000 ppm) or formalin (15 to 25 ppm) solution to exclude all potentially infectious agents prior to stocking holding tanks (Masser and Rouse, 1997). The fundamentals of a good survival rate among hatchery juveniles are size uniformity, stocking density, water quality, shedding, and nutrition (Masser and Rouse, 1997). A temperature-controlled recirculating system with biological filtration is used to maintain suitable water quality for hatchery production at 26-29 °C, >5 ppm (mg/L) dissolved oxygen, pH 7.5-8.0, <5 ppt salinity, >50 ppm hardness, >100 ppm (mg/L) alkalinity, 50 mg/L chloride and 0.1 mg/L heavy metals (Jones and Ruscoe, 1996; Masser and Rouse, 1997). Warmer water (26-29 °C) and long photoperiods (12-14 h light) are used to increase the rate of spawning amongst broodstock, with the greatest spawning events achieved at 28 °C and 14 h light:10h dark (Masser and Rouse, 1997). Dark-coloured tanks can reduce the light stress and

fibreglass or stainless-steel tanks with smooth surfaces can reduce abrasion injury to animals. Adult broodstock are stocked in holding tanks at 1 male to 3 females per 0.1 m^2 of bottom area (Masser and Rouse, 1997). Good spawning rates are achieved using circular (4.5 m diameter) or rectangular (1.4-1.8 m^2) tanks with 0.3-0.9 m deep water (Masser and Rouse, 1997). Broodstock are fed daily a complete diet (shrimp feed containing 28% protein) at 3% body weight (BW) every evening, when they actively forage for food. Some hatchery operators prefer supplementary diets including fresh vegetables, seeds, and beef or chicken heart or liver (Masser and Rouse, 1997). After spawning, berried females are carefully transferred to hatching tanks to avoid egg loss. At a water temperature of 28 °C the eggs hatch within 30 days (Masser and Rouse, 1997). Using this hatchery system an average of 10 eggs per gram of female are obtained, but about 30% of these eggs are lost during incubation due to handling stress, resulting in an average of 7 eggs eventually hatched per gram of female (Masser and Rouse, 1997). Adult females are removed from hatching tanks approximately 7-10 days after hatching, when early juveniles leave their mother. This post-hatch period in the hatchery is very sensitive and important for the survival of early juveniles. As such, handling of juveniles for the first few weeks should be avoided, with greater emphasis given to the administration of formulated feed, and provision of adequate substrates to prevent cannibalism and increase overall growth and survival. Substrates such as shade-cloth, fibreglass window frames, and onion/citrus fruit mesh bags are effective for allowing sufficient water flow, while increasing surface area for juveniles to access for bottom-feeding (Masser and Rouse, 1997). As an important aspect of juvenile health, nutrition is strictly maintained on a commercial shrimp diet (containing >33% protein) at 40% of their biomass fed 3-5 times/day. Improved growth and survival can also be obtained by supplementing one of the daily formulated meals with newly hatched brine shrimp nauplii during the first week (Masser and Rouse, 1997). Overfeeding should be avoided, with excess food particles siphoned from the bottom. On average 50 to 70% juvenile survival is possible in the hatchery by maintaining these rearing principles (Masser and Rouse, 1997). Once juveniles attain a length greater than 5 cm (>1 g) they have a high chance of survival and can be stocked in grow-out ponds when water temperature >20 °C.

Given the 30% loss of eggs observed in hatchery females (rate of egg loss is still unknown in pond-based systems but likely to be higher) there is a critical need for an advanced egg incubation system to improve hatching rates and early juvenile survival (Masser and Rouse, 1997). AquaVerde Redclaw Hatchery and Farm (AquaVerde) and Australian Crayfish Hatchery (ACH) in Queensland, have been testing a crayfish egg incubator originated from Finland (Hemputin egg incubation system) to prevent such egg loss and produce high-quality disease-free redclaw eggs and juveniles. In the Hemputin egg incubation system, eggs stripped from the female's pleopods (swimmerets) are placed in batches in specially crafted 100 ml plastic baskets

inserted into a stainless-steel rack (Figure 2.11; Rudge and O'Sullivan, 2007). The rack sits inside a fibreglass tank (3 m long, 550 mm wide, 100 mm deep) and is connected to an electric motor that rocks the baskets in a circular motion back and forth. This movement allows the egg surface to take-up sufficient oxygen to hatch and is thought to imitate the gentle water flow across the eggs caused by the female redclaw fanning her pleopods swimmerets (Rudge and O'Sullivan, 2007). The juveniles produced from the incubation system have an exceptionally good health status because eggs can be disinfected to remove potential pathogens before incubation. As such, the egg incubation system coupled with optimised monitoring and management strategies can reduce egg mortality, increase hatching and survival rates, and produce healthier, pathogen-free redclaw juveniles for the benefit of redclaw producers, stakeholders and consumers (ACH, 2020). With such a system, juveniles of the same age can also be stocked into nursery ponds, which results in less size variation at the grow-out harvest. A 100-fold weight increase can be achieved (reaching an average weight of 2 grams) with approximately 20,000 juveniles produced over 6 weeks in 8 juvenile ponds with around 70% survival and an average weight of 2 grams.

Figure 2.11. Hemputin egg incubator (AquaVerde, 2024).

2.5.6 Limitations and Future Research Priority

Further research is required to control the timing of female reproduction and maximise natural and induced spawning to improve redclaw aquaculture. In particular, optimisation of factors to improve ovarian maturation and oocyte quality; understanding and controlling the timing and kinetics of ovulation and oviposition; optimisation of factors to improve embryo quality and maximise post-hatch survival of juveniles; and developing means of artificial embryo production.

External environmental factors such as stress, temperature, photoperiod and nutrition still need to be refined to consistently improve gonad maturation and oocyte quality. And there is not

sufficient knowledge on the morphological structure of redclaw eggs (Rodríguez-González et al., 2006b). Since research only clarified the role of dietary lipid (soybean oil) and other nutrients in maturing gonad at early stages of vitellogenesis, it is essential to know whether these nutrients can satisfy the nutritional requirements at latter stages (secondary vitellogenesis) of gonadal development (Li et al., 2011). From studies in decapod crustaceans, it is found that egg and embryo quality can be sufficiently increased through the supplementation of *n*-3 series fatty acids into diets (Churchill, 2003; Harlioğlu et al., 2013). For example, supplementation of *n*-3 series fatty acid in broodstock diet reportedly improved the pleopodal egg and stage-1 larval quality in freshwater crayfish, *A. leptodactylus* (Harlioğlu et al., 2013). Nevertheless, there is no information on potential of *n*-3 series fatty acids on inducing gonadal maturation and improving the quality of egg and embryo in redclaw. So based on the available studies 3 and 6% *n*-3 series fatty acids could be used in broodstock diet in combination with temperature (27 °C) and photoperiod (14L:10D) treatment to induce sexual maturity in redclaw. The biochemical composition of redclaw eggs revealed the presence of increased level of essential amino acids (EAA) in eggs which suggests the significance of this nutrition in oocyte maturation (Luo et al., 2004). However, dietary requirements of EAA have been determined for juveniles only (Muzinic et al., 2004) and studies are absent on the optimum requirement of EAA for redclaw broodstock (Harlıoğlu and Farhadi, 2017). Therefore, additional studies are required to determine the optimum level of EAA to ensure adequate nutrients and energy to the broodstock diet. Despite having a relatively high sterol content, crustacean tissues are unable to synthesise sterols independently, similar to other arthropods. As a result, they rely on obtaining these essential nutrients from their diet to support their growth, development, and overall survival (Wacker and Martin-Creuzburg, 2007). Moreover, it has been observed that the supplementation of cholesterol in the diet of juvenile redclaw can enhance their growth and survival, but there is a lack of studies examining the effects of cholesterol supplementation on the reproductive capacity of redclaw broodstock (Hernández et al., 2004). Furthermore, astaxanthin plays a crucial role in promoting gonadal maturation, spawning, improving egg quality, and enhancing embryo survival in crustaceans (Barım-Öz and Şahin, 2016; Paibulkichakul et al., 2008). However, there is a lack of research investigating the optimal requirements for astaxanthin supplementation specifically in redclaw broodstock. Further studies are needed to determine their optimum levels on reproduction of female redclaw.

Furthermore, the hormonal cascades of stress response in crayfish are not well known which is well known in vertebrates. For example, Crustacean Hyperglycemic Hormone (CHH) is a crustacean stress neuropeptide. Since redclaw are often exposed to extreme conditions such as capture, long-time handling, holding in captivity that may elicit stress response and whether these stressors have any influence on reproduction of redclaw need to be assessed. Thus, a

method to monitor stress level in redclaw is also needed to determine the influence of physiological stress on the egg and embryo quality.

It is also still unclear about the precise timing of ovulation and egg release relative to mating in redclaw. Greater emphasis should be given on studying the sexual differentiation and maturation of crustaceans, as well as their general endocrinology (Ford, 2012). Again, it is unknown whether redclaw female carries any chemical stimuli in their eggs that might help to synchronise the release of sperm and eggs to make the fertilisation more effective. For example, the freshwater astacids (*Austropotamobius pallipes)* was found to release their eggs at a slower pace on the spermatophore and the egg mucous contain chemical substances allowing more time (72-96 h) to exude the sperm from spermathecal for egg fertilisation (McLay and van den Brink, 2016). A similar study could be performed for a better understanding of the reproduction of redclaw.

Moreover, research is urgently needed to induce oocyte maturation, ovulation and egg release in order to maximise both natural and artificial spawning opportunities. In female *C. quadricarinatus,* reproductive efficiency can be improved by adapting proper husbandry protocols, manipulating environmental factors, dietary nutrition and optimisation of feeding regimes of broodstock (Harlıoğlu and Farhadi, 2017; Saoud et al., 2013). But there are still deficits of knowledge on these factors.

2.6. Artificial Control of Gonadal Maturation, Ovulation and Spawning in Decapod Crustaceans

2.6.1 Artificial Control of Gonadal Maturation

Ovarian maturation in crustaceans is regulated by the production of crustacean hyperglycaemic hormone (CHH), mandibular organ-inhibiting hormone (MOIH), moult-inhibiting hormone (MIH) and vitellogenesis-inhibiting hormone (VIH) or gonad-inhibiting hormone (GIH) from the X-organ-sinus gland (XO-SG) complex (Figure 2.12; Chen et al., 2020; Nagaraju, 2011; Swetha et al., 2011). These eyestalk hormones are collectively known as CHH (CHH-A and CHH-B) family peptides that are structurally similar but functionally diversified (Chen et al., 2020). While GIH exerts its inhibitory effect directly on vitellogenin (Vg) synthesis, MOIH and MIH can inhibit vitellogenesis indirectly by suppressing the secretion of methyl farnesoate (MF) synthesised from mandibular organ (MO) and ecdysteroids synthesised from Y organs, respectively (Subramoniam, 2017a). Although, the role of CHH in the regulation of vitellogenesis is not yet established, several studies suggest it triggers vitellogenesis in decapods (De Kleijn et al., 1998; Duangprom et al., 2017; Fu et al., 2016; De Kleijn and Van Herp, 1998). In female American lobster *Homarus americanus*, the total CHH in the haemolymph significantly increased during maturation while a low level of GIH mRNA in the X-organ and a

low amount of the GIH I isoform in the sinus gland were observed only in the immature stage. In contrast, GIH levels in the haemolymph were high during the immature and previtellogenic stages (De Kleijn et al., 1998).

In addition to the XO-SG, the brain and thoracic ganglia of the central nervous system secretes vitellogenesis-stimulating hormone (VSH) or gonad-stimulating hormone (GSH), via a series of intermediate steps, that promotes ovarian development in decapod crustaceans (Figure 2.12; Swetha et al., 2011). Thus, neurohormones in crustaceans play a dual role in gonadal maturation, one inhibitory (VIH or GIH or CHH) and the other stimulatory (VSH or GSH). During the breeding season, GIH levels decrease with a concomitant increase in secretion of GSH; a trend that is reversed by the end of the season (Zapata et al., 2003) while GIH tends to dominate during the pre-maturational phases.

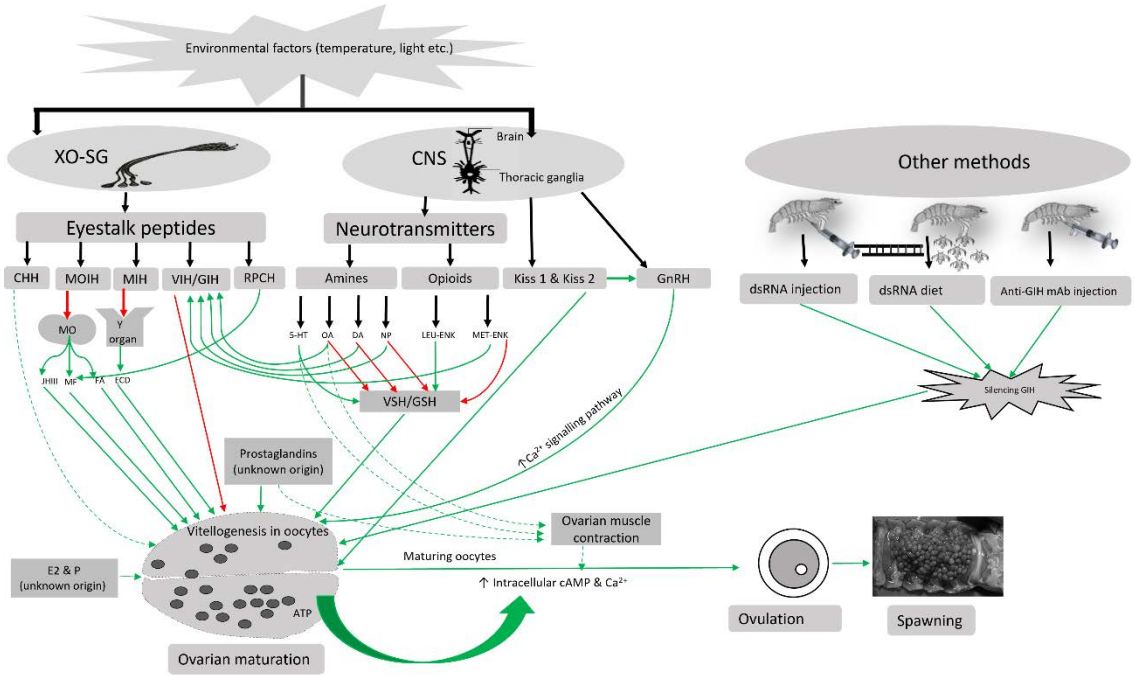


Figure 2.12. Putative endocrine pathway and other factors regulating ovarian maturation, ovulation, and egg release in decapods. Anti-GIH mAb, Anti gonad inhibiting hormone monoclonal antibody; ATP, Adenosine triphosphate; CHH, Crustacean hyperglycemic hormone; CNS, Central nervous system; DA, Dopamine; dsRNA, Double-stranded RNA; ECD, Ecdysteroids; E2, estradiol 17β; FA, Farnesoic acid; GIH, Gonad inhibiting hormone; GnRH, Gonadotropin releasing hormone; GSH; Gonad stimulating hormone; 5-HT, 5-hydroxy tryptamine; JHIII, Juvenile III hormone; Kiss 1 and Kiss 2, Kisseptin 1 and kisseptin 2; LEU – ENK, Leucine Enkephalin; MET ENK, Methionine Enkephalin; MF, Methyl farnesoate; MIH, Moult inhibiting hormone; MO, Mandibular organ; MOIH, Mandibular organ inhibiting hormone; NP, Norepinephrine; OA, Octopamine; PGs, Prostaglandins; P Progesterone; RPCH,

Red pigment concentrating hormone; VIH, Vitellogenesis inhibiting hormone; VSH, Vitellogenesis stimulating hormone; XO-SG, X-organ-sinus gland. Stimulatory and inhibitory effects are indicated using green and red arrows, respectively. The green dashed arrow indicates that the effect is not yet established.

Vitellogenesis is the central process that results in the formation of yolk during the crustacean oogenic cycle when complex yolk proteins are stored in oocytes to supply adequate energy for the developing embryo (Jimenez-Gutierrez et al., 2019; Subramoniam, 2011). In mature females, vitellogenesis occurs between moulting events, with both physiological processes being essential for growth and increased fecundity (Subramoniam, 2011). In crustaceans, multiple hormonal factors that actively control vitellogenesis might have a species-specific role or function synergistically with other hormones. Although the molecular mechanisms of these hormone-mediated pathways are obscure, some hormones appear to be involved in the activation of vitellogenesis (Jayasankar et al., 2020; Subramoniam, 2000). For example, (i) biogenic amines e.g. 5-HT (5 hydroxy tryptamine) also known as serotonin and endogenous opioids e.g. leucine enkephalin (LEU-ENK) secreted from the central nervous system, (ii) juvenile hormone III (JHIII), MF (structural homologue of insect JHIII) and farnesoic acid (FA) secreted by the MO, (iii) ecdysteroids (ECD), originating from Y-organ, and (iv) a variety of vertebrate-type sex steroids, including oestradiol and progesterone of uncertain origin (Figure 2.12; Nagaraju, 2011; Subramoniam, 2000; Swetha et al., 2011). In decapods, a coordinated control of moulting and vitellogenesis is imperative to accomplish continuous growth and increased fecundity and is likely needed to ensure that sufficient nutrients are available for vitellogenesis when it is needed (Subramoniam, 2011). MIH is an important endocrine hormone in crustaceans, which concurrently inhibits moulting and induces ovarian maturation (Nagaraju, 2011). Although the interplay between GSH and GIH is elusive, GSH upregulates vitellogenesis, while GIH suppresses vitellogenesis (Subramoniam, 2017a). Since most inhibitory hormones are primarily produced by the XO-SG in the eyestalk, they can be inactivated using eye-stalk ablation, a practice commonly used to induce ovarian maturation and spawning in commercially important crustaceans in aquaculture settings (Liu et al., 2014a,b; Palacios et al., 1999; Subramoniam, 2017a; Swetha et al., 2011). Other methods that have been used to induce ovarian maturation in commercially important decapods include exogenous hormonal treatment, RNA interference (RNAi) and monoclonal antibody technology (Table 2.2).

Table 2.2: Different methods to induce ovarian maturation in decapod crustaceans.

Methods	**Species**	**Delivery method**	**Dosage**	**Maturation time**	**Results**	***P* value**	**References**
Bilateral eyestalk ablation	*Panuliru homarus*	N/A	N/A	Stage IV ovary in 16d	Greater GI in ablated female	NR	Radhakrishnan and Manambrakat, 1984
	Potamon persicum	N/A	N/A	Moulted in 28d, ovary maturation by 14d	Greater GI in ablated female	<0.05	Khazraeinia and Khazraeinia, 2009
Unilateral eyestalk ablation	*Penaeus semisalcatus*	N/A	N/A	Moulting to stage IV ovary: 5.6 ± 1.9d Moulting to spawning: 8.3 ± 2d	Increased moulting and spawning rates Increased no. of eggs, nauplii and zoea production	<0.001 <0.01	Browdy and Samocha, 1985
	Penaeus semisalcatus	N/A	N/A	Moult: 16.8 ± 1.3d (mean ± SEM) at 28 °C	At 28 °C eyestalk ablation, either at 10 or 14 h of illumination successfully induced maturation and multiple spawns	NR	Aktaş et al., 2003
	Penaeus monodon	N/A	N/A	Ovary maturation in wild and pond females: 5d and 14d,	Larger oocytes in both wild and pond female ovary	NR	Tan-Fermin, 1991

Methods	Species	Delivery method	Dosage	Maturation time	Results	*P* value	References
				respectively			
	Penaeus monodon	N/A	N/A	Vg level and energy production genes: 7d later	Increased GSI, Vg transcript, progesterone, energy production genes and previtellogenic oocytes to mature oocytes	<0.05	Uawisetwathana et al., 2011
	Penaeus vannamei	N/A	N/A	11.2 ± 2.0 and 7.2 ± 0.8d (mean ± SE) between mating for one and more than one spawner, respectively	Increased spawning frequency, significantly higher GSI, larger oocytes, higher acyl glycerides and higher HR; 74.9 ± 16.4% (mean ± SE)	<0.05	Palacios et al., 1999
	Cherax quadricarinatus	N/A	N/A	Moult: previously spawned and first-time spawners 28d and 42d later, respectively	Increased moulting frequencies	<0.01	Sagi et al., 1997
	Procambarus clarkii	N/A	N/A	GI and GSI: 15d later	Increased GI and GSI	<0.05	Guan et al., 2013
	Scylla olivacea	N/A	N/A	Maturation after 35d,	Enhanced ovarian	<0.05	Muhd-Farouk et

Methods	Species	Delivery method	Dosage	Maturation time	Results	*P* value	References
				highest oocyte diameter after 56d	maturation with increased oocyte diameter		al., 2019
17 α-hydroxypro-gesterone	*Procambarus clarkii*	Immersion (60 sec)	0.2% solution	Ovarian maturation in 90d	Induced 50% highest ovarian maturation rate	<0.05	Liu et al., 2014a
		Injection	0.05 mL/crayfish solution)	Ovarian maturation in 90d	Induced 30% highest ovarian maturation rate	<0.05	
		Topical	0.05 mL/crayfish	Ovarian maturation in 90d	Induced 20% highest ovarian maturation rate	<0.05	
MF	*Procambarus clarkii*	Feeding	2 µg/crayfish	Ovarian maturation in 30d	Yielded 10 times larger ovaries	<0.05	Laufer et al., 1998
MF + 17 β-oestradiol	*Procambarus clarkii*	Injection	10 nM + 0.1 µM/crayfish	Ovarian maturation in 21d	Yielded higher GSI	<0.05	Rodríguez et al., 2002
Serotonin (5-HT)	*Procambarus clarkii*	Injection	15 µg/g BW	Ovarian maturation in 15d	Significantly increased ovarian index and oocyte diameter	NR	Kulkarni et al., 1992
	Macrobrachium	Injection	50 µg/g BW	Ovarian maturation in	Increased OI (5.7 ± 0.1%) at 1 µg/g BW as compared	<0.05	Meeratana et al.,

Methods	Species	Delivery method	Dosage	Maturation time	Results	*P* value	References
	rosenbergii			15d	to control (1.5 ± 0%; mean ± SE)		2006
	Macrobrachium rosenbergii	Injection	25 µM and 2.5 µM/prawn	Ovary maturation: 14, 25 and 49d Embryo development: 20 and 19d	Shortened maturation and embryo development time, increased GSI and oocyte diameter	<0.05	Tinikul et al., 2009
	Penaeus vannamei	Injection	50 µg/g BW	Ovary maturation: 42d	Induced greater spawning compared to control	<0.05	Vaca and Alfaro, 2000
	Penaeus monodon	Injection	50 µg/g BW	Spawned at 10.1 ± 2.7d	Greater hatching rate (81.7 ± 5.4%) and nauplii production (83,993 ± 8310)	<0.05	Wongprasert et al., 2006
Serotonin (5-HT) + Spiperone	*Macrobrachium rosenbergii*	Injection	25 µM 5-HT/prawn + 0.27 µM spiperone /prawn	Ovary maturation: 14, 25 and 49d Embryo development: ~16d	Shortened maturation and embryo development time, increased GSI and oocyte diameter	<0.05	Tinikul et al., 2009
Spiperone	*Macrobrachium rosenbergii*	Injection	0.27 µM/prawn	Ovary maturation: 14, 25 and 49d	Shortened maturation and embryo development time, increased GSI and oocyte	<0.05	Tinikul et al., 2009

Methods	**Species**	**Delivery method**	**Dosage**	**Maturation time**	**Results**	***P* value**	**References**
				Embryo development: ~20d	diameter		
EI-DOM	*Procambarus clarkii*	Injection	0.5 mg/crayfish	32d	Induced synchronous maturation of ovaries	<0.05	Liu et al., 2014b
Progesterone	*Metapenaeus ensis*	Injection	0.1 μg/g BW	30d	Induced moulting and mating and stage III, IV, and V ovarian development	NR	Yano, 1985
Naloxone	*Cherax quadricarinatus*	Feeding	1 nM/g BW	13 weeks	Produced bigger oocytes	<0.05	Cahansky et al., 2008
Salmon gonadotropin -releasing hormone (s)GnRH	*Penaeus monodon*	Injection	500 ng/g BW	23.2 ± 2.5d	Shortened ovarian maturation time	<0.05	Ngernsoungnern et al., 2008
					Induced spawning: 71.4%	NS	
					No. of eggs/spawn: 1.0×10^6 ± 2.8×10^5	NS	
					FR: 81.2 ± 4.7%	NS	
Lamprey gonadotropin -releasing	*Penaeus monodon*	Injection	500 ng/g BW	22.8 ± 2.1d	Shortened ovarian maturation time	<0.05	Ngernsoungnern et al., 2008
					Induced spawning: 71.4%	NS	

Methods	Species	Delivery method	Dosage	Maturation time	Results	*P* value	References
hormone (l)GnRH-I					% eggs/spawn: $1.0\times10^6 \pm 1.3\times10^5$	NS	
					FR: 82.0 ± 3.9%	NS	
Octopus (oct)GnRH	*Macrobrachium rosenbergii*	Injection	500 ng/g BW	22.5 ± 1.5d	Shortened ovarian maturation time	<0.05	Ngernsoungnern et al., 2009
					Greater GSI; 4.6 ± 0.9%	<0.05	
lGnRH-I and lGnRH-III	*Macrobrachium rosenbergii*	Injection	500 ng/g BW	25.5 ± 4.0 and 25.5 ± 4.0d, respectively	Shortened ovarian maturation time	<0.05	Ngernsoungnern et al., 2009
					Greater GSI; 6.0 ± 1.2% and 8.9 ± 1.0%, respectively	<0.05	
GnRH analog GnRHa	*Macrobrachium rosenbergii*	Injection	1,000 ng/g BW	26.6 ± 4.0d	Shortened ovarian maturation time	<0.05	Ngernsoungnern et al., 2009
					Greater GSI; 3.8 ± 1.3%	<0.05	
Kiss 1 and Kiss 2	*Macrobrachium rosenbergii*	Injection	50, 250 and 500 ng/g BW	N/A	Shortened ovarian maturation time, greater GSI, spawned eggs and Vn content	<0.05	Thongbuakaew et al., 2016

Methods	Species	Delivery method	Dosage	Maturation time	Results	*P* value	References
RPCH	*Procambarus clarkii*	Injection	1 µM	10d	Significantly increased ovarian index and oocyte diameter	0.05	Sarojini et al., 1995b
	Scylla paramamosain	Injection	10^{-6} or 10^{-7} mM/crab	16d	Significantly increased GSI and Vg levels in ovary and hepatopancreas	0.05	Zeng et al., 2016
	Litopenaeus vannamei	Injection	0.5 or 5 µM	20d	Increased ovarian Vg level and oocyte diameter	<0.05	Chen et al., 2018
GIH-dsRNA	*Penaeus monodon*	Injection	3 µg/g BW	30d	Induced ovarian maturation and spawning (14% in domesticated and 63% in wild shrimp)	NR	Treerattrakool et al., 2011
	Penaeus monodon	Feeding	0.3 g wet weight of GIH-dsRNA-enriched Artemia	14d	Decreased GIH mRNA level and induced ovarian maturation	<0.05	Treerattrakool et al., 2013
Anti-GIH	*Penaeus*	Injection	20 µg GIH	31d	Induced higher maturation	NR	Treerattrakool et

Methods	Species	Delivery method	Dosage	Maturation time	Results	*P* value	References
mAb	*monodon*		mAb/shrimp		and spawning (67%)		al., 2014

Values mean ± standard deviation unless stated otherwise.

Abbreviations: d, days; F, fecundity; FR, fertilisation rate; GI, gonadal index; GSI, gonadosomatic index; N/A, not applicable; HR, hatching rate; NR, not reported; NS, no significant difference; OI, ovarian index.

2.6.1.1 Maturation Induction by Eyestalk Ablation.

Unilateral or bilateral eyestalk ablation can be used to induce ovarian maturation by extirpation of one or both X-organ sinus glands (Muhd-Farouk et al., 2019; Palacios et al., 1999). Excision prevents secretion of GIH; thereby disinhibiting secretion of GSH from the brain and thoracic ganglia (TG; Nagaraju, 2011), which stimulate gonadal maturation and egg production in female crustaceans (Browdy and Samocha, 1985). Ablation causes synchronised endogenous stimulatory hormone secretion that results in a predictable peak of maturation and spawning that facilitates the establishment of harvesting schedules, compared to unpredictable and scattered spawns that occur in unablated females (Browdy, 1992).

The synchronised maturation and spawning of *P. semisulcatus* in captivity were achieved 85 days after unilateral eyestalk ablation in which spawning rate was 91.6 *vs.* 66.7% in unablated females (Browdy and Samocha, 1985). Maturation time was significantly shortened, and the number of spawns increased in every moult cycle, with ablated females showing significantly more spawns (4.6 ± 2.7 *vs.* 1.1 ± 0.9; mean ± SD; $P<0.001$) and producing greater numbers of eggs (248,097 ± 160,185 *vs.* 76,476 ± 68,749; $P<0.001$) per female respectively. The total number of nauplii (184,743 ± 99,715 *vs.* 84,883 ± 44,232; mean ± SD; $P<0.01$) and zoea produced (159,569 ± 87,284 *vs.* 72,154 ± 38,492; $P<0.01$) were significantly greater in ablated compared to unablated females, however there was considerable individual variation between prawns (Browdy and Samocha, 1985).

Reproductive output of Pacific white shrimp *P. vannamei*, increased after eyestalk ablation with greater gonadosomatic index (GSI; 2.6 *vs.* 2.0%; $P<0.05$), more frequent spawning (2.9 ± 0.4 *vs.* 0.6 ± 0.1 spawns/female/month) and larger oocyte diameter (278.6 ± 11.9 *vs.* 222.9 ± 10.3 µm; $P<0.05$) than unablated females respectively (Palacios et al., 1999). Moreover, ablated females had significantly higher levels of acylglycerides in their ripe ovaries than unablated females (11.3 ± 1.2 *vs.* 7.9 ± 1.5 mg/g respectively; $P<0.05$).

The effects of unilateral eyestalk ablation on ovarian development were examined in wild and pond-reared *P. monodon* (Tan-Fermin, 1991). Time to spawning was faster in ablated (wild: 5 and pond-reared: 14 days) compared to unablated (wild: l and pond-reared: 2.2 months) females. Moreover, large oocytes (wild: 110 to 200 µm and pond-reared: 210 to 300 µm in diameter) were only present in the ovaries of ablated shrimps at spawning.

Unilateral eyestalk ablation in black tiger shrimp inhibited the secretion of GIH and induced the production of previtellogenic oocytes by stimulating the gonadotropin-releasing hormone (GnRH) and the calcium signalling pathways (Figure 2.12; Uawisetwathana et al., 2011). Ablation led to vitellogenesis in the ovary confirmed by increased Vg transcription, a reliable marker of ovarian maturation in other decapods (Okumura et al., 2006). Vg gene expression

increased 240-fold (P<0.05) 7 days after ablation, with ovaries maturing to the late cortical rod stage. Stimulation of GnRH and the calcium signalling pathways simultaneously activated the release of progesterone within the ovary, which then induced progesterone-mediated oocyte maturation (Figure 2.12). Increased transcription of Vitellogenin and energy-production genes (cytochrome C oxidase, NADH dehydrogenase, and ATP synthase F0), improved the development of previtellogenic oocytes to mature pre-ovulatory oocytes with germinal vesicle break down (GVBD; Uawisetwathana et al., 2011).

In adult female crabs *Potamon persicum*, bilateral eyestalk ablation significantly enhanced ovarian development with gonadal indices of 1.5 ± 0.3 *vs.* 0.6 ± 0.1 (P<0.05) in ablated *vs.* unablated crabs, respectively (Khazraeinia and Khazraeinia, 2009). Moreover, in orange mud crab *Scylla olivacea*, 56 days after unilateral eyestalk ablation a greater percentage of gonads (12.5 *vs.* 0%; P<0.05) were classified as reaching stage 4 of ovarian maturation. Oocyte diameter was also significantly greater compared to unablated control crabs, reinforcing the stimulatory effect of unilateral eyestalk ablation on maturation of the female gonad (Muhd-Farouk et al., 2019).

The effect of unilateral eyestalk ablation on reproduction and moulting was recorded in two groups (previously spawned and virgin) of female *C. quadricarinatus* during the winter season when maturation is normally arrested (Sagi et al., 1997). Spawning rate was significantly greater in ablated virgin (74%) and previously spawned females (38%) than intact virgin (28%) and previously spawned females (29%). Virgin females exhibited uniform early vitellogenic ovaries with small uniform-sized oocytes while the ovaries of previously spawned females contained two distinctly sized (small and large) coloured oocytes, indicating that early vitellogenic ovaries containing smaller uniform-sized oocytes are more receptive to the ablation treatment. Females with eyestalk ablation moulted considerably more frequently (virgin: 22% and previously spawned:48%) than unablated controls (virgin: 0% and previously spawned: 13%; P<0.05).

Fifteen days after eyestalk ablation of female *Procambarus* broodstock, body length (13.5 ± 0.4 to 14.8 ± 0.7 cm; mean ± SD), body weight (BW; 35.2 ± 3.8 to 40.5 ± 6.8 g), ovarian weight (0.3 ± 0.1 to 1.9 ± 0.6 g) and GSI (0.8 ± 0.2 to 4.4 ± 2.3%; P<0.05) increased significantly (Guan et al., 2013). Unablated crayfish remained smaller and white, whereas ablated crayfish increased in size and changed from white to yellow on the first day after ablation, to brown on Day 7 and dark brown on Day 15. Collectively, these physiological changes indicate how rapidly growth and ovarian maturation occurs following eyestalk ablation in crayfish. Moreover, calmodulin gene expression increased 8.7-fold 1 day after eyestalk ablation in *P. clarkii* (P< 0.05; Guan et al., 2013). Early induction of this small highly conserved protein that binds Ca^{2+}

and transduces Ca^{2+} signals into downstream effects is thought to be crucial during eyestalk ablation-induced ovarian maturation of this species.

Although eyestalk ablation accelerates early spawning, it can result in poor quality spawns with lower fertilisation and hatching rates, total viable nauplii and overall fecundity in some decapods (Emmerson, 1980; Magaña-Gallegos et al., 2018). Eggs from eyestalk ablated pink shrimp *Farfantepenaeus brasiliensis* had reduced fertilisation rate (45.1 ± 0.9 *vs.* 71.6 ± 4.6%; $P< 0.05$) and produced nauplii of shorter body length (438.3 ± 1.1 *vs.* 448.2 ± 1.4 µm; $P< 0.05$) than unablated females (Magaña-Gallegos et al., 2018). Ablation can adversely affect broodstock mating behaviour, with a significant decrease in mating rate observed in ablated *vs.* unablated *P. semisalcatus* females (77.0 *vs.* 95.5%; $P< 0.05$) (Browdy and Samocha, 1985). Ablation may also cause mortality in broodstock (Liu et al., 2014b; Magaña-Gallegos et al., 2018; Sainz-Hernández et al., 2008). Mortality was significantly higher in unilaterally (35%) and bilaterally (68%) ablated *L. vannamei* shrimp than in unablated females (2%) (Sainz-Hernández et al., 2008). This may cause loss of valuable broodstock and reduce productivity and economic returns. Poor-quality spawns may occur immediately after ablation due to the associated stress of the procedure or in subsequent spawning (Palacios et al., 1999). A decrease in offspring quality is also associated with several other factors including the increased frequency of spawning observed in ablated females, which could divert the available energy mostly to reproduction (Magaña-Gallegos et al., 2018; Palacios et al., 1999; Racotta et al., 2003). Oocytes of ablated females develop more rapidly and, as such, receive less energy-rich biomolecules, and nutrients (Palacios et al., 1999). This has been associated with embryo malformation, lower hatching rates and overall declines in fecundity (Palacios et al., 1999). This may also be reflected in the viability of the next generation of spawners, which preliminary evidence suggests may have reduced immunity and fertility. In Indian spiny lobster *Panulirus homarus*, total haemocyte number and prophenoloxidase activity decreased 1 week after the ablation (Verghese et al., 2008). Moreover, ablation appears to suppress important sets of immune-relevant genes in decapod crustaceans (Uawisetwathana et al., 2011). Alternate strategies to stimulate ovarian maturation are warranted to avoid some of these deleterious effects on production and induce predictable gonadal maturation and spawning in commercially important decapods (Uawisetwathana et al., 2011).

2.6.1.2 Sex Separated Stocking to Induce Gonadal Maturation.

In decapod crustaceans, such as of shrimps and prawns the reproduction can be influenced by various factors, including the presence of males (Bauer, 1979; Peeters and Diter, 1994; Tropea et al., 2018). While direct interaction between males and females is often necessary for successful reproduction, there are techniques where the indirect presence of males can influence their reproductive behaviour and potentially improve reproductive outcomes under certain

circumstances. In such method, female individuals are usually stocked separately from males while yet allowing chemical cues or pheromones to mix in a recirculatory system with an intention to simulate natural spawning conditions and cause egg release (Amagai et al., 2022; Huertas et al., 2014; Kurtzman et al., 2010; Soyano et al., 2022). Sexually segregated rearing technique has been found effective in several species such as tilapia, groupers, sea cucumbers, *Penaeus indicus* and *Heptacarpus pictus* (Amagai et al., 2022; Bauer, 1979; Huertas et al., 2014; Marquet et al., 2018; Peeters and Diter, 1994) for inducing maturation as a natural means of reproduction. Although there is no study on sex-separated reproduction in female redclaw, this could be attempted as a non-invasive strategy to enhance reproductive performance and improve production efficiency.

2.6.1.3 Maturation Induction by Hormone Administration.

In decapods, several studies have investigated the use of exogenous hormone treatment to control ovarian maturation, as an alternative to eyestalk ablation to produce high-quality juveniles (Alfaro-Montoya et al., 2019; Laufer et al., 1998; Liu et al., 2014b; Nagaraju et al., 2002; Ngernsoungnern et al., 2008; Treerattrakool et al., 2013). Candidate molecules such as neurotransmitters, neurotransmitter antagonists, steroids, juvenoids and prostaglandins have been tested (Alfaro-Montoya et al., 2019). The establishment of an adaptable technique that would allow the production of high-quality eggs without complications would be of immense advantage to the redclaw industry (Benzie, 1998).

Ovarian maturation in crustaceans is controlled by the steroid hormone, progesterone (Figure 2.12; Liu et al., 2014a; Yano, 1985). In greasyback shrimp *M. ensis*, injected with 0.1 µg/g BW progesterone, ovarian development reached advanced stages (stage III, IV, and V) after 1 month and all females successfully moulted and mated, compared to controls that only reached early ovarian stage II (Yano, 1985). In female *P. clarkii* subjected to different methods of 17 α-hydroxyprogesterone treatment, ovarian maturation was highest (50%) after abdominal immersion for 60 sec, followed by injection (40%), topical application (30%), then control (20%) treatments ($P<0.05$; Liu et al., 2014a).

Crustaceans and insects are both arthropods and share certain hormones. Crustaceans like redclaw are vulnerable to some insecticides and that includes analogues of insect juvenile hormone. Methyl farnesoate (MF) is a sesquiterpenoid insect juvenile hormone, produced in the mandibular organ (Figure 2.12) that plays a significant role in regulating crustacean reproduction (Laufer et al., 1987). In female *P. clarkii*, administration of MF in pelleted food at a rate of 2 µg/crayfish/day for 30 days stimulated gonadal maturation yielding ovaries 10 times larger than controls ($P<0.05$) (Laufer et al., 1998). The effect of MF alone or in combination with other Vg hormones, such as juvenile hormone III (JHIII), 17 α-hydroxyprogesterone and

17 β-oestradiol was evaluated in female *P. clarkii* (Rodríguez et al., 2002). Approximately 10 nM (~2.5 µg) MF was injected per crayfish, with or without 0.1 µM/crayfish of either JHIII, 17 α-hydroxyprogesterone or 17 β-oestradiol twice a week for 3 weeks. The mean increase in GSI was significantly greater in crayfish injected with MF alone (1.1%) or MF plus 17 β-oestradiol (1.3%) compared to MF plus JHIII (0.6%), MF plus 17 α-hydroxyprogesterone (0.4%) and control animals (0.2%; $P<0.05$) (Rodríguez et al., 2002). When intermoult stage female crabs, *Oziotelphusa senex senex,* were injected with 15 ng/crab MF on Day 1, 7, 14 and 21, and sacrificed on Day 28 an increased ovarian index (1304.3% *vs.* -18.1%) and mean oocyte diameter (236.3% *vs.* 5.1%; $P<0.0001$) compared to control animals was observed (Reddy et al., 2004).

The neurotransmitters such as 5-HT, dopamine, enkephalin, norepinephrine, and octopamine are known to be involved in the release of neurohormones and control vitellogenesis in crustaceans (Kulkarni et al., 1992; Lorenzon et al., 2005; Wongprasert et al., 2006). While 5-HT and leucine enkephalin upregulate ovarian maturation, octopamine, dopamine, norepinephrine and met-enkephalin inhibit ovarian development by stimulating GIH release from the XOSG and/or by simultaneously inhibiting GSH release from the brain or thoracic ganglia (Figure 2.12; Kulkarni et al., 1992; Sarojini et al., 1996; Subramoniam, 2017a; Tinikul et al., 2009). *P. monodon* injected with 50 µg/g BW of 5-HT had significantly greater hatching rates (81.7 ± 5.4 *vs*. 65.5 ± 14.5 %; mean ± SD) and nauplii production (83,993 ± 8310 *vs*. 71,344 ± 16621; mean ± SD), compared to eyestalk ablated animals, respectively (Wongprasert et al., 2006). In female *C. quadricarinatus* (4 to 14 g), dopamine injection suppressed gonadal maturation resulting in a greater proportion of females with underdeveloped ovaries (Tropea and López Greco, 2013). Mature *M. rosenbergii* injected with, either 0.25, 2.5 or 25 µM dopamine and octopamine showed decreased ovarian maturation and oocyte diameter compared to controls (Tinikul et al., 2009). In *P. clarkii,* dopamine, norepinephrine and octopamine did not affect ovarian development, whereas injection of 15 µg/g BW 5-HT on Days 1, 5 and 10, significantly increased ovarian index (OI) and oocyte size at Day 15 by 30.5% and 34.0%, respectively (Kulkarni et al., 1992). Also, eyestalk interventional injection (EI-DOM - prepared using a *Bletilla striata* polysaccharide gelatin containing tranexamic acid and domperidone, a dopamine antagonist) induced synchronous ripening of ovaries in *P. clarkii* when injected at a dose of 0.5 mg/crayfish every 3 days for a period of 32 days (Liu et al., 2014b). The rate of ovarian maturation was 66.6 ± 9.6% in treated compared to 22.0 ± 3.0% ($P<0.05$) in control animals. Naloxone (an enkephalin antagonist), spiperone and domperidone (dopamine antagonists) treated pellets were fed twice a week at 10^{-6} mM/g BW to *C. quadricarinatus* for 13 weeks throughout the post-reproductive period (Cahansky et al., 2008). Naloxone treatment produced significantly larger oocytes at the near-mature secondary vitellogenic stage (~830 µm diameter),

compared to spiperone and dimperidone (both ~550 µm diameter), and control treatments (~430 µm diameter; $P< 0.05$) (Cahansky et al., 2008). Thus, naloxone appears to have greater potential than spiperone and domperidone in promoting ovarian growth and maturation in crustacean aquaculture.

Kisspeptin peptides (Kiss l and Kiss 2) synthesised in the central nervous system (Figure 2.12) of decapods could stimulate the proliferation of oogonia, maturation of ovaries and spawning (Thongbuakaew et al., 2016). Kiss l and Kiss 2 were co-localised with GnRH and shown to regulate the synthesis of GnRH from central nervous system (Figure 2.12; Thongbuakaew et al., 2016). Mature female freshwater prawns, *M. rosenbergii* were injected with 50, 250 or 500 ng/g BW of Kiss 1 and Kiss 2 on days 1, 4 and 8 after spawning (Thongbuakaew et al., 2016). The ovaries were dissected from one in seven animals' post-mortem on days 12, 16, 20, 24, 28, 32 and 36, and remaining animals were allowed to spawn. GSI increased significantly on days 12, 16 and 20 among all treatment's groups compared to controls ($P\leq 0.05$). The mean (± SD) time to spawning decreased significantly for Kiss 1 (18.9 ± 1.6, 18.0 ± 1.7 and 16.0 ± 1.7 days) and Kiss 2 animals (22.9 ± 1.1, 18.6 ± 1.9, and 18.0 ± 1.1) treated at doses of 50, 250 and 500 ng/g BW, respectively compared to controls (36.0 ± 2.0 days). By Day 16, the ovaries of Kiss 1 and 2 treated groups developed to stage 4 and had high quantities of Vn in the cytoplasm of their oocytes compared to ovaries of controls that remained at stage 2 and did not show Vn expression. Moreover, the mean (± SD) number of spawned eggs were significantly greater for Kiss 1 ($4.0 \times 10^5 \pm 1.5 \times 10^4$, $4.4 \times 10^5 \pm 2.6 \times 10^4$, $5.0 \times 10^5 \pm 2.9 \times 10^4$) and Kiss 2 animals ($3.4 \times 10^5 \pm 1.9 \times 10^4$, $4.0 \times 10^5 \pm 1.7 \times 10^4$, $4.5 \times 10^5 \pm 1.7 \times 10^4$) treated at doses of 50, 250, and 500 ng/g BW, respectively compared to controls ($1.5 \times 10^5 \pm 0.6 \times 10^4$; $P< 0.05$).

Red pigment-concentrating hormone (RPCH) is a neuropeptide hormone (Figure 2.12) that also appears to function in crustacean reproduction (Chen et al., 2018a; Jayasankar et al., 2020; Landau et al., 1989; Sarojini et al., 1995). This neuropeptide was discovered in the eyestalks of caridean shrimp *Pandalus borealis* (Fernlund and Josefsson, 1972), and has a primary amino acid structure that is conserved across multiple species of decapod (Marco and Gäde, 2019; Sathyanandam et al., 2008). Downregulation of RPCH in the eyestalk and nerve tissue of freshwater shrimp *M. nipponense*, resulted in ovarian degeneration (Fu et al., 2019). RPCH appears to upregulate MF synthesis from the mandibular organs of crustaceans (Figure 2.12; Landau et al., 1989). In *P. clarkii*, *in vitro* incubation of the mandibular organ in 1 µM RPCH-enriched medium stimulated the synthesis and release of MF from this tissue (Landau et al., 1989). Moreover, injection of *P. clarkii* with 1 µM RPCH on Days 1, 6 and 11 resulted in a significant increase in OI (~2.5 *vs.* ~1.5%) and oocyte diameter (~900 *vs.* ~200 µm) compared to controls (P=0.05; Sarojini et al 1995b). In mud crab *S. paramamosain*, repeated injections of 10^{-5} or 10^{-4} mM/crab of RPCH on Day 1, 6 and 11 resulted in significantly greater GSI,

hepatosomatic index, hepatopancreatic Vg transcript levels, and ovarian Vg transcript levels at Day 16 than controls (P=0.05; Zeng et al., 2016). *L. vannamei* injected with 0.5 and 5 µM synthetic RPCH on Days 1, 5, 10, 15 and 20 had more advanced ovarian development determined by histology, and a significant increase in ovarian Vg levels and oocyte area compared to controls (P<0.05; Chen et al., 2018b). These positive results suggest that further investigation of RPCH is warranted for the promotion of ovarian maturation in decapod crustaceans including redclaw crayfish.

2.6.1.4 Maturation Induction by Double-Stranded RNA Interference.

Use of RNAi technology can limit the secretion of GIH by suppressing the endogenous gene. For example, in *P. monodon*, silencing of Pem-GIH expression by GIH-dsRNA injection (Figure 2.12) resulted in increased Vg expression, suggesting that this could be an alternate approach to stimulate ovarian maturation in female decapod broodstock (Treerattrakool et al., 2011). Pem-GIH mRNA transcript was identified in the brain, eyestalk, thoracic and abdominal nerve cords of *P. monodon* (Treerattrakool et al., 2008). The dsRNA was synthesized from coding sequence of mature Pem-GIH which has been linked to a decrease in the transcript levels of Pem-GIH in eyestalk ganglia and abdominal nerve cord explant culture of female *P. monodon* broodstock (Treerattrakool et al., 2008). In *P. monodon with* previtellogenic ovaries, injection of 3 µg GIH-dsRNA/g BW through the arthrodial membrane of the second walking leg inhibited Pem-GIH expression in optic lobes for at least 30 days (Treerattrakool et al., 2011). In wild shrimp, GIH-dsRNA injection induced similar rates of spawning (63 *vs.* 72%) as eyestalk ablation. By contrast in domestic shrimp, GIH-dsRNA injection induced lower rates of spawning (14 *vs.* 53%) than eyestalk ablation, with lowest spawning occurring in controls (6%) (Treerattrakool et al., 2011).

Oral administration of *P. monodon* with dsRNA-expressing *Escherichia coli* fed to them via an *Artemia* carrier (Figure 2.12), can be an alternative means to deliver dsRNA and silence GIH expression (Treerattrakool et al., 2013). Although, the most significant reduction (68%) in GIH mRNA expression occurred in shrimp directly injected with 0.3 µg/g BW GIH dsRNA, shrimp fed with 0.3 g wet weight GIH-dsRNA-enriched Artemia 4 times/day for 14 days still had a significant decrease (28%) in GIH transcript levels compared to the non-enriched Artemia control diet (P<0.05; Treerattrakool et al., 2013). Collectively, these preliminary results indicate that dsRNA interference can alter GIH mRNA levels, which might potentially influence ovarian maturation (Figure 2.12). However, the route of administration, dosage, and frequency of administration warrant further investigation in order to optimise treatments such that they may be as effective in decapods as exogenous hormone treatment or more invasive eyestalk ablation techniques (Treerattrakool et al., 2013).

2.6.1.5 Maturation Induction by Monoclonal Antibody.

Monoclonal antibodies can bind and inhibit circulating GIH in the haemolymph (Figure 2.12) or GIH stored in the sinus gland, leading to vitellogenesis in shrimp (Treerattrakool et al., 2014). An anti Pem-GIH monoclonal antibody (anti-GIH mAb) purified from extracts of *P. monodon* optic lobe, neutralised approximately 58% of GIH activity in primary cultures of ovarian tissue (Treerattrakool et al., 2014). Peptide mapping demonstrated that 16 to 20 (MYNKV) amino acids of mature Pem-GIH could be used as an anti-GIH mAb epitope. A 20 µg injection of purified anti-GIH mAb into the arthrodial membrane of the second walking leg of wild *P. monodon* with previtellogenic ovaries induced a rate of spawning (67%) like eyestalk ablation (60%).

2.6.1.6 Maturation Induction by Gene Regulation.

In the early phases of gonadal development in crustaceans, various genes are dynamically (up/down) regulated. The majority of the genes in most studied species were upregulated in previtellogenic females. For instance, gene expression analyses results showed that Fem-1B, CTSL and CqFtz-f1 were highly expressed in the ovary during ovarian development (Chen et al., 2022; Shun et al., 2024; Zheng et al., 2022) while VGR, Cdc2 genes were downregulated as the ovaries mature in redclaw (Shun et al., 2024; Wang et al., 2013a). Thus, gene regulation study could serve as a cutting-edge tool in determining ovary maturation in redclaw.

In summary, given that crustacean reproduction is highly dependent on hormone regulation, further research using gene silencing (dsRNA interference), or gene editing (CRISPR technologies) may help elucidate the different roles hormones play in ovarian maturation. Based on the studies reviewed in Table 2.2, eyestalk ablation induces ovarian maturation rapidly (5 days) in *P. monodon* (Tan-Fermin, 1991), with a hatching rate of 74.9 ± 16.4% in *P. vannamei* (Palacios et al., 1999). However, because mortality occurs in a significant number of valuable broodstock, eyestalk ablation is becoming increasingly undesirable as a management technique, given the emergence of safer alternatives like exogenous hormone treatment or RNA interference. Among several potential hormones, GnRH (Table 2.2) appears to induce the highest rates of spawning (71.4%) and fertilisation (82.0 ± 3.9%; mean ± SD) in *P. monodon* (Ngernsoungnern et al., 2008). Additionally, other hormones including MF, oestradiol, 5-HT, progesterone, naloxone, Kiss 1 and 2, EI-DOM and RPCH, as well as GIH dsRNA and anti-GIH mAb (Table 2.2) have great potential to stimulate gonadal maturation in decapod crustaceans. These hormones can be administered to decapods by feeding, immersion, or injection. While feeding of hormones is non-invasive, induction of ovarian maturation is slower, taking up to three months in *C. quadricarinatus* (Cahansky et al., 2008) compared to intramuscular administration which can occur within 10 to 15 days in *P. clarkii* (Kulkarni et al.,

1992; Sarojini et al., 1995b) and 20 days in *L. vannamei* (Chen et al., 2018a; Kulkarni et al., 1992). While researchers have employed a variety of strategies to deliver molecular compounds to crustaceans, it is unlikely that each approach is equally successful in stimulating the target organ. Moreover, effect of some potential hormones such as 5-HT and progesterone on ovarian maturation in several commercially important decapods such as redclaw is still unknown. Thus, further study is required comparing both the effect of different hormones, dosages, and their mode of delivery on the success of ovarian maturation of redclaw. Moreover, we know that several aspects of reproduction in decapod crustaceans are intimately related to water temperature and photoperiod (Jones, 1990; Karplus et al., 2003a; King, 1993a; Yeh and Rouse, 1994; Yeh and Rouse, 1995), a combination of environmental manipulation and hormonal treatment could be used to facilitate year-round gonadal maturation and healthy embryo production in redclaw.

2.6.2 Artificial Control of Ovulation and Spawning

Beyond being able to induce ovarian maturation, control of the timing of ovulation and egg release from the gonopore is a prerequisite for successful natural mating and development of AF techniques in redclaw crayfish. Exogenous treatments can help stimulate development of follicles that results in more uniform stages of follicular maturation and improved synchrony of ovulation (Żarski et al., 2019). Moreover, induction of ovulation could enable harvesting of a larger number of viable eggs in every cycle, thereby improving the efficiency of production. The dose, type and timing of exogenous hormones that can induce ovulation have already been established in many vertebrates (Drori et al., 1994; Mansour et al., 1994; Mugnier et al., 2000; Sahoo et al., 2007). For example, in Japanese eel *Anguilla japonica* and chub mackerel *Scomber japonicas*, induction of ovulation following parenteral administration of human chorionic gonadotropin (hCG) occurred within 11-14 h and 33 h, respectively (Dou et al., 2007; Shiraishi et al., 2008). For invertebrates, particularly in decapods however, much of this information is currently unavailable. In order to harness the potential to control the timing of spawning using artificial treatments in decapod crustaceans, it is important to understand the mechanisms of ovulation induction that exist in other species.

2.6.2.1 Ovulation Induction by Hormone Treatment.

In vertebrates, fatty acid derivatives such as eicosanoids, especially prostaglandins (PGs), play an important role in ovulation (Spaziani et al., 1993). During the pre-ovulatory period, PGs are formed in granulosa cells of the follicle in response to luteinising hormone (LH) and follicle stimulating hormone (FSH; Spaziani et al., 1993). PGs facilitate ovulation by inducing tissue oedema and contraction of ovarian smooth muscle resulting in follicle rupture (Behrman, 1979). Prostaglandin E_2 and $F_{2\alpha}$ reach a maximum concentration in follicular fluid just prior to

ovulation in vertebrates (Caldwell and Behrman, 1981), with PGE_2 mainly involved in increasing follicle pressure (via ovarian tissue oedema) and $PGF_{2\alpha}$ predominantly involved in promoting follicle rupture via ovarian smooth muscle contraction (Spaziani et al., 1993).

Prostaglandins are also associated with ovulation in invertebrates, but not to the same extent (Spaziani et al., 1993). In the scallop *Patinopecten yessoensis* (Matsutani and Nomura, 1987), mussel *Dreissena polymorpha* (Fong et al., 1994), and clams *Supisula solidissima* and *S. sachalinensis* (Hirai et al., 1988), PGE_2 enhances the stimulatory effect of 5-HT on egg release. PGE_2 alone cannot initiate the egg release, but it can hinder the synthesis of aspirin (an antagonist of PG) known to inhibit the induction of spawning by 5-HT injections (Matsutani and Nomura, 1987). When 5-HT is used in combination with PGE_2, each at a concentration of 1 μM, nearly 2-fold more eggs are released than after 5-HT treatment alone (Matsutani and Nomura, 1987). The 5-HT induces spawning via receptors on the ovary and the presence of serotonin-containing nerve fibres along the germinal epithelium and gonoduct of *P. yessoensis* has been proven histologically (Matsutani and Nomura, 1987). Therefore, 5-HT released from these serotonergic nerve fibres may be responsible for the induction of ovulation. Also, 5-HT appears to induce GVBD in oocytes of the mussel *D. polymorpha* (Fong et al., 1994), and clams *S. solidissima* and *S. sachalinensis* (Hirai et al., 1988). The direct effect of 5-HT on *P. yessoensis* oocytes could not be examined, since they lyse immediately after being detached from the germinal epithelium (Matsutani and Nomura, 1987). However, the *in vitro* addition of 5-HT increases the number of oocytes undergoing GVBD, suggesting a similar role of 5-HT for *P. yessoensis* (Matsutani and Nomura, 1987). Exposure of zebra mussel *D. polymorpha*, to 1 mM 5-HT at 23 °C for 10 min intervals over 50 min induced oocyte maturation and GVBD (Fong et al., 1994). GVBD was visible in almost 50% of oocytes after 30 min and in almost all oocytes by 40 to 45 min. The spindle apparatus of metaphase chromosomes were numerous, indicating oocytes were about to ovulate (Fong et al., 1994). GBVD might be one of the major actions of 5-HT in the induction of spawning in marine bivalves and PGE_2 appears indispensable in 5-HT induced egg release (Spaziani et al., 1993).

Like vertebrates, PGs may induce ovulation by promoting smooth muscle contraction in the ovary of decapods (Spaziani et al., 1993; Spaziani et al., 1995) but to a lesser extent. Despite their different structures compared to vertebrates and other crustaceans, lobster and crayfish ovarian smooth muscles contain numerous microtubules necessary for contraction (Howard, 1991; Talbot, 1981). In lobster *H. americanus*, the main function of the ovarian muscle is to extrude oocytes during spawning (Talbot, 1981). *In vitro*, octopamine and 5-HT induced muscle contraction in *H. amaricanus* ovaries (Howard and Talbot, 1992). However, *in vivo* in *M. rosenbergii*, octopamine appeared to play a conflicting role, with injection decreasing ovarian maturation and oocyte diameter (Tinikul et al., 2009). Although the mode of action and discreet

timing of these neurohormones on ovarian muscle contraction is not confirmed, the effects are thought to be mediated by increased intracellular concentrations of both cAMP and Ca^{2+} (Figure 2.12; Howard and Talbot, 1992). Moreover, it is known that PGs increase intracellular concentration of Ca^{2+}, that results in myosin phosphorylation and contraction of muscle filaments (Suematsu et al., 1991). Thus, PGs and other neurohormones, like octopamine and 5-HT, may be involved in microtubule-based ovarian muscle contraction during ovulation in decapod crustaceans (Spaziani et al., 1993).

The potential involvement of PGE2 and PGF2α has been investigated *in vitro* during vitellogenesis using ovarian homogenates of the crayfish, *P. paeninsulans* (Spaziani et al., 1993; Spaziani et al., 1995). In one study, PGE_2 was observed to increase gradually from stage I (previtellogenic) to stage V (early vitellogenic) ovaries. Concentrations of $PGF_{2\alpha}$ did not increase until stage V when, along with PGE_2, concentrations started to increase dramatically (Spaziani et al., 1993). In another study, the *in vitro* incubation of ovarian tissue from *P. paeninsulans* in 100 µM $PGF_{2\alpha}$ for 20 min increased intracellular cAMP levels that was strongly correlated with expulsion of oocytes at ovulation by promoting smooth muscle contractions of the ovary (Figure 2.12; Spaziani et al., 1995). These findings suggest that $PGF_{2\alpha}$ could play a role in ovulation and egg release in decapod crustaceans especially in redclaw. However, factors that initiate ovulation *in vivo* in decapod crustaceans, have not been examined (Schroeder and Talbot, 1985). Therefore, further studies are required to determine the effects and neurohormonal mechanisms of $PGF_{2\alpha}$ on oocyte ovulation and spawning in decapods including redclaw.

2.6.2.2 Oocyte Extrusion by Electroejaculation.

In *H. americanus*, oocytes were extruded from females by electrical stimulation (Talbot and Goudeau, 1988) using an electroejaculation protocol developed for males (Kooda-Cisco and Talbot, 1983). Electrical stimulation was performed on mature females with fully developed tegumental glands on their pleopods since this is commonly associated with fully developed ovaries (Aiken and Waddy, 1982; Johnson and Talbot, 1987). Females were placed in dorsal recumbency, and a pair of electrical probes applied to the gonopores situated at the base of the third pair of walking legs. A variable autotransformer with alternating current was used to deliver a gradual increase in voltage stimulation to a maximum of 12 V. Electrical stimulation caused the gonopore flap membrane to open, resulting in oocyte extrusion in some females. These oocytes were already ovulated and free in the oviduct, suggesting that electrical stimulation can promote oocyte ovipositing but not necessarily ovulation (Kooda-Cisco and Talbot, 1983; Talbot and Goudeau, 1988). Similarly, the electrical stimulation protocol developed to collect spermatophore from male freshwater yabbies *C. destructor* (Jerry, 2001) and trialled successfully in male redclaw (Aquino, personal communication) could be adapted to

induce egg release in female crayfish. According to this protocol, animals are anaesthetised by immersing in iced water (10 °C) for 10 min before being secured in a restraining tube with their ventral side facing the operator. An AC variable transformer and a pair of electrodes are used to deliver 10 mA, 50 Hz electrical stimulation for 10 sec to the base of the sternal keel close to the coxa of the fourth pair of walking legs, resulting in spermatophore expulsion (Jerry, 2001). However, such an electroejaculation system has yet to be tested for oocyte extrusion from female redclaw crayfish.

2.6.2.3 Staging of Gonadal Maturation as a Means to Predict Egg Release.

Determining the stage of ovarian development is an important tool to identify mature females that are ready to spawn. In crayfish, the ovary can be divided into multiple stages of maturation based on morphology and colour (Kulkarni et al., 1991). In *P. clarkii*, the immature ovary has small oocytes, and at the onset of maturation, the ovary increases in size and changes from a lucid white to a pearl colour. Eventually, the mature ovary appears deep green to brown immediately before spawning (Kulkarni et al., 1991).

In *C. quadricarinatus*, stages of ovarian development have been described by Jones (1990). Ovarian growth was visualised dorsally through the translucent membrane between carapace and abdomen by holding a concentrated light source against the ventral wall of the abdomen. Using this technique validated by subsequent dissection of animals, the ovary was categorised into three stages: immature, where no ovary is visible; maturing, where the ovary becomes visible, but no solitary ova can be seen; and mature, where the ovary and solitary ova are visible.

In *P. monodon,* the method for determining different stages of ovarian maturation was established based on the relative size of the ovary observed externally from the dorsal exoskeleton (Tan-Fermin and Pudadera, 1989; Treerattrakool et al., 2014; Treerattrakool et al., 2011). Five stages of ovarian development have been determined: (i) undeveloped ovary (S0) that is invisible under light beam; (ii) previtellogenic ovary (S1) that appears as a thin line; (iii) developing or early vitellogenic ovary (S2) that is observed as a linear band through the exoskeleton; (iv) late vitellogenic ovary (S3) that is visualised as a dark band through the exoskeleton with larger growth at the posterior thorax; and (v) mature ovary (S4) that is viewed as a dark band through the exoskeleton with a diamond shape in the first abdominal segment (Treerattrakool et al., 2014; Treerattrakool et al., 2011).

2.6.2.4 Ovarian Biopsy to Predict Egg Release.

Ovarian biopsy was used in ridgeback prawn *Sicyonia ingentis* to estimate the timing of post-vitellogenic oocyte formation and spawning (Anderson et al., 1984). Ovarian biopsies were easier to perform when the ovaries had reached a mature stage of development, with detrimental

effects on shrimp reported from biopsies of undeveloped or partially spawned ovaries. By holding shrimp in a flexed position, biopsies were extracted from the ovary by puncturing the dorsal body wall of the carapace with a needle fixed to a syringe filled with approximately 0.5 mL of crustacean Ringers solution, to help prevent adhesion of oocytes and release of jelly from cortical specialisations (CS) within oocytes after collection (Anderson et al., 1984). Biopsies were stained with haematoxylin and eosin and observed under the microscope. In mature ovaries, oocyte cytoplasm was enlarged and contained yolk spheres enclosed by numerous vesicles of variable size that eventually formed CS. Prawns were regarded to be in CS phase (the fourth stage of oocyte maturation) if the vesicles that form CS had united at the oocyte cortex. Prawns were classified as reaching the GVBD phase if most oocytes had completed nuclear breakdown and were surrounded by follicular cells. Finally, ovulated oocytes could be recognised as lacking surrounding follicular cells. The average time from the initiation of the CS stage to spawning was 97 ± 53 h and 84 ± 22 h (mean ± SD) in two groups of prawns exposed to recurrent ovarian biopsies. It was proposed that the appearance of CS stage oocytes was a definitive marker for impending spawning activity (Anderson et al., 1984; Anderson et al., 1985). Although, the biopsy technique had no significant effect on survival, number of spawns, time of spawning or egg quality compared with controls, it still may be detrimental to females with underdeveloped ovaries, which could potentially compromise their future fertility.

2.7 Methods to Collect and Assess the Quality of Eggs and Embryos in Decapod Crustaceans

To evaluate egg and embryo quality, reliable harvesting, and handling of oocytes from the ovary, or eggs and embryos from the female's pleopods is essential. However, limited reports are available on effective collection and handling methods among commercially valuable decapods, and these appear to differ greatly between species and hatcheries (Bycroft, 1986; Choy, 1985; Gardner, 2007; Policar et al., 2011).

2.7.1 Egg and Embryo Collection and Handling Methods

A range of oocyte, egg and embryo collection methods, handling media and fixatives used among decapods are listed in Table 2.3. The most common method involves the collection of eggs and embryos by manual removal from female pleopods using tweezers/forceps (Table 2.3). In the ovigerous giant crab *Pseudocarcinus gigas*, pleopodal fertilised eggs are recovered by splitting the setae at the joint of the pleopods (Gardner, 2007). In freshwater crayfish *Austropotamobius pallipes*, fertilised eggs are collected from the pleopods by gently stripping with dissecting tweezers or clasps (Policar et al., 2011). While in redclaw *Cherax quadricarinatus*, fertilised eggs are collected using forceps from the pleopods (Yeh and Rouse, 1994).

Chemical treatment is occasionally used to facilitate the collection of pleopodal eggs (Bycroft, 1986; Choy, 1985). For example, in the shrimp *Crangon crangon*, lobsters *Galathea intermedia*, *Homarus vulgaris* and crabs *Carcinus maenas*, *Liocarcinus puber*, *Porcellana platycheles*, sodium hypochlorite rapidly releases all pleopodal eggs without rupture within 2 to 5 min (Choy, 1985). Pleopods are first removed from the female then immersed in 10 to 30% hypochlorite (0.4 to 1.2% available Cl_2) and shaken at 30 sec intervals until fertilised eggs are liberated. The hypochlorite is then drained and neutralised with sodium thiosulphate solution before washing the fertilised eggs with water (Choy, 1985). In female rock lobster *Jasus edwardsii*, 1 N KOH solution efficiently released all fertilised eggs from the pleopods after shaking at regular intervals for 12 h (Bycroft, 1986). Once detached from the setae, eggs were rinsed with seawater to remove debris. Although short-term chemical treatment facilitates egg collection, its impact on embryo hatching rates, survival and deformity in decapod crustaceans has not been determined. However, sodium hypochlorite treatment of finfish eggs causes concentration-dependent reduction in hatching rates and survival of fry (Vincent-akpu and Edafe, 2018). Furthermore, susceptibility to sodium hypochlorite toxicity may vary by strain and species (Peneyra et al., 2020), suggesting that chemical treatment could impede the viability of decapod embryos that warrants further study to determine the optimum concentration for each species of decapod.

Techniques to stimulate the spawning and collection of freshly oviposited unfertilised eggs are yet to be developed in decapod crustaceans. As such, collection of unfertilised oocytes directly from the ovary or oviduct by post-mortem dissection is another common approach. Animals are anaesthetised in an ice bath for 1 to 2 min or euthanised by overdose with ethyl 3-aminobenzoate, methanesulfonic acid (anaesthetic MS-222) before dissection (Hernández-Abad et al., 2018; Wang et al., 2013a). Given this lethal sampling technique causes loss of valuable broodstock, non-lethal methods need to be developed to induce oviposition of unfertilised eggs in the absence of males such that they can be more easily collected from female pleopods using tweezers/forceps.

After collection, oocytes, fertilised eggs, and embryos are placed either in appropriate holding medium or straight into fixatives. For freshwater decapods, NaCl solution, sodium hypochlorite (3% NaClO) solution or ethanol are used as handling media and Bouin’s solution and 10% formalin are used as fixative (Table 2.3). While for marine decapods, seawater is used as handling media, and 4 to 10% formalin, Bouin's solution, 70% ethanol and ethanol glycerine are used as fixatives (Table 2.3).

Table 2.3: Collection methods, handling media/fixatives and predictive markers used to assess egg and embryo quality in decapod crustaceans. NR means not reported. For other abbreviations see table footnote.

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
Freshwater crab								
Scylla serrata	Tweezers	Fertilised eggs	NR	TF (million eggs/batch)	No. of eggs in a known mass x total egg mass	4.0 ± 1.2 to 7.9 ± 1.8 and positively correlated with BW	0.03	Churchill, 2003
				RF (eggs/g female)	Total eggs/female wt.	Decreased with BW	0.004	
				ED (mm)	Microscopy	Had no relation with BW	≥0.05	
				HR (%)	Empty egg cases: un-hatched eggs on egg strands	84.0 ± 6.0% and weakly correlated with BW	≤0.02	
				Egg colour	Photographing egg batches	NS effect on egg quality	≥0.16	
				FA	Gas Chromatography	EPA and ARA increased during embryonic development while	≤0.05	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						DHA and DHA: EPA decreased		
Oziothelphusa senex senex	NR	Fertilised eggs and ovaries	Bouin's fixative	F (eggs/g BW)	No. of eggs in brood	Positively correlated with BW	<0.001	Girish, 2015
				OI (μm)	(OI)=$W_1/W_2 \times 100$	0.18 to 1.97 and affected by seasons	NR	
				OD (μm)	Histology	Range between 53.0 ± 9.2 to 198.0 ± 8.9 at different ovary stages	NR	
Freshwater prawn								
Macrobrachium rosenbergii	Tweezers	Fertilised eggs	NR	F (eggs/g female)	Total eggs/female wt.	Range between VW, VP, HP and CP sources: 1055 ± 260 to 1178 ± 364	NS	Nhan et al., 2009
				ECSI	Egg clutch wt./total wt. of crab	Range between VW, VP, HP and CP sources: 9.3 ± 2.0 to	NS	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						10.2 ± 3.0		
				GSI	Gonad wt./crab somatic wt.	Range between VW, VP, HP and CP sources: 6.9 ± 1.5 to 8.1 ± 2.6	NS	
				In vitro HR (%)	No. of live larvae and dead eggs 24 h after hatching	Range between VW, VP, HP and CP sources: 65 ± 19 to 72 ± 16	NS	
				In vivo HR (%)		Range between VW, VP, HP and CP sources: 49 ± 0 to 54 ± 0	NS	
				Survival to PL (%)		Range between VW, VP, HP and CP sources: 18 ± 8 to 66 ± 4	<0.05	
Macrobrachium	Tweezers	Fertilised eggs	10% formalin, at -25 °C	Egg colour	Photography	Orange to deep brown prior to hatch	NR	Habashy et al., 2012

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
rosenbergii				ED (µm)	Micrometre	Increased with embryogenesis	<0.05	
				PGC_S	Histology	Present at dors-medial region of D6 embryos	NR	
				Eyestalk	Histology	Formed in D22 embryos	NR	
				Protein and moisture (µg/egg)	Spectrophotometry	Increased with embryonic development	<0.05	
				Lipid and carbohydrate (µg/egg)	Spectrophotometry	Decreased with embryonic development	<0.05	
Macrobrachium rosenbergii	NR	Fertilised eggs	NR	F (eggs/spawn)	Microscopy	40,096 to 46,131 at 0, 2, 4, 6 and 8% calcium dose	>0.05	Khasani et al., 2012
				HR (%)	Hatched eggs/total eggs x 100	Increased from 26.5 ± 9.9 to 50.8 ± 10.3% with increase	<0.05	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						in calcium concentration from 0% to 8%		
Macrobrachium rosenbergii	Tweezers	Fertilised eggs	NR	GSI	Proportion of stage V ovary wt. to total BW	Higher GSI with ARA and LOA diet than ARA diet alone	<0.05	Kangpanich et al., 2016
				HSI	Proportion of HP wt. to BW	Higher HP with ARA and LOA diet than ARA diet alone	<0.05	
				F (g/female)	Total no. of eggs/BW of females	Higher F with ARA and LOA diet than ARA diet alone	<0.05	
				Egg clutch wt.	Difference between a gravid female's wt. before and after hatching	Higher with ARA and LOA diet than ARA diet alone	<0.05	
				HR	No. of larvae per total no. of brownish eggs	Higher with ARA and LOA diet than ARA diet alone	<0.05	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
Macrobrachium rosenbergii	Tweezers	Fertilised eggs	3% NaClO	5-HT-ir and DA-ir	HPLC and Bio-Rad Protein Assay, IHC	Higher 5-HT-ir and DA-ir at later embryo stages, 5-HT treated females had shorter embryo development than DA treated females	<0.05	Tinikul et al., 2016
Macrobrachium acanthurus	Post-mortem	Oocytes	NR	Oocyte no. (oocytes/female)	Microscopy and micrometer	Females fed diets with 20% lipid produced higher no. oocytes than at 12.5% and 10%	<0.05	Hernández-Abad et al., 2018
				Oocyte V(mm^3)	$V = \pi \times length \times width^2$	10, 12.5, 15, 17.5 and 20% lipid inclusions had no effect on oocyte V	NSD	
				Oocyte protein	Micro Lowry	10, 12.5, 15, 17.5 and 20% lipid inclusions had no effect on oocyte	NSD	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						protein		
				Oocyte lipid	Bligh and Dyer,1959	15, 17.5 and 20% lipid inclusions increased oocyte lipid content compared with control, 10 and 12.5% lipid inclusions	<0.05	
Neocaridina davidi	Tweezers	Fertilised eggs	NR	F	Egg no. per brood per female wt.	NS in F between COFs and TOFs animals	0.43	Baliña et al., 2018
				V (mm^3)	V= 4/3 x *r*1 x *r*2 x *r*2	NS in V between COFs and TOFs animals	0.11	
				Glycogen, lipid (µg/mg) and energy (J/mg)	Spectrophotometry	NS in glycogen, lipid and energy content between COFs and TOFs animals	0.25, 0.98 and 0.88	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
Freshwater crayfish								
Astacus leptodactylus	Tweezers	Fertilised eggs	NR	Egg no.	Manual counting	Greater F in crayfish fed with diets containing 80 mg/kg and 120 mg/kg of vitamin E than 20 mg/kg	<0.001	Harlioglu et al., 2002
Astacus leptodactylus	Post-mortem	Oocytes	NR	Oocyte no. and size	Manual counting and microscopy	Greater oocyte no. and size at a dietary inclusion of 150 mg/kg vitamin E than at 66 mg/Kg	<0.001	Barım öz, 2009
Astacus astacus	Tweezers	Fertilised eggs and juveniles	NR	C:P, N:P in eggs and juveniles	Spectrophotometry	Higher C:P in eggs than in juveniles	<0.0001	Færøvig and Hessen, 2003
Cherax quadricarinatus	Tweezers	Eggs	NR	F (eggs/g female)	Manual counting	NS difference at 22, 27, 32 and 37% crude protein	>0.05	Rodríguez-González et al.,

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
				EA (mm^2), V (mm^3), wt. (µg) and ED (mm)	NR	Significantly greater EA, V, wt., and ED at 32% crude protein	0.05	2006a
				Protein, lipid, carbohydrate and energy in eggs	Spectrophotometry	Unaffected by 22, 27, 32 and 37% dietary protein	>0.05	
Cherax quadricarinatus	Tweezers	Fertilised eggs	1.2% NaCl solution	F (eggs/g female)	Removing all eggs from female	9.7 to 11.6 and 4, 8 and 12% lipid levels had no effect on F	>0.05	Rodriguez-Gonzalez et al., 2009b
				EA (mm^2), V (mm^3), wt. (mg) and ED (mm)	NR	Greater EA, V, wt. and ED in response to addition of lipids in the diet	<0.001	
				Protein, lipid, carbohydrate (µg/g) and energy input (kcal/egg)	Barnes and Blackstock, 1973, Bradford, 1976, Van Handel, 1965 and applying conversion factors,	4, 8 and 12% dietary lipid levels had NS effect on egg protein, lipid, carbohydrate and energy contents	>0.05	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
					respectively			
Cherax quadricarinatus	Post-mortem	Ovarian tissues	In liquid N_2 at -80 °C	*Cdc2* mRNA	qRT-PCR and IHC	Higher *Cdc2* in ovaries than other tissues, stage I and II had highest levels while lowest in stage IV ovaries	<0.05	Wang et al., 2013a
Marine crab								
Scylla serrata	NR	Fertilised eggs and larvae	NR	F (eggs/g BW)	No. of eggs (zoea + unfertilised eggs)/BW of females	NS difference in F between crabs fed with NF, NF + AD or AD	>0.05	Millamena and Quinitio, 2000
				FR (%)	Total fertilised eggs/(fertilised + unfertilised eggs) x 100	NS difference in FR between crabs fed with NF, NF + AD or AD	>0.05	
				Larval stage index	A (absolute value x no. of eggs)/10	NS difference in stage index between crabs fed with NF,	>0.05	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						NF + AD or AD		
				Total no. zoea	Manual counting	Significantly more zoea from crabs fed with NF + AD	0.05	
Scylla serrata	NR	Freshly hatched zoea	NR	No. Zoea	NR	Higher no. zoea in crabs fed with NF + AD12 or NF alone	<0.05	Alava et al., 2007
				Zoeal lipid (mg/g DW)	Chromatography	Higher zoeal lipid in crabs fed with NF + AD12 than AD alone	<0.05	
Scylla serrata	Siphoning from tank	Larvae	NR	HR and SR (%)	Poor quality survived until early zoea and good quality metamorphosed to instar	Higher HR and SR in good quality larvae than poor quality larvae exposed to formalin	<0.05	Quinitio et al., 2018
Scylla paramamosain	NR	Fertilised eggs,	NR	F (million eggs/female)	No. of zoea and unfertilised eggs	NS difference in F between crabs fed fresh food or	NSD	Djunaidah et al., 2003

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
		larvae				formulated diets		
				FR (%)	Total fertilised eggs/(fertilised + unfertilised eggs) ×100	NS difference in FR between crabs fed fresh food or formulated diets	NSD	
				HR (%)	Counting	Crabs fed formulated diets had higher HR	<0.05	
Pseudocarcinus gigas	NR	Fertilised eggs	In seawater and at -60 °C	ED (µm)	Image analysis	Increased with development	<0.001	Gardner, 2001
				Egg dry mass (µg)	Counting, rinsing, drying and weighing	Decreased with development	<0.001	
				Egg moisture (%)	Drying, cooling and weighing	Increased with development	<0.001	
				Protein (µg/egg)	Lowry method	Decreased with development	<0.001	
				Lipid and carotenoid	Gravimetry and chromatography	NS change with development	>0.15	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
				(µg/egg)				
Uca rapax	Tweezers	Fertilised eggs	4% formaldehyde	Egg no.	Image-J software	With CW no. of Stage III eggs increased per ovigerous female	<0.01	Figueiredo and Narciso, 2008
				V (mm^3)	$V = (\pi D^3)/6$	Increased during embryonic development	0.00	
				Moisture content (%)	Drying, cooling and weighing	Increased during embryonic development	0.04	
				Lipid and FA content (g/g DW)	Chromatography	Decreased during embryonic development	0.00	
Uca annulipes	NR	Fertilised eggs	Seawater	F	No. of newly extruded stage I eggs	Significantly different F between two populations	<0.001	Penha-Lopes et al., 2009
				V (%)	$V= 4/3(\pi R^3)$	Range from 87.4 to 97.3 between three	NR	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						populations during wet seasons		
				FA	Chromatography	Higher SFA and MUFA in one population than other two during wet season	<0.05	
Callinectes sapidus	Tweezers	Fertilised eggs	NR	F	DW calculation	Increased with the increase of CW	0.00	Graham et al., 2012
				ED (µm)	Microscopy	Increased with CW	0.00	
Callinectes sapidus	Tweezers	Fertilised eggs, Zoea	NR	Energy content, lipid and FA	Calorimetry, Folch et al., 1957 and GLC	Egg energy, lipid and FA content had NS relationship with CW but with date of female collection	>0.86, >0.53, 0.17 and 0.004	Koopman and Siders, 2013
Marine shrimp								
Penaeus monodon	Collection from tank	Fertilised eggs	NR	FR (%)	Egg no. with symmetrical cleavage/total no.	Male size with which females mated had NS effect on F	NS	Hall et al., 2003

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
					of eggs × 100			
				HR (%)	NR	Male size with which females mated had NS effect on HR	NS	
Litopenaeus vannamei	Tweezers	Fertilised eggs	4% formalin, at -70 ˚C	F (eggs/spawn)	No. of eggs from each spawn per female	Range between 1st, 2nd, 3rd and 4th time spawners: 108.5 x 10^3 to 135.0 x 10^3	0.04	Arcos et al., 2004
				FR (%)	Observing normal cleavage and embryonic development	Range between 1st, 2nd, 3rd and 4th time spawners: 84.4 to 88.7	0.51	
				ED (µm)	Image analysis	Range between 1st, 2nd, 3rd and 4th time spawners: 248.4 to 275.0	0.00	
				Protein (mg/g)	Bradford, 1976	Range between 1st, 2nd, 3rd and 4th time spawners: 75.8 to	0.04	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						97.7		
				Lipids (mg/g)	Sulphophosphovanillin	Range between 1st, 2nd, 3rd and 4th time spawners: 41.7 to 47.4	0.72	
				Triacylglycerides (mg/g)	Colourimetry	Range between 1st, 2nd, 3rd and 4th time spawners: 9.3 to 12.0	0.53	
				Egg vitellin (mg/g)	ELISA	Range between 1st, 2nd, 3rd and 4th time spawners: 3.9 to 6.9	0.03	
Neomysis japonica	Post-mortem	Ovary and hepatopancreas	Bouin's fluid	ERα antibody	IHC	Localisation of ERα in nuclei of hepatopancreas, oocytes and follicles	NR	Yang et al., 2012
Marine lobster								
Jasus edwardsii	NR	Larvae (phylloso	Buffered formalin,	Phyllosoma BL	Microscopy	Warm incubated phyllosoma was 4.4% and 3.9%	<0.05	Smith et al., 2002

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
		ma)	liquid N_2			smaller than ambient and cold incubated phyllo soma, respectively		
				Phyllosoma CW and CL	Microscopy	Warm incubated phyllosoma was 2.7% and 3.7% smaller in CW and CL, respectively than ambient incubated phyllosoma	<0.05	
				Hatching time (D)	Times from capture to hatch	Significantly different between warm, ambient and cold treated phyllosoma	<0.05	
				Ascorbic acid (µg/g DW) and lipid classes (%)	HPLC and TLC	Significantly different between warm, ambient and cold treated	<0.05	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						phyllosoma		
				FA (%)	Gas chromatography	Significantly different MUFA and PUFA between warm, ambient and cold treated phyllosoma	<0.05	
Sagmariasus verreauxi	NR	Fertilised eggs	NR	Appearance of median eye	Microscopy	Affected by temperature	<0.001	Moss et al., 2004
				EI (µm)	Length and width for each eye	Prior to hatching EI ranged 150 to 165	NR	
				Hatching time (D)	Days to hatch	Affected by temperature	NR	
Homarus americanus	NR	Fertilised eggs and larvae	at -80 ˚C	ED (mm)	Digitalised imaging	Different between female sizes and years	<0.008	Ouellet and Plante, 2004
				DW (mg)	Electro-microbalance	Lower egg DW for both small and large females in one year	<0.008	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
						relative to other years		
				Stage I CL at hatching (mm)	Digitalised imaging	Lower in small females than in large females for different years	<0.001	
Homarus americanus	Tweezers	Fertilised eggs	at -80 ˚C, ethanol-glycerine	ED (mm)	Digitalised imaging	Increased during embryo development (1.7 to 1.8)	NS	Sibert et al., 2004
				Egg water (%)	NR	Increased during embryo development (15 to 86)	NR	
				Egg DW (µg)	Electro-microbalance	Higher for largest females than smallest	NR	
				PI_e (mm)	Length and width of embryo's lateral eyes pigment spot	Increased during embryo development (0.06 to 0.13)	NR	
				Yolk protein (µg/index)	Colorimetry	Decreased during embryo development	NR	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
				Lipid (µg/ind)	TLC	Decreased during embryo development	NR	
Homarus americanus	Tweezers	Fertilised eggs	10% formalin to 70% EtOH	Egg size	Microscopy	Diseased lobsters' eggs are bigger than non-diseased eggs	0.002	Miller et al., 2013
				DW	Micro balance	DW varied between diseased and non-diseased eggs	0.004	
				Total C	Gas Chromatography	Total C varied between diseased and non-diseased eggs	0.001	
Homarus americanus	Tweezers	Fertilised eggs	Ethanol-glycerine, at -20 °C	F (no. of embryos)	Currie, Schneider and Wilke, 2010	Positively correlated with female size	<0.001	Koopman et al., 2014
				ED (mm)	Microscopy	Significantly different between seasons	<0.001	
				Energy density (kJ/g wet mass)	Calorimetry	Significantly different between seasons	<0.001	

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
				Lipid (%)	Folch et al. 1957, TLC	Significantly different between seasons	<0.01	
				Fatty acid profile (% wet mass)	Gas chromatography	Significantly different between years and lobster size	0.01, <0.001	
Homarus americanus	Tweezers	Fertilised eggs	4% formalin solution	Clutch quality	% of eggs covering abdomen	Smaller females were more likely to carry abnormal clutches	NR	Tang et al., 2018
Homarus americanus	Tweezers	Fertilised eggs	NR	F (eggs/lobster)	Gravimetric dry wt. and depth gauge	NS between the methods	NS	Coleman et al., 2019
				ED (mm)	Microscopy	Increased with embryo development	<0.001	
				ESI	Long and short axes of embryo's eye pigment crescent	Increased with both egg stage and egg size	NR	
Panulirus argus	Tweezers	Fertilised	NR	F	Microscopy	Female size and spermatophore size	NS	Butler et

Species	Collection method	Samples	Handling media/fixative	Predictive biomarker	Assessment methods	Results	*P* value	Reference
		eggs				had NS effect on F		al., 2015
				EA (mm^2)	Microscopy	Female size had no influence on egg area	0.27	
				C:N content	Elemental analyser	Female size did not influence C:N	0.22	
				Fertilisation success	No. of fertilised eggs/total eggs extruded	Female size and spermatophore size influenced fertilisation success	0.001	
Pleuroncodes monodon	Tweezers	Embryos	Dried in lyophiliser	Lipids (mg)	Gravimetrically	Winter embryos had higher lipids than summer embryos	<0.001	Bascur et al., 2018
				FA (mg/g DW)	Gas chromatography	Winter embryos had higher SFA, MUFA and PUFA than summer embryos	<0.001	

Values are mean ± standard deviation unless stated otherwise.

Abbreviations:

AD, artificial diet; ARA, arachidonic acid; BW, body weight; CL, carapace length; C:N, carbon: nitrogen; COFs, control ovigerous female at 28 ± 1 °C; CP, China pond-reared breeders; CW, carapace width; D, diameter; DA, dopamine; DHA, docosahexaenoic acid; DW, dry weight; DW calculation, the ratio of the dry weight of the entire egg mass including the dry weight of the subsamples to the estimated dry weight of one egg; EA, egg area; ECSI, Egg clutch somatic index; ED, egg diameter; ELISA, enzyme linked immunosorbent assay; EPA, eicosapentaenoic acid; ESI, eyespot index; FA, fatty acid; F, fecundity; FR, fertilisation rate; GLC, gas liquid chromatography; GSI, gonadosomatic index; HR, hatching rate; HP, Hawaii pond-reared breeders; HP, hepatopancreas; HIS, hepatosomatic index; HPLC, high performance liquid chromatography; IHC, immunohistochemistry; LOA, linoleic acid; MUFA, monounsaturated fatty acids; NR, not reported; NS, no significant difference; NF, natural food, NF + AD12, natural food + artificial diets containing 12% lipid levels; N:P, nitrogen: phosphorus; OD, oocyte diameter; OI, ovarian index; PGCs, Primordial germ cells; PI_e, Parkinson eye index; PUFA, ploy unsaturated fatty acids; PL, post larva; RF, relative fecundity; SFA, saturated fatty acids; SR, survival rate; TF, total fecundity; TLC, thin layer chromatography; TOFs, transferred ovigerous females from 33 ± 1 °C to 28 ± 1 °C; Tweezers: manual removal of fertilised eggs from pleopods or abdomen using tweezers, spatula or scalpel; V, volume; *r*1 and *r*2, radii of major and minor axes of fertilised eggs, respectively; VP, Vietnam pond-reared breeders; VW, Vietnam wild breeders; W_1, wet weight of the ovary and W_2, total wet weight of the crab

2.7.2 Markers to Assess Egg and Embryo Quality

Common methods to determine the reproductive performance of female decapods include estimating fecundity through the measurement of fertilisation rates, hatching success, and juvenile survival (Arcos et al., 2003; Arcos et al., 2004; Castro-Longoria, 2003; Pinchuk and Hopcroft, 2006). However, these predictive criteria are also impacted by the fertility of males and thus are not the sole variables in determining female fertility independently. Consequently, evaluation of unfertilised oocyte quality is the only direct measure of female fertility free of male factors. Given such gametes currently require post-mortem methods of collection, indirect measures of fertility between different females can be derived by evaluating pleopodal eggs and embryos fertilised by the same male. In such a scenario, embryos with reduced viability might arise, for example, due to differences in female genotype, body condition, husbandry practices, diet, or other biological or environmental factors. Several advanced fertility tools developed in mammals could be optimised to enable egg and embryo quality to be evaluated in decapod crustaceans explicitly in redclaw. Typically, markers for egg and embryo quality can be divided into three categories: i) morphological markers, ii) biochemical markers, and iii) advanced functional markers. A summary of these markers and their method of assessment in decapod crustaceans is shown in Table 2.3.

2.7.2.1 Morphological Markers.

Evaluation of normal egg and embryo morphology is the most widely used method to assess quality, which is non-invasive and requires only a few pieces of equipment (Vanderwall, 1996). Some commonly used morphological parameters are outlined below.

2.7.2.1.1 Size, Volume, and Weight.

Size, volume, and weight are useful traits to assess the quality of fertilised eggs (Fischer et al., 2009; George, 1996; Herring, 1974). The size of fertilised eggs substantially varies across species and affects the progression of embryo development (Herring, 1974; Levitan, 2006; Rosa et al., 2007). Generally, larger embryo takes longer to develop. For example, lobsters produce larger embryos with fewer larval stages (3-4) that take 6 to 11 months to develop, compared to crab embryos which are small and have 5 larval stages but take 4 months to develop (Rosa et al., 2007). However, exceptions exist in caridean shrimp species that produces medium sized embryos with up to 11 stages that grow rapidly (2 to 4 weeks) from spawning to hatching (Rosa et al., 2007). In addition, within each species of decapod, embryo size is an indicator of the amount of energy reserves within the embryo (Herring, 1974). Larger embryos produced by the western population of *Plesionika martia martia* shrimp exhibited a higher fatty acid content during hatching, which could reduce the embryo's dependence on the external environment for nourishment, increasing the chance of larval survival (Rosa et al., 2007).

In decapod crustaceans, there is a 50 to 159% increase in embryo volume during development (Figueiredo and Narciso, 2008; Oh and Hartnoll, 2004). However, embryo volume decreases under adverse rearing conditions. For example, in *Macrobrachium acanthurus,* embryo volume decreased significantly from 0.11 ± 0.02 to 0.09 ± 0.01 mm^3 (mean ± SD here and throughout the manuscript unless otherwise stated) with increasing salinity from 0% to 1.7%, respectively ; $P < 0.05$ (Fukuda et al., 2017).

Dry weight of fertilised eggs is another predictor of embryo quality. In lobster *H. americanus*, initial mean dry weight of fertilised eggs is positively correlated with embryo growth efficiency index. This implied that under identical experimental conditions (time and temperature), larger embryos in terms of dry weight had a higher growth rate and used their yolk triacylglycerol reserves more efficiently than smaller embryos (Sibert et al., 2004).

In redclaw crayfish *C. quadricarinatus*, the size, volume, and weight of freshly released fertilised eggs were used as indicators of egg quality in response to dietary treatment of broodstock. Redclaw fed 32% crude protein demonstrated significantly greater egg area (mm^2), diameter (mm), volume (mm^3) and weight (μg) than those fed 22%, 27% or 37% crude protein (Rodríguez-González et al., 2006a). However, it is yet to be reported in decapods whether the size, weight and volume of fertilised eggs is directly associated with hatching success of embryos and late-stage survival of juveniles.

2.7.2.1.2 Fertilisation Rate.

The fertilisation rate of eggs influences the number of viable embryo that develop and hatch (Bardon-Albaret and Saillant, 2017). Traditionally, fertilisation rate is determined by estimating the proportion of eggs that develop after spawning under a compound microscope (Zhang and Lin, 2004). For example, in freshwater prawn *Macrobrachium rosenbergii*, fertilised eggs are confirmed by the appearance of the black eye spot visible under light microscopy (Ngernsoungnern et al., 2009). Broodstock derived from wild-caught female marine shrimp *Penaeus vannamei* had higher fertilisation and nauplii production rates (72.9 ± 5.1% and 73.4 ± 3.9%, respectively) than pond-reared females (68.0 ± 3.3% and 51.8 ± 4.9%, respectively) (Palacios et al., 2000). However, the problem with traditional methods that focus on cell cleavage within embryos as a measure of fertilisation rate, is that they are unable to distinguish normal cleavage development from parthenogenic activation (oocyte cell division without fertilisation by sperm) (Kuris, 2020; Pillai and Clark Jr., 1987). Alternative methods exist that rely on detecting 2nd polar body (PB) extrusion from eggs; a process uniquely triggered by fertilisation with spermatozoa (Garnica-Rivera et al., 2004; Liu et al., 1999; Ye et al., 2009).

2.7.2.1.3 Hatching Rate.

Hatching rate is a frequently used predictive biomarker for estimating embryo quality in decapod crustaceans (Arcos et al., 2003; Arcos et al., 2004). Usually, at hatching, the decapod embryo develops all thoracic appendages, which occupy 3/4 of their whole ventral surface. As the embryo hatches the chorion is expelled and pereopods extend (García-Guerrero et al., 2003b). Hatching rate is typically calculated using the following formula:

$$\text{Hatching rate (\%)} = \frac{\text{total number of hatchlings (juveniles)}}{\text{total number of fertilised eggs}} \text{ x } 100 \text{ (Khasani et al., 2012)}$$

Hatching rate in decapods is strongly influenced by the quality of fertilised eggs. For example, in *Penaeus monodon*, good quality fertilised eggs had greater than 50% hatching rate compared to between 1 and 10% among poor quality eggs (Hall et al., 2003). However, optimum hatching rate in decapod crustaceans varies by species and is known to be impacted by changes in culture conditions such as temperature, salinity, nutrition, and exposure to chemicals and hormones (Lee et al., 2000; Moss et al., 2004; Vargas-Ceballos et al., 2019; Vogt, 2007). For example, spiny lobster *Sagmariasus verreauxi* raised at 20, 17 and 13 °C had a declining hatching rate of 98%, 81% and 38%, respectively (Moss et al., 2004). Embryos of *M. tenellum* treated with 0, 10 and 30 practical salinity units had significantly different hatching rates of 60%, 82% and 0% respectively (Vargas-Ceballos et al., 2019). Grass shrimp *Palaemonetes pugio* embryos showed different hatching rates when exposed to 172 µg/L 2-methyl-1,2-naphthoquinone genotoxicant at different stages of embryo development (Lee et al., 2000). Development of stage 4 embryos was more affected by methyl-1,2-naphthoquinone exposure than stage 7 embryos with the hatching rates of 0 and 90%, respectively (Lee et al., 2000). Exposure of marbled crayfish *Procambarus alleni* embryos to 0, 1, 10 and 100 µg/L 17 α-methyl testosterone was negatively associated with hatching rates of 100%, 100%, 87% and 67%, respectively (Vogt, 2007).

2.7.2.1.4 Embryo Heartbeat.

Regulation of cardiorespiratory function is essential to an organism's survival and ultimately involves many homeostatic processes (Reiber, 1997). Moreover, fluctuations in cardiac function can be a sign of abnormal development or detrimental physiological or external environments (Reiber, 1997). During embryonic development, the cardiovascular system facilitates oxygen supply to ensure the viability of developing tissues (Harper and Reiber, 2004; Reiber, 1997). In zebrafish *Danio rerio*, heart rate is a useful marker of advanced stages of embryo development (Kimmel et al., 1995; Ugwuagbo et al., 2019). Zebrafish embryos microinjected with PGE_2 demonstrated substantially higher average heart rate and hatching rate (120 bpm and 33%, respectively) compared to controls (90 bpm and 5%, respectively; Ugwuagbo et al., 2019). Videos for embryonic heartbeat can easily be recorded using a stereo microscope mounted with a digital camera and analysed using DanioScope™ software (Noldus Information Technology

bv, Wageningen, Netherlands). This software has been extensively used to measure heartbeat in zebrafish embryos (Fuad et al., 2018; Grone et al., 2016). Atrial or ventricle beat frequencies can be examined by using the software to select a tracking zone over the atrium or ventricle respectively (Fuad et al., 2018).

In decapod embryos, sub-optimal culture conditions can significantly affect heartbeat and development (Ceballos-Osuna et al., 2013). Porcelain crab *Petrolisthes cinctipes* embryos had 37% slower heart rate and 15% less growth when cultured in seawater at lower pH (7.6) compared to higher pH (7.9) (Ceballos-Osuna et al., 2013). The heartbeat in crayfish begins mid-way through embryonic development (E-Stage XIII) and increases in frequency during development (Harper and Reiber, 2004). A decline in cardiac activity before hatching (E-Stage XVI) indicates oxygen depletion in the developing embryo (Reiber, 1997). The heart rate of *Procambarus clarkii* embryos incubated at 25 °C were observed by video microscopy across four embryonic and three larval stages: E-Stage XIII (12-13 days), E-Stage XIV (14-17 days), E-Stage XV (17-19 days), E-Stage XVI (19-21 days), and larval stages L-Stage I (21-23 days), L-Stage II (23-25 days), and L-Stage III (25-27 days) (Harper and Reiber, 2004). Mean heart rate remained unchanged at 160 ± 3 bpm at E-Stage XIII and 163 ± 2 bpm at E-Stage XIV. Heart rate increased significantly to 192 ± 6 bpm at E-Stage XV, then decreased significantly to 149 ± 3 bpm throughout E-Stage XVI and remained unchanged until hatching. At hatching, the heart rate increased dramatically to 255 ± 9 bpm at L-Stage I and remained at this rate throughout the next two larval stages (Harper and Reiber, 2004). Due to the translucent nature of decapod crustacean embryos such as redclaw, *C. quadricarinatus* from Stage 5 (13 to 15 days) onwards (García-Guerrero et al., 2003b), heart rate can also be measured using DanioScope™ software as an indicator of embryo health. Further research, however, is needed to validate this software for use in decapods.

2.7.2.1.5 Late-Stage Larval Survival.

Survival to later stages of larval development is an important marker and ultimate goal for hatchery operators. Pre incubation of estuarine crab *Chasmagnathus granulata* embryos at 1.5, 2.0 and 3.2% salinity until hatching followed by 1.0% saline thereafter, resulted in 85, 90 and 30% larval survival, respectively (Luis and Klaus, 2003). Moreover, zoea took longer (10 days) to develop from stage I to II in 3.2 than 1.5 or 2.0% saline (~7 days each) (Luis and Klaus, 2003). Unfortunately, baseline rates of late-stage larval survival are unknown in commercially important decapods such as prawns, lobster, crayfish, and crab. Post larva of *M. rosenbergii* showed highest survival (93% *vs.* 88%) when stocked at larger sizes (0.52 g *vs.* 0.27 g) in commercial nurseries (Hulata et al., 1990). Redclaw *C. quadricarinatus* juveniles <0.5 g tend to be delicate and difficult to handle without causing mortality (Medley, 1994). Collectively, these

data suggest that the proportion of hatched embryos that develop into 0.5 g juveniles could be a valuable threshold for assessing the quality of late-stage decapod juveniles.

Morphological assessment of eggs and embryos involves skilled personnel, but techniques usually rely on subjective visual appraisal. This can yield variable results between different laboratories and even among researchers in the same laboratory (Vanderwall, 1996). Alternative methods outlined below may be more definitive.

2.7.2.2 Biochemical Markers.

In crustaceans, egg yolk, is the major source of nutrients for the developing embryo and consists of proteins (primarily lipoprotein; lipovitellin), lipids, carbohydrates and carotenoids (Vinagre et al., 2007). These components in fertilised eggs are considered useful biochemical indicators for decapod embryo quality (Arcos et al., 2003; Arcos et al., 2004; García-Guerrero et al., 2003a; Palacios et al., 2002). Protein plays an important role in maturing gonads as well as egg and embryo quality (García-Guerrero et al., 2003a; Wouters et al., 2001). Lipids and their constituent fatty acids are considered fundamental dietary elements for the successful reproduction and survival of progeny in crustaceans, since they are a major energy source for gonadal development and are indispensable components of cellular structures in the embryo (Hernández-Abad et al., 2018). Triglycerides are primarily a storage lipid for energy reserves, with females having a different triglyceride composition than males that is associated with differences in reproductive function (Cuzin-Roudy et al., 1999). Carotenoids are transported from the hepatopancreas to the ovaries during secondary vitellogenesis via the haemolymph (Vincent et al., 1988). Carotenoids, mainly astaxanthin, are powerful foragers of free radicals and protect eggs from oxidative degradation (Wouters et al., 2001). Carotenoids have a direct impact on reproductive metrics such as egg quantity, hatching rate, and total nauplii production (Wouters et al., 2001).

Content of protein, lipids and triacylglycerides in fertilised eggs of *Litopenaeus vannamei*, have been shown to vary across spawning cycle. For example, the fertilised eggs produced during the 4th round of spawning had significantly higher amounts of protein (125 ± 9.6 *vs.* 100 ± 5.0 mg/g), lipid (49.0 ± 2.7 *vs.* 41.4.0 ± 1.7 mg/g) and triacylglycerides (22.8 ± 1.0 *vs.* 18.0 ± 0.6 mg/g) than fertilised eggs produced during the 3rd round of spawning; P<0.05 (Arcos et al., 2003). Fertilised eggs of *P. vannamei* that yielded >80% survival at zoea III stage had significantly higher (mean ± SD) triglycerides (30 ± 3 *vs.* 23 ± 4.0 mg/g) and carotenoids (153 ± 38 *vs.* 137 ± 16 µg/g) than fertilised eggs that yielded <60% survival (Palacios et al., 2002). Fertilised eggs of wild-caught white shrimp *Fenneropenaeus indicus* had higher triacylglycerol (21.3 ± 1.1 *vs.* 18.7 ± 1.7 mg/g) and carotenoids (6.9 ± 1.0 *vs.* 5.1 ± 0.6 g/g) and produced more viable nauplii (61.4 ± 12.4 *vs.* 53.6 ± 12.8%) than eggs of pond-reared shrimp; P<0.001

(Regunathan, 2008). Yolk protein in the form of vitellin (Vn) is an important source of nutrition for crustaceans throughout embryonic development (Ghekiere et al., 2005; Subramoniam, 2017b). The concentration of Vn in oocyte can influence fertilisation rate and is vital for the production of healthy juveniles (Arcos et al., 2003). In fertilised eggs of mysid shrimp *Neomysis integer*, Vn concentration decreased as embryos developed from stage I (104.6 ± 41.0 g/mL), II (40.2 ± 23.6 g/mL) and III (11 ± 8.6 g/mL) (Ghekiere et al., 2005).

During embryonic development of *C. quadricarinatus*, variation in the biochemical composition of fertilised eggs influence the quality of the spawn (García-Guerrero et al., 2003a). The most abundant constituent is protein 63.2%, followed by lipids 32.3% and carbohydrates 4.4%. A gradual decrease in the protein and lipid content of fertilised eggs occurs during development, indicating use of protein as a structural component of cells and increased lipid utilisation as a major energy source (García-Guerrero et al., 2003a). Although lipids are not the most abundant component, they contribute to over 50% of the total energy content of freshly spawned fertilised eggs (García-Guerrero et al., 2003a). Lipids and proteins account for on average 4 and 2 calories/day respectively to the energy consumed by developing embryos. The limited starting amount of carbohydrates present in fertilised eggs indicates that they are not predominantly used for energy generation during embryo development. Lastly, water uptake increases during embryonic development associated with the formation of new cells and constitutes 52 and 85% of total embryo and juvenile weight respectively (García-Guerrero et al., 2003a).

While assessment of the biochemical composition of embryos may provide some measure of embryo quality the main disadvantage of using biochemical markers is that analysis of chemical composition requires multistep sample preparation that risks losing parts of the sample during conventional processing. Moreover, more elaborate assays can be time-consuming, complex, and costly, requiring specialised expensive equipment (Byliński et al., 2017; Moran and McAlister, 2009). Validation of the measurements and guides to optimum concentrations may, however, still be lacking which require further research.

2.7.2.3 Advanced Markers.

Recently, methods that assess cellular organelle integrity and function have been applied more frequently in decapods to evaluate egg and embryo quality more objectively. These include assays that assess cell viability, mitochondrial function, nuclear maturation, DNA damage and the measurement of reactive oxygen species.

2.7.2.3.1 Nucleic Acid Markers to Determine Cell Number, Oocyte Nuclear Maturation and Egg Fertilisation.

DNA-specific fluorescent stains are generally used to investigate nuclear maturation and chromosome migration during cell cycle progression (Klonisch et al., 2010) and as a stain to

assess cell number to evaluate egg and embryo quality. Hoechst (33258 and 33342) and DAPI (4', 6-diamidino-2-phenylindole) are the most commonly used nucleic acid stains (Buttino et al., 2003; Kalinowski et al., 2004; Khosravi-Farsani et al., 2010; Masci and Monteiro, 2005; Zirbel et al., 2007). Multiple nuclei in actively dividing cells can be easily visualised due to the strong affinity of these stains for DNA-binding proteins and their specificity to the main groove of DNA and its A-T-rich sequence (Dervan, 1986).

Hoechst and DAPI can bind to the DNA of live or dead cells, causing the nucleus to fluoresce blue at 461 nm under the fluorescent microscope after excitation at 350 nm by UV light (Crowley et al., 2016a). Fertilised eggs of rotifer *Brachionus plicatilis* had bright blue nuclei when stained with Hoechst in PBS for 30 min at room temperature (Gotesman, 2016). In marbled crayfish, freshwater shrimp *Caridina multidentata*, and peppermint shrimp *Lysmata boggessi*, nuclear labelling with Hoechst and DAPI was used to characterise chromosomal segregation, blastomere formation, gastrulation, and germ disc formation during early embryonic development (Alwes and Scholtz, 2006; Klann and Scholtz, 2014; Romero-Carvajal et al., 2018). In *Macrobrachium olfersi*, the mitotic index (the ratio between the number of cells in mitosis and the total number of cells) was investigated by staining UV-B irradiated 6 day-old embryos with DAPI (Nazari et al., 2010). DAPI was also used to visualise metaphase (symmetrical alignment of chromosomes prior to anaphase) in freshwater prawn and mangrove crab embryos undergoing cell division (Sobieh and Darwish, 2020; Phimphan et al., 2019). Completion of nuclear maturation by the process of meiosis is critical for the final maturation of haploid oocytes to facilitate fertilisation and normal development of diploid embryos. Oocyte nuclear maturation involves the resumption of prophase I of meiosis I (also known as GVBD), extrusion of the first PB, and progression of the cell cycle to metaphase II of meiosis II where it arrests, until fertilisation by spermatozoa causes extrusion of the second PB (Liu et al., 1999; Ye et al., 2009). Extrusion of the first PB is a crucial marker of nuclear maturation which can be used to identify oocyte competence in sub-fertile female decapods or females subjected to artificial methods to accelerate gonadal maturation and induce egg release (discussed later). In mice, the use of Hoechst 33342 allows the rapid identification of meiotically mature oocytes by the extrusion of first PB for downstream use in IVF (Cavalera et al., 2019). Hoechst 33258 and/or histone H1 immunoassay together with fluorescent confocal microscopy were used to identify GVBD in human oocytes during *in vitro* maturation prior to assisted reproduction (Combelles et al., 2002). Maturation status of more than 200 bovine oocytes per batch could rapidly be determined using DAPI (as an anti-lamin A/C-DAPI conjugate), and identified GVBD and metaphase I stage oocytes with a reduced chance of classification error during observation (Prentice-Biensch et al., 2012). Unfertilised *P. monodon* eggs spawned into artificial seawater (20% Mg^{2+}) and stained with Hoechst 33258 showed that their pronuclei

arrest at prophase I prior to fertilisation (Pongtippatee et al., 2010). Moreover, freshly spawned fertilised eggs of Pacific white shrimp *L. vannamei*, were collected every 2 min for 1 h and stained with 50 ng/mL DAPI. The highest number of eggs with second PB extrusion (a marker of fertilisation) occurred 18 min after fertilisation (Garnica-Rivera et al., 2004). Despite these limited studies in shrimp, the use of fluorescent nucleic acid markers to identify nuclear maturation of oocytes and subsequent fertilisation is yet to be applied to commercially important decapods such as crabs, lobsters, and crayfish.

There are several other nucleic acid stains that can be used to evaluate nuclear maturation, ploidy, and embryo development in decapods. Propidium iodide (PI; excitation: 493 nm and emission: 536 nm; red) is a nucleic acid stain widely used in diverse cell types which can be evaluated by different types of fluorescent microscopy and flow cytometry (Rosenberg et al., 2019). Green tiger shrimp *P. semisulcatus* embryos incubated in PI (1 mg/mL) at room temperature for 15 min in the dark were assessed for cellular ploidy by flow cytometry (Kır et al., 2015). Sytox green (excitation: 488 nm and emission: 523 nm; green), is another fluorescent nucleic acid dye to stain embryos in both vertebrates (Bedzhov and Zernicka-Goetz, 2014) and invertebrates (Hajihassani and Dandurand, 2018; Jeffery, 2002; Klann and Scholtz, 2014). Incubation of freshwater shrimp embryos *Caridina multidentata*, in Sytox green for 3 h stained embryonic nuclei to identify the process of gastrulation and development from the egg to Nauplius stage by confocal laser scanning microscopy (CLSM) (Klann and Scholtz, 2014). A combination of Hoechst (0.9 mg/mL) and Sytox green (5 mM), was used to study early cleavage of *P. monodon* embryos by laser scanning microscopy and 3D image software (Biffis et al., 2009). Apart from their above applications, these nucleic acid stains can also be used as nuclear counterstains to evaluate cell viability, mitochondrial function, DNA damage, embryonic stress, and the concentration of reactive oxygen species in eggs and embryos.

2.7.2.3.2 Viability (Membrane Integrity) Assays.

Viability staining differentiates live cells from dead cells by analysing cell membrane integrity (Sousa et al., 2014). Viability assays have been used to evaluate female gametes in both vertebrates (Sousa et al., 2014; Yazdanpanah et al., 2013) and invertebrates (Gorokhova, 2010; Le Goïc et al., 2014). Viability is typically assessed using fluorescent microscopy following staining with combination of Hoechst 33342/PI (Khosravi-Farsani et al., 2010), SYBR-Green I/PI (Le Goïc et al., 2014), or using single staining protocols with fluorescein diacetate, 7-aminoactinomycin D (7-AAD) or Sytox green (Buttino et al., 2003). PI and 7-AAD are red fluorescent double stranded DNA stains and Sytox green is a green, fluorescent nuclear stain which do not penetrate intact membranes of living cells, but only damaged membranes associated with dead cells (Buttino et al., 2003; Crowley et al., 2016b; Le Goïc et al., 2014). By contrast, Hoechst 33342 (blue) and SYBR-Green I (green) penetrate and stain the nucleus of

living and dead oocytes and, as such, are used as fluorescent counter stains. Mouse oocyte viability was assessed based on plasma membrane integrity by staining oocytes with Hoechst 33342 and PI (each 10 μg/mL) for 10 min before examination under a fluorescent microscope (Khosravi-Farsani et al., 2010). The nucleus of PI-positive dead cells fluoresced red due to ruptured membranes while the nucleus of membrane-intact PI-negative viable cells fluoresced blue (Khosravi-Farsani et al., 2010).

Hydrolysis of fluorescein diacetate (6 μM) by living cells was used as a viability indicator to assess the cytotoxicity of cryoprotectants during cryopreservation of Pacific oyster *Crassostrea virginica* eggs (Paniagua-Chávez et al., 2006). Samples were excited by 450 to 490 nm light and the number of viable (green) and dead (unstained) eggs observed by fluorescent microscopy (Paniagua-Chávez et al., 2006). The viability of copepod *Calanus helgolandicus* embryos was compared using fluorescein diacetate, 7-AAD and Sytox green stains (Buttino et al., 2003). Sytox green was more effective than the other two stains because it did not show auto-fluorescence, was less toxic to embryos, longer lasting and resulted in accurate estimation with homogenous staining of 76 ± 31% (mean ± SD) dead embryos (Buttino et al., 2003).

Oocyte viability was evaluated in oyster *C. gigas* using SYBR-Green I and PI double staining. Live oocytes fluoresced bright green, while dead oocytes acquired both green and red fluorescence (Le Goïc et al., 2014). PI was also used to stain the isolated nuclei from fertilised tiger prawn *P. monodon* eggs (Hall et al., 2003). This is because staining of whole intact *P. monodon* eggs was difficult because of their large size and impermeable envelope that surrounds the egg after fertilisation (Hall et al., 2003). Therefore, removal of the outer egg envelope by enzymatic digestion might be necessary to adapt this protocol to assess viability in eggs of some other decapod species. However, egg viability of several crustacean species *Brachionus plicatilis*, *Daphnia magna*, *Nitocra spinipes, Acartia tonsa*, could be analysed without chitinase treatment to lyse the eggshell, by staining eggs with the fluorescent nucleic acid stain TO-PRO-1 iodide (Gorokhova, 2010). To date, protocols to detect viability of eggs and embryos have not been developed for decapods. Therefore, validation and optimisation of these existing methods to redclaw could provide valuable tools to assess egg and embryo quality in these species.

2.7.2.3.3 Mitochondrial Function Assays.

Mitochondria in eukaryotic organisms are extremely dynamic, varying in quantity, size and function depending on a cell's need for energy production (Agnello et al., 2017). Mitochondria, as the cell's powerhouse, directly participate in several cellular functions providing energy (adenosine triphosphate; ATP) for oocyte and embryonic development (Agnello et al., 2017; Suzuki et al., 2005). The segregation of mitochondria within different blastomeres in the

cleaving embryo during development is rigorously maintained. Mitochondrial number is thought to play a role in determining a blastomere's long-term survival. Mitochondrial malfunction may not only impair developmental processes but also cause embryonic apoptosis. Mitochondrial function is thus critical for proper embryonic development (Dumollard et al., 2007). During oocyte maturation, mitochondrial membrane potential (Δφm) increases significantly (Van Blerkom and Davis, 2007), which is associated with increased metabolism (Motta et al., 2000). Significantly higher rates of fertilisation and blastocyst formation occur from oocytes with higher ATP levels (Nagano et al., 2006). In pig oocytes, it has been demonstrated that the Δφm is an important regulator of ATP production, which is critical for their maturation and subsequent embryo development (Lee et al., 2014). In mice, *in vivo* fertilised embryos demonstrated higher Δφm in blastocysts than zygotes, 4 and 8 cell stage embryos (Acton et al., 2004). By contrast, reduction of the mitochondrial Δφm resulted in lower rate of blastocyst formation in mice (Lee et al., 2014).

Given Δφm is impaired in response to stress and can predict subsequent cell death, it has been used as a measure of cell stress and apoptosis (Witte and Horke, 2011). In eggs, Δφm can be detected using a variety of dyes, allowing for more versatility in wavelengths and combination with other fluorescent indicators. Detection is possible with conventional fluorescent microscopy or CLSM (Van Blerkom, 2008). At present, a number of mitochondria-specific fluorescent probes have been used to determine mitochondrial number and function in both vertebrate and invertebrate oocytes, including rhodamine123 (r123), 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1) (Ge et al., 2012; Suzuki et al., 2005; Van Blerkom, 2008), MitoTracker™ Orange (MTO) and MitoTracker™ Green (MTG) (Agnello et al., 2017; Morici et al., 2007). As positive control, FCCP (carbonyl cyanide p-(tri-fluromethoxy) phenyl-hydrazone), CCCP (carbonyl cyanide m-chlorophenyl hydrazone) and Valinomycin are used to induce the death of live cells by blocking oxidative phosphorylation in mitochondria. By contrast, ddC (2'3-dideoxycytidine) can be used to reduce mitochondrial copy number (Lee et al., 2014; Salvioli et al., 1997; Sivandzade et al., 2019). Although the association of mitochondrial dysfunction with reduced fertility of oocytes in female vertebrates is well documented, there are surprisingly few studies in invertebrate species.

MTG and MTO are two powerful stains in fertilised sea urchin *P. lividus* eggs to determine mitochondrial mass and oxidative activity, respectively by CLSM (Agnello et al., 2017; Morici et al., 2007). MTG staining works independently of Δφm (Pendergrass et al., 2004) while MTO staining is Δφm dependant (Tomkova et al., 2018). When these dyes are co-localised, they fluoresce yellow and represent oxygen consumption in the mitochondrial population; and when mitochondrial activity is increased, they change to red (Agnello et al., 2017; Morici et al., 2007). During oogenesis in *P. lividus*, mitochondrial mass, distribution and oxidative

phosphorylation activity were studied by incubating oocytes with cell-permeable MTG and MTO dyes (Agnello et al., 2017). MTG green fluorescence was more localised in the cytoplasm of smaller oocytes (20 to 40 μm) and scattered in larger oocytes (60 to 90 μm). Red fluorescence was very weak in smaller oocytes due to low mitochondrial activity but increased considerably in the germinal vesicle of larger oocytes. Throughout oogenesis, mitochondrial number and activity increased in larger oocytes up to 90 μm. Whereas, in fully grown mature oocytes (>90 μm), oxygen consumption (mitochondrial activity) decreased, probably to a basal metabolism (Agnello et al., 2017). The optimum concentration and incubation time for MTG and MTO has not yet been optimised for decapod eggs and embryos.

Rhodamine (r123) has been used as a sensitive marker of mitochondrial function in mammalian oocytes (Salehnia et al., 2013; Thouas et al., 2004). In mice, metaphase II oocytes were photosensitised (visible light for 60 sec to induce mitochondrial damage) and stained with r123 before excitation at 520 nm to detect green fluorescence using CLSM. Zygotes generated from photosensitised oocytes had a diffuse, homogenous ooplasmic staining pattern, compared to zygotes generated from non-irradiated oocytes, which had a punctate fluorescein staining pattern similar to that of functional, undamaged mitochondria (Thouas et al., 2004). Mitochondria of *in vitro* matured bovine oocytes stained with r123 exhibited three types of staining pattern: (i) intensely stained mitochondria distributed throughout the ooplasm; (ii) weakly stained mitochondria at the periphery of the ooplasm; and (iii) strongly stained mitochondria in-between type (i) and (ii) in terms of intensity and distribution (Nagano et al., 2006). The majority of the oocytes with type (iii) staining, had first PB extrusion, indicating a high level of maturity and developmental capacity (Nagano et al., 2006). Mitochondrial distribution in Sea urchin *Lytechinus pictus* eggs was examined by incubating with 10 to 20 μM r123 for about 1 h (Lee and Aarhus, 2000). To date however, r123 has not been used to assess mitochondrial function in eggs or embryos of crustaceans let alone decapods.

Another stain to analyse Δφm is the lipophilic green dye, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) which identifies mitochondria expressing high Δφm and accumulates in mitochondria at low concentration Δφm (Terasaki et al., 1984). To serve as a positive control for zero Δφm, CCCP is added to cells in combination with $DiOC_6(3)$ and incubated at 37 ˚C for 15 min (Barry et al., 2000). Stained samples can be observed using CLSM with excitation at 488 nm and emission at 492 to 629 nm (Witte and Horke, 2011). $DiOC_6(3)$ labelling identified the vegetal RNAs in mature oocytes of the amphibian *Xenopus that* contain clusters of mitochondria (Chang et al., 2004). The distribution and function of hamster oocyte mitochondria were studied using $DiOC_6(3)$ during maturation and fertilisation by fixation in 0.25% glutaraldehyde in sucrose buffer, followed by staining with 5 ng/mL $DiOC_6(3)$ for 30 to 60 sec (Suzuki et al., 2005). GV oocytes showed uniform cytoplasmic fluorescence, while MI

oocytes demonstrated increased intensity of mitochondrial fluorescence. In zygotes the mitochondria were arranged in a dense cluster surrounding the pronuclei and little fluorescence around cortical cytoplasm (Squirrell et al., 2003; Suzuki et al., 2005). To date however, $DiOC_6(3)$ has not been validated to analyse Δφm in the eggs or embryos of decapods eggs.

While all mitochondrial probes mentioned above can be used to detect Δφm, r123 and $DiOC_6(3)$ appear to exhibit inconsistent staining in human cell lines (Salvioli et al., 1997). r123 binds to mitochondria regardless of mitochondrial energy level producing variable fluorescent intensities or occasionally no staining even on energised mitochondria; potentially causing misleading results (Ludovico et al., 2001). Furthermore, r123 was less effective for assessing Δφm by CLSM (Sun et al., 2020). $DiOC_6(3)$ appears to non-specifically bind several membranes of cellular organelles other than mitochondria, which can influence the total cellular fluorescence (Salvioli et al., 1997). JC-1, however, has been shown to be a reliable fluorescent dye for monitoring Δφm changes in both vertebrate and invertebrate species (Picone et al., 2013; Salvioli et al., 1997; Wilding et al., 2001). JC-1 assembles in mitochondria and indicates Δφm across the oocyte matrix (Reers et al., 1995). The dye crosses the membrane when the mitochondrion has a high Δφm and forms J-aggregates that appear red under UV light. When Δφm is low, the dye remains in its monomeric form and fluoresces green (Acton et al., 2004). Mitochondrial Δφm is calculated as a ratio of red florescence to green fluorescence, which corresponds to activated mitochondria (J-aggregates) and less-activated mitochondria (J-monomers), respectively (Ge et al., 2012).

Use of JC-1 dye in human oocytes demonstrated that mitochondrial activity was strongly associated with the rate of embryonic development following fertilisation (Wilding et al., 2001). Since mitochondrial metabolic activity is important for nuclear maturation of oocytes, a reduced rate of mitochondrial respiration may negatively impact subsequent embryo development (Wilding et al., 2001). Staining by JC-1 of fresh human metaphase II (MII) oocytes was associated with a higher number of red fluorescent mitochondria at the periphery of oocytes, indicating a higher mitochondrial Δφm than those of vitrified oocytes (Chen et al., 2012). Mitochondrial Δφm was assessed by JC-1 staining in porcine oocytes treated with the Δφm inhibitor, FCCP (Lee et al., 2014). Greater concentrations of FCCP (2000 μM) significantly inhibited mitochondrial Δφm, resulting in the reduction of ATP synthesis and no first PB extrusion compared to controls. This demonstrated that poor mitochondrial function in oocytes impairs completion of meiotic maturation (Lee et al., 2014). While changes in mitochondrial membrane potential have been studied using JC-1 in the eggs of sea urchin *P. lividus* (Picone et al., 2013), it's use is yet to be validated to determine the quantity and activity of mitochondria during maturation, fertilisation and development of redclaw eggs.

2.7.2.3.4 DNA Damage Assays.

Within living cells, DNA is constantly subjected to different endogenous and exogenous factors that may cause damage (Chatterjee and Walker, 2017; Stringer et al., 2018). DNA damage can be divided into two categories: lesions and strand breaks, both of which may have serious cellular ramifications. Lesions are changes in DNA base structure that can disrupt its chemical and/or physical assembly. Lesion can result in point mutations which could disrupt DNA transcription and translation of functional proteins (Sturmey et al., 2008). Endogenous agents, such as reactive oxygen species, or sporadic events may trigger this type of DNA damage (García-Rodríguez et al., 2018; Sturmey et al., 2008). The resistance of oocytes to DNA damage has been less extensively studied compared to that of spermatozoa, owing to the difficulties of collecting oocytes for research (García-Rodríguez et al., 2018). In addition, the prolonged arrest of maturing oocytes in meiotic prophase I and subsequent pre-ovulatory arrest at meiotic metaphase II prior to fertilisation, are specific periods in which oocytes are particularly susceptible to foreign agents that can induce DNA damage (García-Rodríguez et al., 2018; Marangos and Carroll, 2012).

Double-stranded breaks are regarded as the most severe form of DNA damage that can induce oocyte death (Winship et al., 2018) or significant abnormalities in subsequent early embryos (Stringer et al., 2018). In mammals, DNA damage can disrupt the expression of developmental genes important for differentiating early cell lineages in the embryo, such as trophectoderm, leading to a developmental delay in blastocyst formation; ultimately resulting in implantation failure and pregnancy loss (Peña et al., 2017). Furthermore, unfertilised oocytes are vulnerable to reactive oxygen species-induced DNA damage during manipulation for assisted reproduction due to exposure to light and fumes from the incubator (García-Rodríguez et al., 2018), mutagens or metabolic toxins (Kopeika et al., 2014). To date, DNA damage has been shown to exhibit deleterious effects in the eggs and embryos of both vertebrates and invertebrates, including crustaceans (Browman et al., 2003; García-Rodríguez et al., 2018; Negron and Lockshin, 2004; Winship et al., 2018; Yűce and Sadler, 2001; Zeni et al., 2015). Following exposure to genotoxic chemicals, a strong link was discovered between DNA strand breaks and embryo deficiencies in blue crabs *Callinectes sapidus* and grass shrimp *Palaemonetes pugio*, in which 50% of embryos failed to hatch (Hook and Lee, 2004; Lee et al., 1999). Freshwater prawn *M. olfersi* embryos exposed to high levels of UV radiation (310 mW/cm^2 for 30 min) had DNA damage in the form of cyclobutane pyrimidine dimer (CPD) or thymine dimers, which significantly decreased the density of proliferating embryonic cells compared to non-irradiated controls (30.8 ± 1.7 *vs.* 40.6 ± 2.2 cells/mm^2; $P<0.05$) (Zeni et al., 2015). Generally, two nearby thymines on the same DNA strand break their hydrogen bonds with the corresponding adenines on the neighbouring strand and then bond with each other. As a result, the term pyrimidine (or

thymine dimer) was coined (Mitchell and Karentz, 1993). Thus, detection of DNA damage in unfertilised eggs is one method that can be used to identify a putative female cause for poor embryo development (independent of male factors) during reproduction (Stringer et al., 2018).

Several methods are available for measuring DNA fragmentation in cells that include high-performance liquid chromatography (HPLC) in combination with electrochemical detection, mass spectrometry, immunoassay, gas chromatography connected to mass spectrometry, comet assay (single-cell gel electrophoresis), and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay (Cooke et al., 2008; Sturmey et al., 2008). However, only comet, immuno, and TUNEL assays have been previously reported to detect DNA damage in crustacean eggs and embryos.

Over the past few decades, the comet assay (which relies on electrophoresis of single cells) has been increasingly applied in genotoxicology (Lee and Steinert, 2003). The comet assay can detect and measure a range of DNA damage including single and double-strand breaks, inter-strand crosslinking of DNA, and damaged bases that arise at endonuclease responsive sites in individual nuclei (Nahon et al., 2008; Olive and Durand, 2005). The performance and results of this assay are influenced by cell type as well as different manipulations during gel preparation, alkaline rinsing, electrophoresis voltage, time and current; and thus a completely standardised protocol is still absent (Azqueta et al., 2011). Generally, for decapods, embryonic cell suspensions are prepared and transferred into the slide and electrophoresed at 25 V, 200 to 300 mA, 8W for 10 to 20 min under alkaline conditions (Hook and Lee, 2004; Lee et al., 2000). During electrophoresis, DNA with strand breaks (alkali-labile sites) spread towards the anode creating a comet shape. Thus, the comet has a distinct head consisting of intact DNA and a tail containing relaxed DNA loops or fragmented pieces of DNA (Berthelot-Ricou et al., 2011). Following electrophoresis, slides are stained with ethidium bromide and image analysis software used to measure several parameters of the comet including percentage of DNA in the comet head and tail, amount of DNA in the cell, tail length, and tail moment. The tail moment is the percentage of DNA in the tail multiplied by the tail length (Hook and Lee, 2004; Lee et al., 2000).

Use of comet assay in grass shrimp *P. pugio* showed that early-stage embryos are more prone to death (even with lower DNA damage) than late-stage embryos exposed to different concentrations of toxicants (Hook and Lee, 2004; Lee et al., 1999). Exposure of stage 4 embryos of *P. pugio* to 86 and 172 µg/L 2-methyl-1,4-naphthoquinone induced 1.3 ± 0.5% and 3.4 ± 1.1% (mean ± SD) DNA tail damage and 50 ± 0.6% and 0% respectively hatching rates, compared to stage 7 embryos which showed 1.1 ± 0.4% and 4.4 ± 0.8% tail damage and 95 ± 6% and 90 ± 9% hatching rates, respectively (Lee et al., 2000). Similarly, stage 4 embryos of the same species had significantly poorer hatching rates (46% *vs.* 89%) than stage 7 despite

lower DNA tail damage (5% *vs.* 10%) after exposure to 37.5 nM benzo[α]pyrene (Hook and Lee, 2004).

UV-induced DNA damage was evaluated in fertilised eggs of sea urchins *Paracentrotus lividus* and *Sphaerechinus granularis* using the comet assay (Nahon et al., 2008). Compared to controls, UV irradiation induced two-fold higher DNA strand breaks in fertilised eggs of *P. lividus* and *S. granularis*; decreased embryo cleavage to the 2-cell stage by eight-fold (10.5 ± 14.8% *vs.* 88.4 ± 7.9%; mean ± SD) in *S. granularis*; and significantly impaired embryonic development of normal plutei from 82% to 38.4% and 5%, respectively (Nahon et al., 2008).

Accurate interpretation of comet assay data depends on optimum slide staining, robust image analysis software and measurement of reliable parameters such as % tail DNA (Kumaravel et al., 2009). The sensitivity of the comet assay is limited to detection of hundreds to several thousand breaks per cell, and is unable to detect DNA damage caused by processes such as apoptosis or necrosis in which DNA fragments are too small to be detected and might diffuse away even before electrophoresis starts (Collins et al., 2008). Moreover, cytotoxicity can lead to false positive/negative results (Collins et al., 2008). Other methods such as immunoassays, however, can measure apoptotic damage in oocytes (Bosco et al., 2005; Rodríguez-Marí et al., 2010).

Immunometric DNA damage assays rely on antigen-antibody reactions that have several advantages in terms of detecting instability, including specificity, selectivity, and the ability to analyse a wide range of cell and tissue types (Boguszewska et al., 2019). Immunofluorescent assays, radio immunoassays, enzyme immunoassays, and heavy metal-labelled antibody assays are basic types of immunoassays that use reporter molecules such as fluorochromes, radioactive isotopes, enzymes, and heavy metal-containing nanoparticles (Boguszewska et al., 2019). Immunoassays detect CPD or thymine dimer (Mitchell and Karentz, 1993). Thymine dimer formation was identified in *M. olfersi* embryos exposed to UV radiation using CPD immunohistochemistry by incubating embryos in anti-thymine dimers IgG-1, followed by a fluorescent secondary anti-mouse IgG-1 antibody and subsequent DAPI staining to detect damaged DNA (Zeni et al., 2015). A CPD assay per megabase of DNA was developed by a chemiluminescent antibody identification system for fertilised eggs of Calanoid copepod *Calanus finmarchicus*, and Atlantic cod *Gadus morhua* (Browman et al., 2003). The identification system is subtle enough to detect DNA damage in individual eggs or larvae and can be used as an indicator of UV induced DNA damage (Vetter et al., 1999).

Although immunoassays are widely used for detecting DNA damage, the risk of cross-reactivity of antibodies with DNA bases is a major drawback (Boguszewska et al., 2019). Moreover, antibodies tend to be species-specific and may not be directly applicable to a new

species, requiring validation for every new species. The TUNEL assay could be a reliable alternative since it is a well-established tool for measuring DNA damage in eggs and embryos of mammals (Fouladi-Nashta et al., 2005; Gambini et al., 2014; Mateusen et al., 2005; Winship et al., 2018; Yuan et al., 2005), finfish (Negron and Lockshin, 2004) and invertebrates (Yűce and Sadler, 2001). The TUNEL assay in human oocytes revealed 84% and 88% DNA damage in metaphase I virgin oocytes (those not microinjected with sperm) and oocytes after intracytoplasmic sperm injection, respectively (Bosco et al., 2005). Fertilisation failure was associated with a greater incidence of DNA fragmentation after microinjection, suggesting that apoptosis of oocytes could explain their inability to be activated following sperm injection (Bosco et al., 2005). The TUNEL assay detects damaged DNA via FIT-C-conjugated dUTP incorporation into exposed 3'-hydroxyl (OH) ends of DNA fragments (Kalo and Roth, 2011; Peña Jr. et al., 2019). The nuclei of TUNEL-positive (DNA damaged) cells appear green under fluorescent microscopy and are often combined with Hoechst (blue fluorescence) or PI (red fluorescence) as counterstains (Kalo and Roth, 2011; Makarevich and Markkula, 2002). After TUNEL, dying sea urchin eggs undergoing apoptosis were brightly labelled with contracted dark brown cytoplasm, compared to viable eggs that were unstained and appeared transparent with light gold homogenous yolk (Yűce and Sadler, 2001). To date however, TUNEL has had limited application to assess DNA fragmentation in decapod embryos. For example, in sections of *H. americanus* embryos, TUNEL confirmed apoptosis by labelling the nuclei of small, pyknotic and fragmented cells (Harzsch et al., 1999).

One potential complication of TUNEL and other nuclear staining techniques is their poor permeability across the external fertilisation membrane of decapod eggs coupled with their relatively large size (~2 mm in diameter), which makes the nucleus of fertilised eggs more resistant to DNA staining (Johnson et al., 2011). Enzymatic pre-treatment could overcome these barriers since the nuclei of early-stage fertilised lobster eggs were successfully stained with Hoechst after pre-treatment with a solution of proteolytic and collagenase enzymes (Johnson et al., 2011).

2.7.2.3.5 Reactive Oxygen Species.

Reactive Oxygen Species (ROS) are produced as a by-product of cellular respiration, and may be a potential marker of egg quality because high concentrations are detrimental to cell activity, function and early embryonic development (Finkel and Holbrook, 2000). ROS include a range of diverse chemical species such as hydroxyl radicals, hydrogen peroxide and superoxide anions, which can be produced from internal or external sources (Finkel and Holbrook, 2000). Some ROS products are highly unstable, such as superoxide or hydroxyl radicals, while hydrogen peroxide is spontaneously diffusible and relatively longer lasting (Finkel and Holbrook, 2000). Mitochondria are thought to produce the bulk of intracellular ROS (Finkel and

Holbrook, 2000), which can cause oxidative changes, including DNA breakage, mitochondrial damage, protein oxidation, and lipid peroxidation.

In mammals, under *in vitro* culture condition, excessive production of intracellular ROS during early embryonic development affects metabolism and is harmful to embryo survival (Guérin et al., 2001). In unfertilised rat oocytes, increased amounts of ROS and cytosolic free Ca^{2+} disrupted maturation-promoting factor, causing spontaneous resumption of meiosis, spontaneous egg activation, and deterioration of egg quality in a number of species (Chaube et al., 2018). These eggs were unsuitable for fertilisation due to dispersed chromosomes in the egg cytoplasm and inadequate extrusion of the second PB (Premkumar and Chaube, 2016). In Pacific oysters *C. gigas*, oxidative damage was induced in oocytes by incubation in 0.1, 1 and 10 nM tert-butyl hydroperoxide before staining with 2′,7′dichlorodihydrofluorescein diacetate probe to measuring ROS by flow cytometry (Le Goïc et al., 2014). Tert-butyl hydroperoxide significantly enhanced ROS generation (increased green fluorescence) in these oocytes in a dose-dependent manner with ~ 6-fold greater ROS at 10 nM compared to control (Le Goïc et al., 2014). In vertebrate oocytes, ROS, identified by staining with 2′,7′dichlorodihydrofluorescein diacetate has also been measured using spectrophotometry (Berger and Wilde, 2013; Zhang et al., 2011), microtiter plate (Shoeb et al., 2013), or western blotting (Berger and Wilde, 2013). The mean ROS fluorescence intensities are measured from the acquired images using ImageJ software (Chaube et al., 2018; Gupta et al., 2010). The fluorescent intensity of ROS in cadmium exposed porcine oocytes was significantly higher (25.3 ± 0.8 RFU) than control (6.2 ± 0.4 RFU) when observed by confocal microscopy after dichlorofluorescein staining (Zhou et al., 2019). These oocytes with higher ROS showed significantly greater DNA damage and apoptosis when further stained with molecular markers γH2AX and Annexin-V, respectively. Oocytes impacted by significantly greater ROS, DNA damage and apoptosis failed to progress through meiotic maturation (Zhou et al., 2019). To our understanding, the effect of ROS on egg quality and overall embryo development (fertilisation and hatching rates, embryo survival) in commercially important decapods including redclaw has not yet been evaluated.

2.7.2.3.6 Heat Shock Protein as a Marker of Biological Stress.

Stress is a complex condition of disrupted homeostasis that affects the endocrine, immunological, and reproductive systems in humans (Li et al., 2015). Chronic stress is associated with female infertility and leads to poor fertilisation success (Ebbesen et al., 2009). Heat shock proteins (HSPs) serve as biomarkers of stress resistance mechanisms within cells (Dhama et al., 2019) and maintain interactions with intracellular polypeptides preventing their incorrect assembly or denaturation (Cimino et al., 2002). Expression of HSPs is upregulated in response to exposure to a range of threats including hyperthermia, heavy metals, free oxygen

radicals, ethanol, inflammation and infection (Neuer et al., 2000). Heat shock factor 1 is highly expressed in oocytes and regulates principal HSPs, such as HSP90, which is necessary for the normal development of embryos (Metchat et al., 2009). Heat shock factor 1 knockout mice (*Hsf1*$^{-/-}$), exhibited HSP90 deficit and generated defective oocytes by disrupting meiosis (Metchat et al., 2009). Thermal stress is known to cause apoptosis in oocytes and embryos (Edwards and Hansen, 1997). HSP70 plays an important role in regulating apoptotic pathways and repairing proteins important for oocyte maturation and, therefore, providing intracellular resistance to thermal stress (Zeron et al., 2001). Common methods to measure HSP concentrations include staining with fluorescent stain coupled with fluorescent microscopy and ImageJ software (Souza-Cácares et al., 2019), antibody probing (Kumar et al., 2018), qRT-PCR (Gao et al., 2014; Metchat et al., 2009; Mohamad et al., 2018), and *in situ* hybridisation (Wu and Chu, 2008). Thus, HSPs offer a noteworthy biomarker of egg and embryo quality in vertebrate and invertebrate species (Kohn et al., 2015; Wu and Chu, 2008). In fertilised sea urchin eggs, incubation in exogenous heat shock cognate protein (Hsc70)/Hsp70 decreased the time until nuclear envelope breakdown by more than 10% compared to the control eggs incubated in artificial seawater (Browne et al., 2007). This implies that extracellular HSP70 exhibits unique functional effects on sea urchin eggs by modifying signal transduction processes involved with the acceleration of mitosis following fertilisation (Browne et al., 2007). Whole mount *in situ* hybridisations were performed on 16 h old Zebra fish embryos employing antisense RNA probes after heat shock for 1 h at 37 °C (treatment) *vs.* incubation at 28.5 °C (control; Krone, et al., 1997). At 37 °C, *hsp90α* and the *myoD* genes were detected in the somites and pectoral fin buds of zebrafish embryos, but not in embryos incubated at the control temperature. This indicates that under environmental stress *hsp90α* gene has a special function in the normal process of myogenesis in zebrafish embryos, in addition to protecting all cells within the embryo (Krone et al., 1997). Additionally, a small HSP (HspB1) was identified as important in regulating the growth and normal development of Zebra fish embryos as quantitative morphometric analysis demonstrated a 47% reduction in the cross-sectional area of myofibers in HspB1 morphant embryos compared to controls (Middleton and Shelden, 2013). Although the function of heat shock proteins during embryonic development of decapods are not yet confirmed, estradiol-treated vitellogenic ovary of *Metapenaeus ensis* showed a dose dependent increase in expression of Hsp90 compared to ethanol-treated control ovaries (Wu and Chu, 2008). Hsp90 was highly expressed in oocytes prior to vitellogenesis, reflecting an active transcription and subsequent translation and function at a later embryonic stage, which suggests that Hsp90 plays a key part in the ovarian maturation process (Wu and Chu, 2008). Larvae of brine shrimp and *M. rosenbergii* pre-treated with the phenolic compound phloroglucinol, showed higher survival by an elevated expression of HSP70 when challenged against pathogenic *Vibrio parahaemolyticus* (Kumar et al., 2018). To date, no studies have yet

examined the expression of HSPs during stress as markers of quality in the eggs and embryos of commercially important decapods specifically redclaw.

2.7.2.3.7 High-Throughput Methods to Detect Egg and Embryo Quality.

Flow cytometry is a valuable technique routinely used for the quantification of sperm quality in both mammals and decapod crustaceans (Lezcano et al., 2004; Seligman et al., 1991). However, because of the large size of some decapod eggs, which in redclaw measure about 2.27 mm in diameter (Rodríguez-González et al., 2006a), intact eggs and embryos are not compatible with the fine tubing that facilitates single-cell flow cytometric analysis. While, flow cytometry has been employed to evaluate some finfish and invertebrate eggs, before analysis, the much smaller cell nucleus had to be extracted by a complex process of mechanical detachment and fed separately through the flow cytometer, thereby limiting functional analyses to nuclear traits only (Lecommandeur et al., 1994). Although invertebrates such as oysters have an egg diameter of <50 μm, lipid droplets from ruptured eggs could potentially pass through the instrument and disrupt sample flow (Paniagua-Chávez et al., 2006). Due to such technical issues, microscopy (stereo and inverted or confocal fluorescence) coupled with cellular organelle-specific stains will likely be more suitable techniques to evaluate egg and embryo quality in decapod crustaceans such as redclaw (Kashir et al., 2012).

2.8 Conclusions

Redclaw, a species with great potential for aquaculture and is highly regarded in both local and international markets. Despite this, the redclaw farming industry is struggling to meet demand due to inadequate and unsteady supply of seedstock for grow-out. This insufficiency is largely thought to be due to variable female fecundity, sub-optimal rearing conditions, and sub-optimal hatching systems for juvenile production (Austin, 1998; Dan and Hamasaki, 2011; Hamasaki et al., 2002; Ikhwanuddin et al., 2012; Leonard et al., 2001). To address these challenges, several strategies can be employed to enhance the production of high-quality juvenile. These approaches include promoting gonadal maturation through natural and hormone-induced methods, enhancing the quality of eggs and embryos through dietary supplementation, evaluate fertility adopting morphological and molecular tools in selecting highly fertile females. Additionally, the development of AF techniques can contribute to accelerate genetic selection and improve intensive production of superior juveniles.

Induction of gonadal maturation and spawning in decapods would facilitate more uniform, year-round production of juveniles. Extensive studies indicate that ovarian maturation of female decapods can be triggered through eyestalk ablation (Palacios et al., 1999; Uawisetwathana et al., 2011), exogenous hormone administration (Kulkarni et al., 1991; Rodríguez et al., 2002), RNAi technology (Treerattrakool et al., 2013; Treerattrakool et al., 2011) and use of

monoclonal antibodies (Treerattrakool et al., 2014) which have potential to implement in redclaw for maturation induction. Although research has evaluated the effect of hormone induction using 5-HT, MF, naloxone, oestradiol, progesterone, and GnRH as potential candidates for gonadal maturation in decapods, the mechanisms regulating ovulation and egg release are still unknown, which is an essential component that facilitates timed AF. Studies in mammals (Spaziani et al., 1993; Spaziani et al., 1995) and invertebrates (Hirai et al., 1988; Matsutani and Nomura, 1987) suggest that administration of certain prostaglandins (PGE2 and PGF2α) could be applied to trigger egg release in redclaw.

Moreover, nutritional deficiency can result in poor reproductive outputs in crustaceans (Hernández-Abad et al., 2018; Thien and Yong, 2017). Various dietary nutrients such as proteins, lipids, minerals, and vitamins affect the reproductive performance of crustaceans (García-Guerrero et al., 2003a; Harlıoğlu and Farhadi, 2017; Rodriguez-Gonzalez et al., 2009a,b; Wouters et al., 2001) and supplementation with *n*-3 fatty acids, cholesterol, and astaxanthin were identified as potential nutrients in promoting gonadal development, spawning, producing higher quality eggs, and increasing embryo survival (Barım-Öz and Şahin, 2016; Harlioğlu et al., 2012; Harlioğlu et al., 2013; Niu et al., 2014). However, there are lack of studies examining the effects of these important nutrients on the reproductive capacity of redclaw broodstock requiring further studies to determine their optimum levels on reproduction of female redclaw.

Although several studies in mammals (Klonisch et al., 2010), finfish (Browman et al., 2003; Kosmehl et al., 2008) and invertebrates (Agnello et al., 2017; Gorokhova, 2010; Le Goïc et al., 2014; Yűce and Sadler, 2001; Zirbel et al., 2007) have identified potential markers for evaluating egg and embryo quality, they have not yet been widely applied to decapod eggs and embryos. Application of advanced cell markers for viability, mitochondrial function and DNA damage may reveal some of the causes of poor juvenile production associated with female infertility in redclaw, which may not be detectable using conventional methods. Further research, however, is needed to optimise these techniques for application in redclaw. Traditionally used morphometric markers such as egg diameter, volume, weight, colour, as well as fertilisation, hatching and survival rates can also help identify the limiting step in the production cycle. Thus, simultaneous development and application of advanced tools coupled with traditional markers could rapidly identify broodstock (in/sub)fertility or suboptimal husbandry practices.

Moreover, the establishment of AF techniques will facilitate complete control of reproduction and accelerate genetic selection. To date, the IVF technique shows high rates of fertilisation (94%) in some species, but relatively low hatching rates (0-3%) in the limited number of decapods tested (Ikhwanuddin et al., 2015; Talbot et al., 1991). The low hatching success of

IVF eggs might be associated with suboptimal methods used to collect and store eggs, aging of eggs, fertilisation media, incubation time and ratio of sperm to eggs, etc. (Byrne et al., 2010; Samarin et al., 2016; Yeates et al., 2014). Thus, limited research directed at developing these technologies is currently impeding its adoption by the redclaw industry.

In summary, productivity in various decapod aquaculture industries, particularly redclaw, is limited by a lack of selection methods for more fecund females, variable production of juveniles, and non-standard husbandry practises. This review explored several strategies to induce ovarian maturation and timed egg release and improving egg quality. Additionally, the review has identified the potential for the use of advanced fertility tools to improve the identification of females producing poor quality eggs and embryos. Furthermore, AF process has been delineated emphasising the possible obstacles that might be responsible for the lower success rates in IVF of decapods. Once optimised, these techniques will dramatically improve the productivity of redclaw aquaculture. While the thesis identified advanced reproductive tools and AF as valuable reproductive techniques for further research, the broad aim of this thesis to induce maturation as well as manipulate fecundity in redclaw was achieved through natural manipulation, hormone administration and supplementation of carotenoid rich diet. For quality evaluation, traditional fertility tools such as egg number, hatching rate and survival rate were also selected from the review in the subsequent chapters.

Chapter 3. Development of Sex-Separated Rearing Strategy to Induce Ovarian Maturation and Spawning in Redclaw, *Cherax quadricarinatus*

3.1 Abstract

Redclaw crayfish *Cherax quadricarinatus*, a tropical freshwater species, native to northern Australia and is regarded as a promising species for global expansion of aquaculture. However, poor female fertility is a hindrance towards the commercial production of redclaw. The present study investigated if the presence of males isolated elsewhere in the same RAS system (dissociated) can stimulate spawning and increase reproductive efficiency in female redclaw. The study was conducted in two phases dissociated (111 days) and associated (34 days). Redclaw were held in vertical recirculating aquaculture systems with each system consisting of 42 individual compartments (45 cm length × 33 cm width × 25 cm height) arranged as six compartments horizontally and seven compartments vertically. The average body weight of females was 66 ± 2.2 g. In the dissociated phase, females were either kept alone (0M, n = 36) or were exposed to either one (1M, n = 36) or two (2M, n = 36) males suspended in the uppermost row of the system. During the associated phase, females (n = 108) were maintained at a sex ratio of 1M:1F. During the dissociated phase, the spawning rate was less in the control compared to the male exposed groups (2.8%, 1/36, 18.1%, 13/72 respectively; $p = 0.026$). However, the moulting rate was greater in the control compared to the male exposed groups (22.2%, 8/36; 5.6%, 4/72 respectively; $p = 0.009$). During the associated phase, there were no significant differences in spawning rate (22.2%, 8/36 and 33.3%, 24/72), mean days to spawning (21.88 ± 3.06 and 16.38 ± 2.35), moulting rate (11.1%, 4/36 and 6.9%, 5/72) and mean days to moulting (24.50 ± 3.97 and 22.40 ± 3.93) in the control and male exposed groups, respectively ($p>0.05$). However, the mean total number of eggs (670.9 ± 26.0 and 507.0 ± 29.36), fecundity (11.00 ± 0.59 and 6.14 ± 0.47), the hatching rate (89.70 ± 1.31% and 77.50 ± 9.24%) and the total number of juveniles produced (545.70 ± 42.50 and 343.30 ± 37.47) during the associated phase were greater in the male exposed groups compared to the control group respectively; $p<0.05$). It was concluded that pre-exposure of redclaw females to males in a dissociated recirculation system, increases spawning rate during a dissociated phase, and after exposure to males improves egg and juvenile production during an associated phase. Such a breeding strategy has potential to increase hatchery productivity.

3.2 Introduction

Redclaw crayfish *C. quadricarinatus*, is a freshwater decapod crustacean endemic to northern Australia and south-eastern Papua New Guinea (Ghanawi and Saoud, 2012; Jones, 1990; Ruscoe, 2002; Saoud et al., 2013; Webster et al., 2004). Redclaw has gained much popularity for tropical aquaculture for its outstanding attributes including robustness, simple reproductive cycle, and high market value (Jones, 1990; Masser and Rouse, 1997; Webster et al., 2004). The domestic and international demand for redclaw is rising tremendously, yet neither conventional pond-based production methods nor relatively new hatchery technologies have been able to consistently produce commercial numbers of healthy juveniles (Rigg et al., 2021). For the industry to expand further, greater juvenile production through effective hatchery management is necessary.

Reproduction in female decapods in commercial hatcheries is traditionally managed by manipulation of environmental stimuli such as photoperiod and temperature (Barki et al., 1997; Karplus et al., 2003), dietary supplementation (Rodríguez-González et al., 2006a, 2009a,b), and through eyestalk ablation (Palacios et al., 1999; Sagi et al., 1997; Uawisetwathana et al., 2011). Additionally, administration of hormones (Cahansky et al., 2008; Sarojini et al., 1995a; Tinikul et al., 2014; Zeng et al., 2016), RNA interference and monoclonal antibody technology (Treerattrakool et al., 2011, 2014) are also employed to achieve ovarian maturation in decapods and results from preliminary research have shown these methods as promising for enhancing reproduction in decapods. However, their practical application is so far limited by being invasive, labour intensive, and resulting in variable reproductive responses. These include asynchronous spawning, variable hatching rates and juvenile mortality (Alfaro-Montoya et al., 2019; Barki et al., 2011; Jones, 1995a, 1995b; Masser and Rouse, 1997; Okumura and Sakiyama, 2004; Stevenson et al., 2013). Moreover, the use of monoclonal antibodies and some prospective hormones such as red pigment concentrating hormones, kisseptins, GnRH and its analogues is constrained by factors such as undetermined signalling pathways, commercial unavailability, and non-optimised dosage rates (Ngernsoungnern et al., 2009; Sarojini et al., 1995b; Thongbuakaew et al., 2016; Treerattrakool et al., 2014; Zeng et al., 2016). Furthermore, the effects of hormones on the environment have not been evaluated and consumer resistance to the use of hormones in food producing animals is growing (Alfaro-Montoya et al., 2019; McEvoy, 2016). Considering these limitations, manipulation of female reproduction in a natural way is preferable as a non-invasive and sustainable strategy.

Crustaceans can exhibit spontaneous breeding where females spawn in response to chemical cues/substances such as pheromones and peptides streamed from the presence of males held in separate tanks while physically dissociated from females but still sharing a recirculated water supply system (Aiken et al., 1984; Peeters and Diter, 1994; Waddy and Aiken, 2011). In

commercial hatcheries, male and female redclaw are usually kept together at a sex ratio of 1 male to 5 females while maintaining optimal water temperatures of 28°C and a photoperiod of 14 h light and 10 h dark (Yeh and Rouse, 1994; Yeh and Rouse, 1995). Under such conditions, the females' mate with males and the released eggs are fertilised by sperm attached to the sternum of females. Spontaneous spawning in the absence of males has not been reported for redclaw. However, unlike redclaw, spontaneous spawning in absence of males has been reported to occur in *Penaeus indicus* (Peeters and Diter, 1994) and in *Heptacarpus pictus* (Bauer, 1979).

Spontaneous spawning has also been reported in fish such as groupers where males and females were housed separately but had a shared water supply system. This suggests that in groupers the presence of males plays an important role in the induction of oocyte maturation and ovulation possibly via exposure to pheromones (Amagai et al., 2022; Soyano et al., 2022). Moreover, tilapia urine from males contains a pheromone that primes the female's reproductive system by increasing the production and release rates of the maturation-inducing steroid 17,20β-P (Huertas et al., 2014). Exposure of female mice and cows to male urine accelerates the onset of puberty and improves the fecundity with larger production of offspring (Mucignat-Caretta et al., 1995; Rekwot et al., 2001).

To these author's knowledge, there is no scientific study reporting spontaneous spawning of female redclaw without the direct presence of a male. However, at the Australian Crayfish Hatchery, staff have observed female redclaw to occasionally spawn when stocked in a recirculating system where males are housed separately to the females but share the same recirculating water. An advantage of being able to induce spontaneous spawning in redclaw would be to facilitate a method of providing AF and cryopreservation of unfertilised eggs. Moreover, the physical presence of males in some decapod species such as freshwater caridean shrimp, *Neocaridina davidi* can enhance the onset of reproductive maturity (Tropea et al., 2018). The objective of the study was to determine if pre-exposure of female redclaw to males in a dissociated, closed recirculating system could induce spawning and enhance reproductive performance. Our hypothesis was that inclusion of 1 or 2 males within the top level of a recirculating culture system will reduce the time interval to spawning and improve the reproductive performance of redclaw females.

3.3 Materials and Methods

3.3.1 Experimental Site and Plans

The study was conducted at the Australian Crayfish Hatchery (ACH; 19° 15' 28.656" S, 146° 43' 31.908" E), located within the tropical region of northern Australia. Redclaw were housed in vertical recirculating aquaculture systems (RAS) with each system consisting of 42 individual

compartments (45 cm length × 33 cm width × 25 cm height) arranged as six compartments horizontally and seven compartments vertically. The volume of each compartment, water source, and flow rate were 14 L per compartment (breeding boxes), full RAS, and full water exchanges every 10 min in each compartment, respectively. A diagrammatic arrangement of the RAS used is illustrated in Figure 3.1. All crayfish had been microchipped prior to the study (Mini Microchips Australia, Merrylands NSW. ISO 11784/11785 FDX-B Microchip) which provided the identification, date, gonadal stage, moult stage, and compartment number.

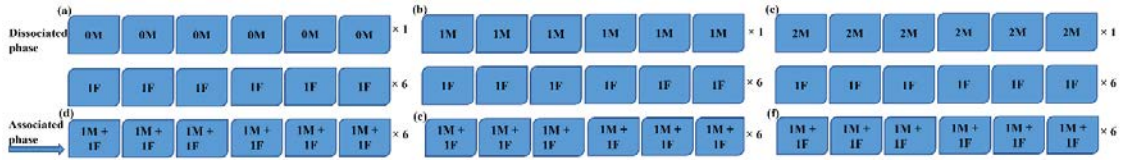


Figure 3.1. Diagrammatic arrangement of the experimental procedure used for the dissociated and associated phases of the study. Dissociated phase: (a) 0M females cultured in water without the presence of males (0M:6F), or (b) 1M 6 males cultured with 36 females (1M: 6F), or (c) 2M 12 males cultured with 36 females (2M:6F) in the dissociated phase. Associated phase: (d), (e) and (f) each female was exposed to 1 male (1M:1F).

Redclaw were held at 26 ± 2 °C and 14L:10D photoperiod. Water quality (temperature, pH, dissolved oxygen, general hardness, ammonia, and nitrite was monitored weekly and animals were fed a combination of Entomix (ACH proprietary feed) and commercial spirulina pellets (multiple commercial sources) once daily. Redclaw were observed daily for mortality and moulting. The average body weight of females used in this study was 66 ± 2.2 g and were classified as being within the early ovarian developmental stage (Stage I). Gonadal maturation in females was assessed using a concentrated light source as previously described in redclaw (Jones, 1990). Briefly, females were taken into a dark room and ovarian development was determined by flashing a submersible light (10,000 Lumen) on both left and right sides and the ventral part of the carapace (Jones, 1990). Females were held in compartments in the first 6 rows and 6 columns in each system. Males were held in the top row, if included, directly above the females. All crayfish were held separately unless otherwise stated. Three such recirculating systems were used one for each treatment.

3.3.2 Dissociated Phase

During the dissociated phase (DP), females had no exposure to males (Control) or females were held separately to males with shared culture water. Females in the control group (n = 36) were held separately in the bottom six rows of an individual RAS. No males (0M) were included in this system. In the dissociated group with one male (1M), females (n = 36) were held separately in the bottom 36 containers of an individual RAS. A total of 6 males were held in individual containers in the top row of the system. In the dissociated group with two males (2M), females

(n = 36) were held separately in the bottom 36 containers of an individual RAS. A total of twelve (12) males were held in individual containers in the top row of the treatment system. The duration of the dissociated phase was 111 days.

3.3.3 Associated Phase

On conclusion of the dissociated phase and during the associated phase (AP) males (n = 108) were added to all females (n = 108) at a sex ratio of 1M:1F in individual compartments. Males were matched with females based on female body weight, with a maximum of 20 g size disparities. Females were checked for spawning every second day and the weight of each animal was recorded every 14 days. The duration of the AP was 34 days.

3.3.4 Determination of Reproductive Parameters

Spawning was assessed by retrieving females using a hand net and visually observing the ventral surface of the abdomen and opening the curled tail for the presence of eggs. Day of spawning was recorded as the day spawning was detected -1. Once spawned, the eggs were collected and counted using the 'CountThings' software (Dynamic Ventures Inc., Cupertino, CA). Eggs were collected from females using forceps and stripping eggs into a container before being photographed for counting using an automated option by the software. Fecundity was measured as eggs per g body weight of female (Rodríguez-González et al., 2006). After collection, the eggs were treated in 70% ethanol for 1 min and finally were placed in the hatching incubator (Australian Hemputin adapted from Finnish design) and monitored until they hatched. The incubator had a volume of 300 L and utilised a RAS as the water source. Hatching was identified with the complete emergence of juveniles from the eggs (García-Guerrero et al., 2003). Hatching rate was determined after initial egg collection by observing all the incubated eggs once daily and counting all the dead eggs until hatching was completed by subtracting the cumulative total number of dead eggs counted from the original number of eggs. Survival rate to the juvenile stage was measured when hatchlings moulted to become juveniles at approximately 16 days after hatching and was recorded as the (number of hatchlings – the number of surviving juveniles)/100. Craylings and juveniles were held in 1000 l tanks. From hatch to craylings no feeding was performed as they relied on their yolk sack for nutritional support. From crayling to juvenile stage of development biological floc was fed.

3.3.5 Data Treatments and Statistical Analyses

Statistical analyses were conducted using IBM SPSS Statistics version 27 for Windows (SPSS Inc., Armonk, NY, USA). A mixed effects model was used to compare the body weights of redclaw exposed to different treatments from day 0 to 145 with individual redclaw as random effects and treatment, phase, and time as the fixed effects. Proportional data were compared with a Pearson's Chi-Square test. Analysis of covariance was used to compare differences

between means (days to moulting, days to spawning, eggs per female and total number of juveniles). The initial body weight of the crayfish was entered as a covariate but only retained if its effect was significant.

A log rank test, using Kaplan-Meier survival curves, was used to determine if there were differences in the survival distribution during the dissociated and associated phases for the different treatments. Cox regression was also used to assess the time to spawning in both phases in relation to treatment, initial weight and whether crayfish moulted or not. Treatment was retained in all models but variables that did not affect the dependent variable (p>0.10) were removed from final models. Initial analyses treated 1M and 2 M treatments (i.e., exposure to 1 and 2 males, respectively) as separate treatments. However, for every variable examined, no significant differences were found between the two treatments. Therefore, data from these two treatments were subsequently combined (Table 3.1 and 3.2) and compared with the results from the control group.

3.3.6 Ethical Statement

Ethics approval was not required for the conduct of this study on crustaceans by the James Cook University, Animal Ethics Committee. On completion of the study, animals were returned to the breeding colony for subsequent reuse in the breeding program.

3.4 Results

The initial body weight of crayfish and carapace length were highly correlated (r^2 = 0.87; F_1 = 698.3, p<0.001) so only body weight was included in statistical models. Using a linear, mixed effect model no significant effect of treatment on body weight was detected (Figure 3.2; F_1 = 0. 043, p = 0.836), however, differences in body weight over time were detected between phases (F1 = 7.32, p = 0.007). Mean body weight was greater in the dissociated compared to the associated phase (65.91 ± 0.777 vs 65.59 ± 1.262, respectively) and changed over time (F1 =7.79, p = 0.005). However, a phase by time interaction was, found indicating a highly significant difference in slope (rate of change in weight) between Phases (F1 = 9.87, p = 0.002). During the dissociated phase, the slope was marginally positive for the control group (b_1 = 0.0024), but slightly negative (b_1 = -0.0003) for the group exposed to males (Figure 3.2). In the associated phase the slope was negative for both control and male exposed group (b_1 = -0.0164 and b_1 = -0.0191, respectively; Figure 3.2) indicating that both treatments lost body weight during the associated phase although the difference was significant the amount weight loss was very small.

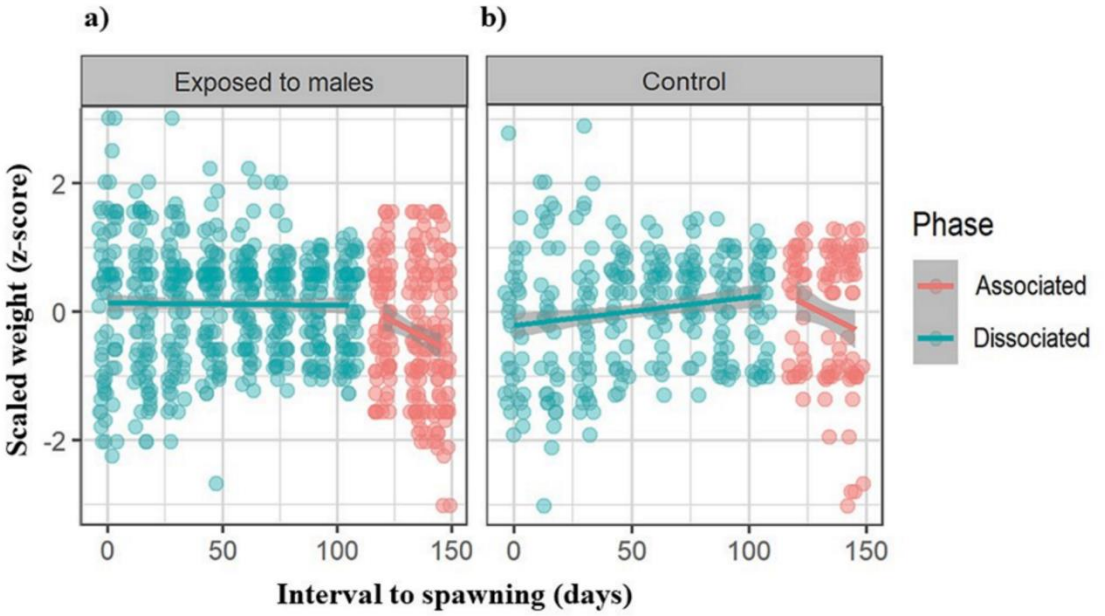


Figure 3.2. Body weight (mean ± SEM) of redclaw crayfish over the duration of the study that were (a; 1 M and 2 M) and were not (b; Control) exposed to males.

3.4.1 Dissociated Phase

During the DP the survival distributions for the days to spawning for the two interventions differed (Figure 3.3; $\chi^2_1 = 4.93$, $p = 0.026$). Using Cox's regression time to spawning during the DP was not associated with initial body weight or moulting (p>0.730) but tended to be affected by treatment ($p = 0.058$, OR: 7.13, 95% CI 0.93 – 54.5). During this phase the survival rate did not differ significantly between the females that were and were not exposed to males (90.3% and 86.1%, respectively; $p = 0.52$). The reproductive characteristics associated with the DP are listed in Table 3.1. The percentages of redclaw that spawned in the DP was significantly greater in the females exposed to males compared to the control group. The percentage of redclaw that moulted was significantly greater and the interval to moulting was significantly shorter in control group when compared with females that were exposed to males (Table 3.1).

Table 3.1: Reproductive characteristics of female redclaw during the dissociated phase.

Variable	Control	Exposure to males	*p*
n	36	72	-
Spawned (%)	2.8 (1/36)	18.1 (13/72)	0.026
Moulting (%)	22.2 (8/36)	5.6 (4/72)	0.009
Days to moulting	23.75 ± 5.66	52.75 ± 6.29	<0.001

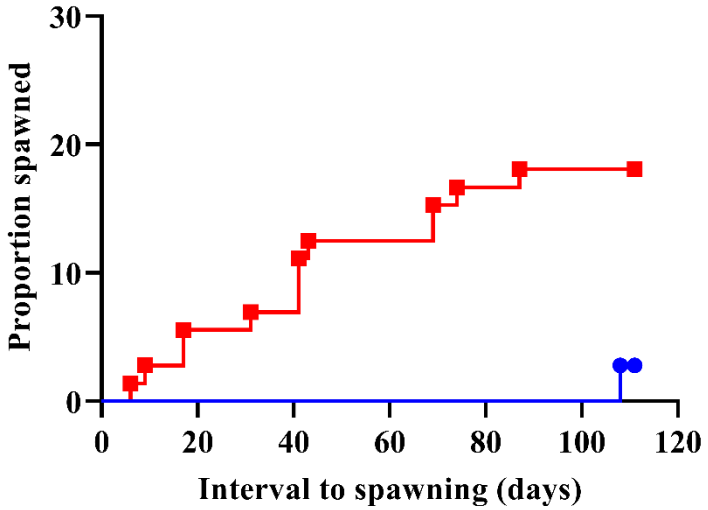


Figure 3.3. Kaplan-Meier survival curve (distribution) of the proportion of redclaw crayfish spawned during the dissociated phase of the study for redclaw crayfish that were (■; 1 M and 2 M) and were not (●; Control) exposed to males.

3.4.2 Associated Phase

During the AP the survival distributions for the two interventions did not differ significantly (Figure 3.4; $\chi^2_1 = 1.59$, $p = 0.207$). Using Cox regression, time to spawning during the AP was not associated with treatment ($p = 0.215$; OR = 1.66; 95% CI = 0.75 – 3.69), or initial weight or whether crayfish moulted or not ($p>0.220$).

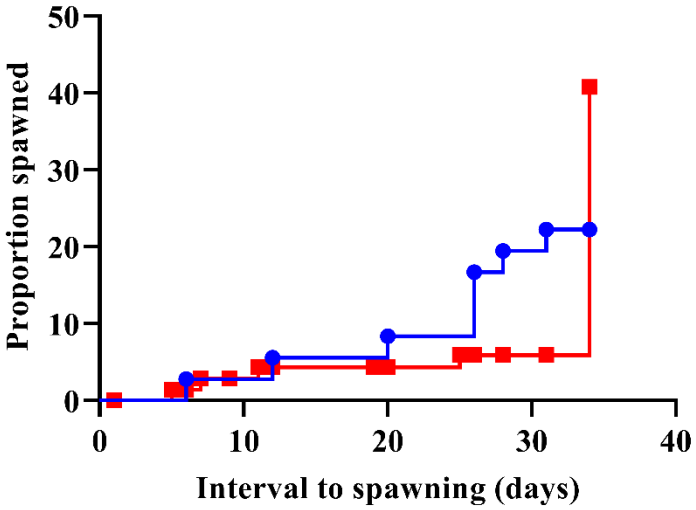


Figure 3.4. Kaplan-Meier survival curve (distribution) of the proportion of redclaw crayfish spawned during the associated phase of the study for redclaw crayfish that were (■; 1 M and 2 M) and were not (●; Control) exposed to males.

The survival rate of the control and male exposed group in the AP was identical to that during the DP; hence again no significant difference was detected. Reproductive characteristics associated with the AP are listed in Table 3.2. The percentage of animals spawning and moulting as well as the days to spawning and moulting were not significantly different between control and the male exposed group. The total number of eggs per female, fecundity, hatching rate and total number of juveniles produced were significantly greater for the male exposed group compared to the control group (Table 3.2). During the AP the initial female body weight did not significantly affect the total number of eggs/female (r^2 = -0.01, p = 0.41), but it did significantly affect fecundity (r^2 = 0.60, p<0.001; Figure 3.5). There was a tendency for initial body weight to affect the total number of juveniles (r^2 = 0.07, p = 0.086) but the regression line only explained 9.5% of the variation and so poorly represented the relationship.

Table 3.2: Reproductive characteristics of female redclaw during the associated phase.

Variable	**Control**	**Exposure to males**	***p***
n	36	72	-
Spawned (%)	22.2 (8/36)	33.3 (24/72)	0.233
Days to spawning	21.88 ± 3.06	16.38 ± 2.35	0.227
Eggs/female	507.0 ± 29.36	670.9 ± 26.00	<0.001
Fecundity (Number of eggs per g female BW)	6.14 ± 0.47	11.00 ± 0.59	0.008
Hatching rate (%)	77.50 ± 9.24	89.70 ± 1.31	0.039
Total number of juveniles	343.30 ± 37.47	545.70 ± 42.50	<0.001
Moulting (%)	11.1 (4/36)	6.9 (5/72)	0.460
Days to moulting	24.50 ± 3.97	22.40 ± 3.93	0.722

The spawing rate in the AP was greater than in the DP for both treatments (Control DP versus AP: 2.8%, 1/36 vs 22.2%, 8/36; $\chi^2_1 = 6.22$, $p = 0.013$; exposed to males DP versus AP: 18.1%, 13/72 vs 33.3%, 24/72; $\chi^2_1 = 4.40$, $p = 0.036$).

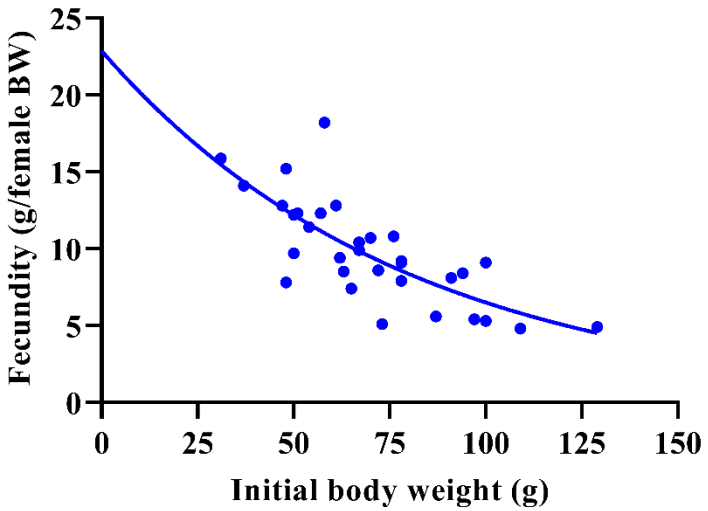


Figure 3.5. Quadratic relationship between the initial body weight and fecundity of redclaw crayfish.

3.5 Discussion

The primary objective of the present study was to determine whether pre-exposure of females by the indirect presence of males stimulates spawning and increases reproductive performance in female redclaw. Our interpretation of the results indicates that pre-exposure of females to males in a dissociated stocking arrangement enhanced the spawning rate during a DP and significantly increased the number of eggs and juveniles produced when the females were subsequently associated directly with males. The significantly greater spawning rates in the DP and juvenile production in the AP in females subjected to pre-exposure to males was most likely induced through external cue stimuli in the form of chemical cues produced by males as reported in Angler fish *Pterophyllum scalare* (Chien, 1973). In female *P. scalare,* the visual and chemical signals from male stimulated the release of gonadotrophin hormones, which resulted in rapid development of the gonads and following ovulation and spawning compared to the group held isolated from any kind of male stimuli (Chien, 1973). This also corroborates the findings of Mozambique tilapia, where exposure of females to male urine augmented the production of the oocyte maturation hormone 17,20β-P to synchronise the spawning and fertilisation by mediating the female endocrine system (Huertas et al., 2014). Additionally, the chemosensory system plays a significant part in the coordination of aggregation and spawning behaviours in sea cucumbers *Holothuria arguinensis*, where water conditioned by males contain labile compounds such as phosphatidylcholines that attracted conspecifics and initiated spawning in both sexes (Marquet et al., 2018). Studies on crustaceans showed that the properties of urinary sex pheromones are crucial for promoting effective partner choice and mating, which raised the possibility that such chemical communication in crustaceans might trigger reproductive responses as well (Barki et al., 2011; Kamio et al., 2005). However, unlike fish, in crustaceans it is yet to be reported that chemical communication promotes spawning and egg production in females that are isolated from males.

In the present study, during the DP, the mean body weight of females in the control group increased slightly, a greater percentage moulted, and a reduced percentage spawned compared to the group indirectly exposed to males within the same recirculating system. Significant weight gain has also been achieved in other decapod crustaceans including crayfish, crabs and prawns following moulting (Cameron, 1989; Hammond et al., 2006; Xu et al., 1993). The percentage of crayfish that moulted in the AP also decreased by 50% from 22.2% during the DP to 11.1% in the AP, whereas, in the male exposed group the moulting percentage increased by a smaller percentage of 23.2% (5.6% to 6.9%) from the DP to the AP indicating that the presence of males either directly or indirectly inhibits moulting. Our results are also consistent with an earlier study on redclaw, which demonstrated that moulting took place after the breeding season when animals were reared at 26 to 28°C temperature and 14 h light and 10 h dark condition

(Barki et al., 1997). Our findings are also in agreement with other studies conducted on *Scyonia ingentis* and *Penaeus indicus*, where moult frequency was greater in immature females in contrast to sexually mature females that spawned multiple times with no moulting (Anderson et al., 1985; Aquacop, 1975; Emmerson, 1980). Moreover, in banana shrimp *Penaeus merguiensis*, it was reported that spawning occurred before moulting and freshly moulted females did not have mature (stage III or IV) ovaries (Crocos and Kerr, 1983). Furthermore, during the non-reproductive period, in female crabs *Chasmagnathus granulata*, the percentage of crabs that moulted was greater than the percentage that spawned indicating that there was a negative association between moulting and spawning (López Greco and Rodríguez, 1999). Our results suggest that moulting and ovarian maturation are two competitive energy demanding processes which often do not occur simultaneously in decapods (Aiken et al., 1980; Zmora et al., 2009). In the AP, females lost body weight in both control and male exposed groups which could be due to females partitioning and expending energy for reproduction and subsequent egg production when they were paired with males. During the AP, the spawning rate, and the mean days to spawning were not significantly different between the treatments perhaps because one-fifth (18.1%) of the animals in the male exposed group had already spawned during the DP. Insufficient time may have been available following spawning for the ovaries of these animals to mature again enabling them to spawn again during the AP which continued for only 34 days. An earlier study conducted on redclaw showed that when reared at a temperature of 25 to 26°C and photoperiod of 12L:12D the average interval between spawning without moulting was 96 days while only 22% (6 out of 27) females spawned (King, 1993). In adult crabs *C. granulata*, the mean interval between spawning was 59.2 ± 6.7 days (López Greco and Rodríguez, 1999) which could suggest that in the present study that insufficient time was available for spawning again during the AP.

The spawning rate in the animals exposed to males indirectly during the DP was less than when they were directly exposed to them during the AP (18.1% vs 33.3%, respectively; $p = 0.036$). The exact reason for this cannot be determined from this study but could related to the longer cumulative exposure that females had to males by the end of the AP and/or the more direct contact that occurred between males and females in the AP compared to the DP. This could include exposure to behavioural and tactile cues that provided a greater stimulus for reproduction during the AP compared to the DP. It could also be that more intimate exposure favoured greater concentrations of hormones released from males in the vicinity of females. The significant increase in spawning in the unexposed females when exposed to males strongly suggests the overall importance of the presence of males to stimulate spawning in female redclaw, which has been previously demonstrated (Huertas et al., 2014; Marquet et al., 2018).

During the AP, female fecundity was found to be inversely associated with body weight, such that at higher initial body weight, fecundity was lower. This finding is concordant with other research in crayfish *Orconectes limosus* Raf., where larger females (in total length and body weight) showed significantly lower fecundity compared to the smaller females (Graczyk et al., 2019). The fecundity (11.3 eggs per g) obtained in the present study was the greatest reported to date in redclaw where fecundity has been found to vary between 8.5 to 10.31 eggs per g female (Rodríguez-González et al., 2006, 2009). In the current study, the greatest fecundity was recorded in females that ranged from 30 to 70 g. Thus, females within this weight range may be preferable for stocking in hatcheries compared to females of greater weight to maximise reproductive potential for the commercial production of eggs. The higher fecundity per unit weight of females suggest that these younger females are at the peak of their reproduction and likely to produce a greater number of eggs. Stocking smaller compared to larger female redclaw might also be beneficial economically by requiring less space and feed for maintaining broodstock.

A significant increase in hatching rate was also observed during the AP in the male exposed group compared to the control group. Although the exact reason for this difference in hatching rate is unknown, a greater hatching rate in male exposed group can be interpreted as an induction of ovarian development in the females that has been pre-exposed to cues from the males during the DP. As a result, the females had longer time to accumulate nutrition for yolk deposition, which could have led to the production of more viable eggs with higher quality.

This study has also identified a number of areas for further study. For example, the induction of a significantly greater percentage of females to spawn that were subjected to the indirect presence of males could be a potential technique in the production of unfertilised eggs from redclaw which may then be utilised for AF and cryopreservation if these techniques become able to be applied in commercial aquaculture. The significant increase of spawning in the dissociated event and the greater production of juveniles in the AP suggests that chemical stimuli are released into water by males that stimulate reproduction in females. However, the identity of those chemicals and by what sensory modalities are stimulatory cues transmitted remain unknown and warrant further research. It is also crucial to determine how long the chemicals may persist for in water. For example, in sea cucumber *H. arguinensis*, when pre-spawned male water (freshly spawned water, 2 h and 4 h aged spawned water) was added to the aquaria containing either males or females, sea cucumbers spawned that received freshly spawned water and 2 h aged spawned water. However, no sea cucumber released gametes that was stimulated with 4 h aged spawned water, indicating that the spawning ingredient had degraded or evaporated (Marquet et al., 2018). Chemical cues often have a broad spectrum, and depending on the compound's polarity, molecular size, and other physicochemical

characteristics, purifying each one requires a unique set of processes and tools (Kamio et al., 2022). To investigate this, mass spectrometry-based metabolomics could be employed to identify molecules or tissue specific chemicals that are present in water that can influence reproduction in females (Jjunju et al., 2020; Marquet et al., 2018). It is also unknown if there is an optimum time and duration of exposure of females to males in a dissociated aquaculture system to enhance the production of juveniles during the associated phase.

3.6 Conclusions

This study is the first to investigate the effect of pre-exposure to males in a dissociated arrangement on spawning and other reproductive parameters in female redclaw and the potential for this system to increase the production of eggs and juveniles. It was found that an aquaculture system that dissociated males from females but shared the same, recirculating water increased the spawning rate and, subsequently when females were directly exposed to males, increased juvenile production in an indoor hatchery system. Production of unfertilised eggs during a dissociated stage could be a crucial component in providing unfertilised eggs for AF and cryopreservation, which should greatly facilitate selective breeding program. Moreover, the demonstration of greater per unit weight fecundity of smaller young females may provide opportunities to reduce space and feed requirements within commercial hatchery systems. The novel aquaculture approach for the improvement of reproduction developed here for redclaw could potentially allow the avoidance of ethical challenges and limitations associated with more invasive reproductive technologies such as eyestalk ablation, endocrine manipulation, RNA interference and monoclonal antibody technology that have been used to induce ovarian maturation in crustaceans. However, additional research is needed to identify potential chemicals in water that stimulated reproduction in redclaw.

Chapter 4. Development of Hormone-Based Method to Induce Ovarian Maturation and Spawning in Redclaw, *Cherax quadricarinatus*

4.1 Abstract

Variable fertility and asynchronous spawning of female redclaw, *Cherax quadricarinatus*, limits the production of juveniles in commercial hatcheries. The present study investigated the effects of intramuscular (IM) injections of methyl farnesoate (MF), serotonin (5-HT) or naloxone on ovarian maturation and reproductive function of female redclaw. Redclaw were housed in vertical recirculating systems where each system comprised 42 separate compartments, organised into a grid of six horizontal and seven vertical compartments. Control (100 µL IM crayfish saline solution) or MF (8.3×10^{-2} µg/g BW IM) treatments were administered on Days 0, 5, 10, 15 and 20 of the study and 5-HT (1.3 µg/g BW IM) or naloxone (6.7×10^{-1} µg/g BW IM) were administered on Days 10, 15 and 20. Males were introduced to females on Day 25. On Day 25 the mean ± SEM gonadosomatic index (GSI) (5.3 ± 0.1, 5.2 ± 0.2, 4.1 ± 0.3 and 4.1 ± 0.4, $p = 0.002$) and diameter of oocytes (1822.8 ± 58.9 µm, 1927.3 ± 84.9 µm, 972.5 ± 26.9 µm and 940.3 ± 75.8 µm, $p<0.001$) were greater in animals treated with 5-HT and naloxone compared to the Control and MF treated animals, respectively ($p<0.001$). Spawning was more prevalent ($p<0.05$) and the interval to spawning was less ($p<0.05$) in crayfish treated with 5-HT and naloxone compared to the Control and MF treated animals. Female redclaw treated with MF had higher moulting rates, lower moult interval but also lower survival than other treatments. The mean number of eggs/female (600.4 ± 34.0, 550.6 ± 17.9, 465.0 ± 36.5 and 453.2 ± 17.7) and fecundity (9.2 ± 0.1, 8.2 ± 0.2, 7.0 ± 0.2 and 6.3 ± 0.1) and hatching rate (87.4 ± 1.0, 85.7 ± 1.2, 77.2 ± 1.8 and 65.2 ± 5.1) were significantly greater in the 5-HT and naloxone treated crayfish compared to those treated with Control and MF, respectively. Intramuscular administration of 5-HT or naloxone can increase egg production and hatching rate in an indoor hatchery setting outside their reproductive season while MF increases moulting in female redclaw, but also increases the risk of mortality without effectively increasing maturation or spawning.

4.2 Introduction

Redclaw, *C. quadricarinatus*, are tropical freshwater crayfish, indigenous to North-Western Queensland and the Northern Territory of Australia and Southern Papua New Guinea, that are commercially produced with many advantageous characteristics (Jones, 1990; Haubrock et al., 2021). Redclaw hold significant economic importance on a global scale, ranking as the second-most cultured and caught crayfish species worldwide (Haubrock et al., 2021). Female redclaw produce a few hundred eggs, whereas penaeid prawns or commercially farmed species produce thousands to millions of eggs. Egg output is therefore a production bottleneck and industrialisation of redclaw farming is contingent on strategies to increase the number and consistency of egg production. Furthermore, other limiting factors within the industry include asynchronous and poor hatching rate among fertilised eggs, and mass mortality of early juveniles (Calvo et al., 2011; Jones, 1995; Masser and Rouse, 1997; Shun et al., 2020). The lack of reliable, intensively produced redclaw juveniles for the stocking of grow-out (production) ponds is a major rate-limiting element of freshwater crayfish farming which is needed to enhance profitability.

In commercial hatcheries, maturation and spawning of female decapod crustaceans including prawns and crabs are induced using the technique of eyestalk ablation (Khazraeinia and Khazraeinia, 2009; Muhd-Farouk et al., 2019; Tan-Fermin, 1991; Uawisetwathana et al., 2011). Although eyestalk ablation can predictably induce ovarian maturation and spawning in redclaw, the permanent removal of eyestalk ultimately disrupts the organism's ability to regulate various physiological processes (Abdu et al., 2001), and is not conducive to repeat spawns in a season (Sagi et al., 1997). Many associated problems have been reported, like reduced lifespan of broodstock, an increase in mortality rates, deterioration in spawn quality and quantity over time, lower fecundity and fertility, poor hatching rates, loss of embryos as well as lower production of juveniles (Browdy, 1992; Magaña-Gallegos et al., 2018; Rodrigues et al., 2022; Zacarias et al., 2019). Therefore, eyestalk ablation is not generally favoured for use as a standard aquaculture practice.

Parenteral such as intramuscular and intraperitoneal administration of hormonal treatments is a common practice in aquaculture to synchronise maturation of gametes and to induce spawning (Black and Black, 2013; Guppy et al., 2022; Song et al., 2020; Tinikul et al., 2014; Tinikul et al., 2023). Several studies have investigated the use of exogenous hormone treatments to control ovarian maturation to produce high-quality juveniles in decapod crustaceans (Alfaro-Montoya et al., 2019; Nagaraju, 2011; Tinikul et al., 2023; Treerattrakool et al., 2013; Wongprasert et al., 2006). Chemicals such as serotonin (5-hydroxy tryptamine, 5-HT), methyl farnesoate (MF), 17β-oestradiol, and naloxone have been tested to induce ovarian maturation in crayfish (Cahansky et al., 2008; Liu et al., 2014b; Rodríguez et al., 2002). MF is an insect juvenile

hormone (the unepoxidated form of JHIII of insects), synthesised and secreted from the mandibular organ (MO), into haemolymph and involved in the regulation of gonadal maturation and moulting in decapods. The neurotransmitter 5-HT is released from the brain and thoracic ganglion of decapod crustaceans and involved in the regulation and release of endocrine factors, including vitellogenin hormones. Additionally, it can act as an intermediary substance that triggers the release of the red pigment-concentrating hormone from the neural tissue in the eyestalk. This in turn, could potentially accelerate the release of the putative gonad stimulating hormone (Fingerman, 1997; Kornthong et al., 2014; Meeratana et al., 2006; Tomy et al., 2016). 5-HT immunoreactivity was also found in crustacean gonads and its stimulatory roles on gonad maturation has been confirmed (Meeratana et al., 2006; Nakeim et al., 2020; Soonthornsumrith et al., 2018; Tinikul et al., 2011). This supports the theory of 5-HT acting through a receptor-mediated autocrine/paracrine mechanism to regulate the maturation of crustacean oocytes. Additionally, naloxone (an antagonist of enkephalin) appears to have greater potential in promoting ovarian growth and maturation in decapods (Cahansky et al., 2008). The ability of naloxone to induce spawning in other decapods including redclaw crayfish has not been previously reported. However, literature suggests that endogenous enkephalins hormones (methionine- and leucine- enkephalins) are functional in the thoracic ganglion of decapod crustaceans and exert an inhibitory effect on gonad stimulating hormone (Cahansky et al., 2008; Fingerman, 1997; Prasad et al., 2014). When naloxone are administered they are thought to antagonise the action of both methionine and leucine, thereby allowing the secretion of gonad stimulating hormones, which in turn stimulate ovarian maturation, enhance spawning and shorten the intervals to spawning (Cahansky et al., 2008; Fingerman, 1997; Prasad et al., 2014). While feeding redclaw females with MF during reproductive arrest period did not show any effect on reproduction (Abdu et al., 2001), injection of MF + 17β-oestradiol twice weekly for 3 weeks in red swamp crayfish *Procambarus clarkii* yielded significantly greater gonadosomatic index (GSI) than controls (Rodríguez et al., 2002). In the same species, injection of 5-HT increased ovarian index and oocyte size by 30% and 34%, respectively after 15 days. Injection of naloxone and 5-HT alone in female freshwater crab *Barytelphusa guerini* increased the ovarian index (Prasad et al., 2014). In *C. quadricarinatus* administration of naloxone orally in the diet, significantly increased mean oocyte diameter compared with experimental control animals (Cahansky et al., 2008). However, no studies on effects of parenteral administration of MF, 5-HT and naloxone on ovarian maturation and spawning in redclaw have yet been reported to our knowledge. The objective of this study was to induce synchronous gonadal maturation and spawning by intramuscular (IM) administration of hormones to female redclaw. Our hypothesis was that IM injection of MF, 5-HT and naloxone in females will increase the mean GSI, oocyte diameter, spawning rate, total number of eggs and decrease the mean interval to spawning compared to saline-treated Controls.

4.3 Materials and Methods

4.3.1 Experimental Animals

The study was carried out at the Australian Crayfish Hatchery, Townsville, Queensland in a tropical region of northern Australia (ACH; 19° 15' 28.656" S, 146° 43' 31.908" E). Experiments were undertaken during June and July 2022, which coincides with a normal period of reproductive quiescence in this species (Sagi et al., 1997). Ethics approval was not necessary for the execution of this study on crustaceans by the James Cook University Animal Ethics Committee. Before stocking, the gonadal maturation in females was assessed using a concentrated light source as previously described for redclaw (Jones, 1990), with all females used in the study being classified as being within the early ovarian developmental stage, no ovary discernible (Stage I - Immature). Briefly, females were taken into a dark room and ovarian development was determined by flashing a submersible light (10,000 Lumen) on both left and right sides and the ventral part of the carapace (Jones, 1990). The ovary development was measured based on the dark shadow on the carapace produced against flashed light. Redclaw (n = 168), with a mean ± SEM body weight (BW) of 65.6 ± 0.9 g were stocked in four, vertical recirculating aquaculture systems (RAS) with each system consisting of 42 individual compartments (45 cm length × 33 cm width × 25 cm height) arranged as six compartments horizontally and seven compartments vertically. Animals (n = 42/treatment) were treated with IM injections of either crayfish saline (Control), MF, 5-HT, or naloxone.

Redclaw were held at 26 ± 2 °C and 14L:10D photoperiod. Water quality (temperature, pH, dissolved oxygen, general hardness, ammonia, and nitrite) was monitored weekly and kept within the optimal range for redclaw. Animals were fed a combination of Entomix (ACH proprietary feed) and commercial spirulina pellets (multiple commercial sources) once daily in accordance with routine management within the hatchery for maintaining breeding and growing animals. Animals were observed once daily for recording mortality and moulting while spawning events were recorded every second day. Spawning was assessed by retrieving females using a hand net and visually observing the ventral surface of the abdomen and opening the curled tail to enable visualisation of eggs. The day of spawning was recorded as the day spawning was detected -1. Once spawned, the eggs were collected and counted with the aid of software (CountThings software, Dynamic Ventures Inc., Cupertino, CA). Eggs were collected from females using forceps by stripping into a container before being photographed for counting using an automated option within the software. Fecundity was measured as eggs/g of BW (Rodríguez-González et al., 2006a). After collection, eggs were treated in 70% ethanol for 1 min and then placed in an incubator and monitored until hatching occurred. The water chemistry for egg incubation was DO > 5 ppm, pH 7.5-8.5, temperature 26 ± 1 °C, ammonia <0.25 ppm, nitrite <0.25 ppm, nitrate 40-80 ppm, GH >50ppm, KH >50 ppm. Hatching was identified as the

complete emergence of juveniles from eggs (García-Guerrero et al., 2003). Hatching rate was calculated by the number of hatched juveniles/original egg number × 100.

4.3.2 Hormone Preparation and Application

A 1.2% crayfish saline solution was prepared by adding NaCl (12.27 g), KCl (0.402 g), $CaCl_2$ (1.47 g), $MgCl_2$ (0.487 g), and $NaHCO_3$ (0.168 g) in 1 L deionised water. After complete dissolution, pH of the solution was adjusted to 7.4 and stored in a refrigerator until use (Goldina, 2011). Methyl farnesoate (Cat # S-0153; Sapphire Bioscience Pty Ltd, NSW, Australia) was first dissolved in 100% ethanol and then diluted to a concentration of 5 mg/mL with crayfish saline solution (Van Harreveld, 1936). Separate stock solutions of 5-HT (Serotonin creatinine sulfate monohydrate; Cat # H7752; Merck Life Science Pty Ltd, Bayswater, VIC, Australia) and naloxone (Naloxone hydrochloride dihydrate; Juno Pharmaceuticals Pty Ltd, Cremorne, VIC, Australia) were prepared by dissolving 24 mg and 0.1 mL, respectively in 1 mL of crayfish saline solution.

Initially a pilot study was undertaken to test the effect of treatment on gonadal development and survival, since optimum dose rates in redclaw were unknown. All hormones were administered intramuscularly into the 3rd pair of abdominal segments using a 0.25 mm × 6 mm (31 G) needle (0.5 mL BD Ultra-Fine insulin Syringe, Becton Dickinson, San Diego, CA, USA). Crayfish (n = 6/treatment) were administered 100 µL of either isosmotic crayfish saline solution (Pilot Control), MF at either 4.3 × 10^{-2} or 8.3 × 10^{-2} µg/g crayfish on Days 0, 5, 10, 15 and 20 (Rodríguez et al., 2002); 5-HT at either 20.0 or 40.0 µg/g crayfish on Days 10, 15 and 20 (Kulkarni et al., 1992); or naloxone at either 3.3 × 10^{-1} or 6.7 × 10^{-1} µg/g crayfish on Days 10, 15 and 20 (Sarojini et al., 1996). Naloxone has previously been dose-optimised for another freshwater crustacean, *P. clarkii*, determining 1 × 10^{-6} mol/individual, followed by 1 × 10^{-7} mol/individual and 1 × 10^{-8} mol/individual (Sarojini, et.al., 1996). Given that both doses successfully induced maturation, in this study 1 × 10^{-7} mol/individual was chosen to maintain injection volumes in similar ranges to the other treatments to about 100 µL/crayfish.

In the pilot study, MF increased GSI at the highest dose 8.3 × 10^{-2} µg/g crayfish, thus this dose was chosen for subsequent experiments. In the case of 5-HT, an unanticipated reaction was observed in animals after administering both doses of 5-HT. This included cessation of movement and inability to ambulate, lateral recumbency and ventral curling of the tail. Therefore, a dose response study was carried out with 5-HT being administered at a dose of 6.3 × 10^{-1} µg/g crayfish, 1.3 µg/g crayfish and 1.7 µg/g crayfish. In this pilot study the greatest GSI was observed after administration of 1.3 µg/g crayfish without any abnormal reaction and so this dose was selected for the main study.

For the main study, crayfish (n = 42/treatment) were injected intramuscularly at the 3rd pair of abdominal segments with either crayfish saline (Control, 100 µL), or MF (8.3×10^{-2} µg/g crayfish) on days 0, 5, 10, 15 and 20, or 5-HT (1.3 µg/g crayfish) or naloxone (6.7×10^{-1} µg/g crayfish) on days 10, 15 and 20. A diagram of the treatment schedule is shown in Figure 4.1. On Day 25, 8 animals from each group with a weight range of between 41 to 86 g were randomly selected, killed by immersing them in an ice slurry at -20 °C for 15 mins as described by Vazquez et al (2008). Then males were paired to the remaining females at a sex-ratio of 1M:1F based on a maximum of 20 g difference in BW for 64 days.

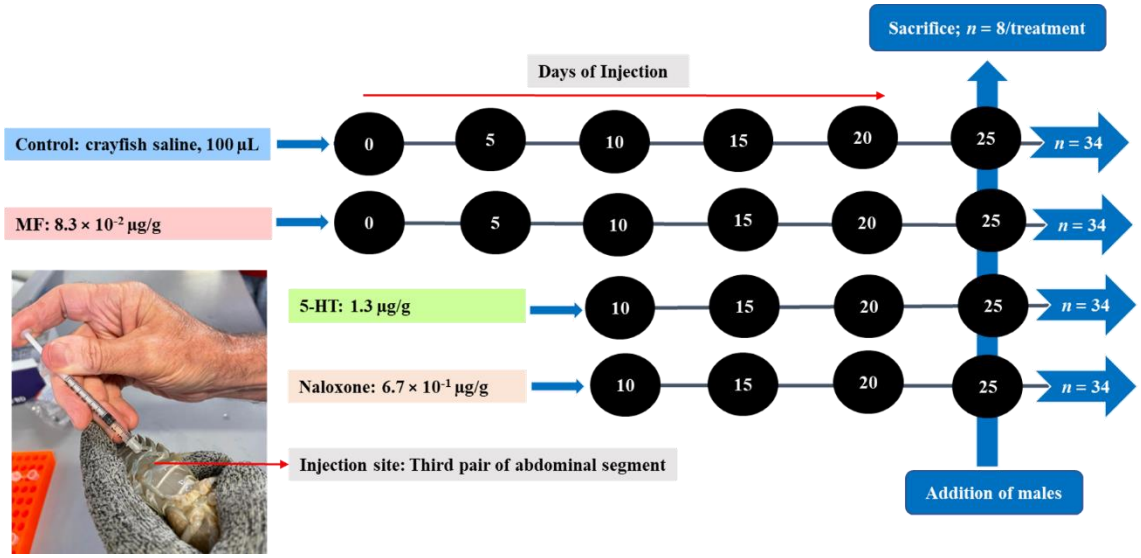


Figure 4.1. Hormone treatment schedule in redclaw, *Cherax quadricarinatus*. Treatments were administered on each day indicated between Days 0 and 20 for crayfish treated with saline or MF and between Day 10 to 20 for crayfish treated with 5-HT or naloxone.

4.3.3 Determination of Gonadosomatic Index, Oocyte Diameter and Histological Preparation

Following euthanasia, crayfish were weighed and the ovaries were removed, weighed and immersed in Davidsons solution (Rodríguez-González et al., 2006b). The GSI was measured using the following equation.

$$GSI = \left(\frac{gonad\ weight}{total\ tissue\ weight}\right) \times 100$$

Ovaries were processed for histological assessment after fixing for a minimum of 48 h and immersion in a series of ethanol concentrations before being embedded in paraffin (Vazquez et al., 2008). A minimum of five microscope slides per histology specimen were prepared and each slide contained three consecutive ovary slices, each 3 µm thick collected from the middle part of the ovary from each female. Sections were subsequently stained with haematoxylin and eosin. Slides were examined in sequential order of collection under light microscopy. Oocytes with a visible complete nucleus were selected for measurement, and ImageJ software was used

to compute the average of two measurements taken perpendicularly to each other in order to determine the diameter of the oocytes (Schneider et al., 2012). For each crayfish, the diameter of at least 30 oocytes were measured and mean oocyte diameter was calculated. The stage of development of the ovary was based on oocyte size as described by Abdu et al. (2000).

4.3.4 Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics version 27 for Windows (SPSS Inc., Armonk, NY, USA). Proportional responses were compared using a Pearson's Chi-square test. Logistic regression and the Wald F test statistic were used to determine the effect of treatment on the odds ratios (ORs) and their 95% CIs for spawning, moulting and survival. Body weight and its interaction with the dependent variable were initially included in the model but removed from the final model, using backwards stepwise regression when $p \geq 0.10$. If an interaction was significant at $p < 0.10$ it was retained in the model. Goodness of fit of the models was assessed using the Hosmer and Lemeshow test. Probability values for all main effects remaining in models were determined using the approximate Chi-Squared distribution of the Wald statistic. Odds ratios with 95% confidence intervals were also calculated for all main effects retained in the models.

Differences between means were compared using analysis of covariance with the initial BW entered as a covariate but this was subsequently removed if the effect of BW was not significant *(*$p > 0.05$). Cox regression was used to model the times to spawning, moulting or death of crayfish and to assess the effects of treatment and initial BW on these variables. Using R version is 4.3.2 for Windows (R Core Team, 2023), a multiple comparison of spawning (%), mean number of eggs/females, fecundity, and days to hatching was performed.

4.4 Results

4.4.1 Gonadosomatic Index and Oocyte Diameter

The mean GSI and diameter of oocytes both differed between treatments ($p < 0.01$; Table 4.1) with both variables being larger in magnitude in animals treated with 5-HT and naloxone compared to Control and MF treated crayfish. However, there was no significant difference between 5-HT and naloxone or Control and MF treatments. Representative images of ovarian tissue for each of the four treatments are shown in Figure 4.2.

Table 4.1: Reproductive characteristics of redclaw (n = 34) after parenteral administration of treatments.

Parameters	**Control**	**MF**	**5-HT**	**Naloxone**	***p*-value**
GSI	$4.1^{b} \pm 0.3$	$4.1^{b} \pm 0.4$	$5.3^{a} \pm 0.1$	$5.2^{a} \pm 0.2$	0.002
Oocyte diameter (µm)	$972.5^{b} \pm 26.9$	$940.3^{b} \pm 75.8$	$1822.8^{a} \pm 58.9$	$1927.3^{a} \pm 84.9$	<0.001
Survival (%)	85.3^{a} (29)	58.8^{b} (20)	88.2^{a} (30)	82.4^{a} (28)	<0.001
Spawning (%)	20.6^{b} (7)	17.6^{b} (6)	52.9^{a} (18)	50.0^{a} (17)	0.002
Days to spawning (D)	$57.6^{a} \pm 1.62$	$60.3^{a} \pm 1.48$	$50.9^{b} \pm 1.16$	$50.1^{b} \pm 1.38$	0.009
Eggs/female	$465.0^{c} \pm 36.5$	$453.2^{c} \pm 17.7$	$600.4^{a} \pm 34.0$	$550.6^{b} \pm 17.9$	<0.001
Fecundity (eggs/g female)	$7.0^{c} \pm 0.2$	$6.3^{c} \pm 0.1$	$9.2^{a} \pm 0.1$	$8.2^{b} \pm 0.2$	<0.001
Moulting (%)	14.7^{b} (5)	50.0^{a} (17)	8.8^{b} (3)	11.8^{b} (4)	<0.001
Days to moulting (D)	$43.6^{a} \pm 6.5$	$24.9^{b} \pm 1.2$	$46.7^{a} \pm 2.9$	$54.5^{a} \pm 1.9$	<0.001
Hatching (%)	$77.2^{b} \pm 1.8$	$65.2^{c} \pm 5.1$	$87.4^{a} \pm 1.0$	$85.7^{a} \pm 1.2$	<0.001
Days to hatch (D)	$30.1^{ab} \pm 0.2$	$30.7^{a} \pm 0.2$	$29.2^{c} \pm 0.2$	$29.4^{bc} \pm 0.2$	<0.001

^{abc} Numbers with different superscripts in the same row differ significantly (p<0.05)

Values within bracket indicate number of crayfish that survived, spawned, or moulted for respective parameters.

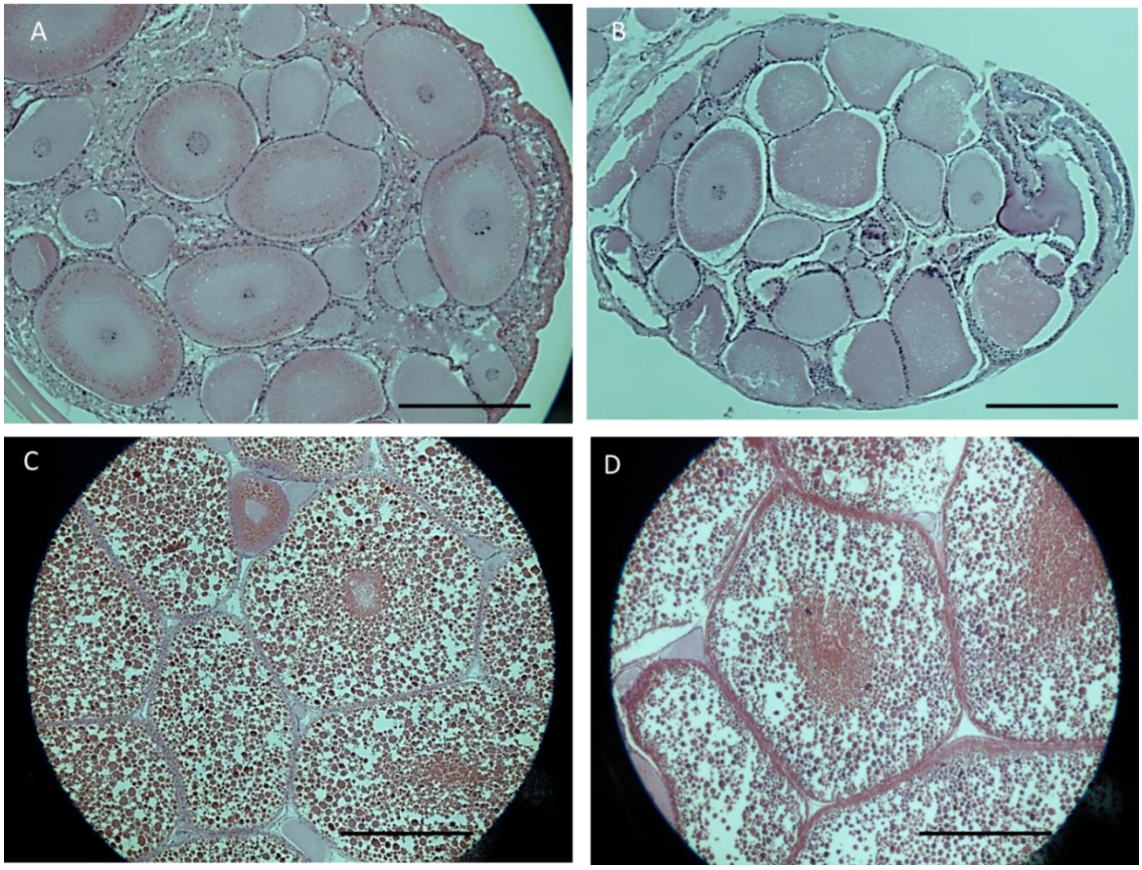


Figure 4.2. Representative haematoxylin- and eosin-stained histological sections of redclaw, *Cherax quadricarinatus*, ovaries on Day 25 of the study stained following treatment with A) crayfish saline (control), B) MF, C) 5-HT or D) naloxone. Scale bar (▬▬) 0.5mm.

4.4.2 Survival

The survival was similar for Control animals and for those treated with 5-HT and naloxone, but the animals treated with MF had a significantly lower survival rate (Table 4.1 and Table 4.2). The survival distribution for the MF treated crayfish significantly differed from the Control, with mortality occurring earlier and to a greater degree (Table 4.3 and Figure 4.3). A significant interaction was also found between treatment and initial BW with smaller crayfish treated with MF tending to die sooner (p<0.05; Table 4.3). However, the effect of BW on the probability of death was not apparent in animals in the Control, 5-HT, or naloxone treatments (Table 4.3).

Table 4.2: Logistic regression results showing log-transformed odds ratio estimates for effects of treatment relative to Control on the proportion of animals spawning, moulting, and surviving.

Parameters	**Treatment**	**B**	**SE**	**Chi-Square (Wald)**	**df**	**Odds ratio**	**95% CI**	***p***	**Reference group**
Spawning	MF	-0.19	0.62	0.09	1	0.83	0.25 - 2.78	0.758	Control
	5-HT	1.47	0.55	7.23	1	4.34	1.49 - 12.77	0.007	Control
	Naloxone	1.35	0.55	6.13	1	3.86	1.32 - 11.24	0.013	Control
Moulting	MF	1.76	0.59	8.78	1	5.80	1.81 - 18.56	0.003	Control
	5-HT	-0.58	0.78	0.56	1	0.56	0.12 - 2.56	0.456	Control
	Naloxone	-0.22	0.72	0.09	1	0.80	0.20 - 3.28	0.721	Control
Survival	MF	-10.30	4.71	4.83	1	0.001	0.00 - 0.33	0.028	Control
	5-HT	-4.70	4.23	1.20	1	0.009	0.00 - 40.71	0.273	Control
	Naloxone	0.26	4.73	0.00	1	1.30	0.00 - 13.80 × 10^3	0.956	Control
	Treatment * BW								
	BW × MF	0.13	0.07	3.84	1	1.14	1.00 - 1.31	0.050	Control
	BW × 5-HT	0.07	0.06	1.41	1	1.08	0.95 - 1.21	0.235	Control
	BW × Naloxone	-0.01	0.07	0.01	1	0.99	0.87 - 1.13	0.912	Control

BW = Initial body weight, MF = Methyl farnesoate, 5-HT = 5-hydroxy tryptamine.

Table 4.3: Cox regression for analysis of odds ratio estimates for effects of treatment on spawning, moulting and survival distributions.

Parameters	**Treatment**	**B**	**SE**	**Chi-Square (Wald)**	**df**	**Odds ratio**	**95% CI**	***p***	**Reference group**
Spawning	MF	-0.19	0.56	0.11	1	0.83	0.30 – 2.50	0.739	Control
	5-HT	1.26	0.45	7.96	1	3.52	1.40 – 8.50	0.005	Control
	Naloxone	1.20	0.45	7.17	1	3.33	1.40 – 8.10	0.007	Control
Moulting	MF	1.60	0.51	9.83	1	4.96	1.80 – 13.50	0.002	Control
	5-HT	-0.54	0.73	0.54	1	0.58	0.14 – 2.44	0.461	Control
	Naloxone	-0.27	0.67	0.16	1	0.76	0.21 – 2.85	0.688	Control
Survival	MF	10.32	4.08	6.42	1	3467.90	$10.30 – 89.60 \times 10^{6}$	0.011	Control
	5-HT	4.13	3.89	1.12	1	61.90	$0.03 – 12.84 \times 10^{4}$	0.290	Control
	Naloxone	0.29	4.13	0.00	1	1.33	$0.00 – 43.41 \times 10^{2}$	0.945	Control
	Treatment * BW								
	BW × MF	-0.14	0.06	5.19	1	0.87	0.78 – 0.98	0.023	Control
	BW × 5-HT	-0.06	0.06	1.34	1	0.94	0.84 – 1.05	0.248	Control
	BW × Naloxone	-0.00	0.06	0.00	1	0.99	0.89 – 1.11	0.974	Control

BW = Initial body weight, MF = Methyl farnesoate, 5-HT = 5-hydroxy tryptamine.

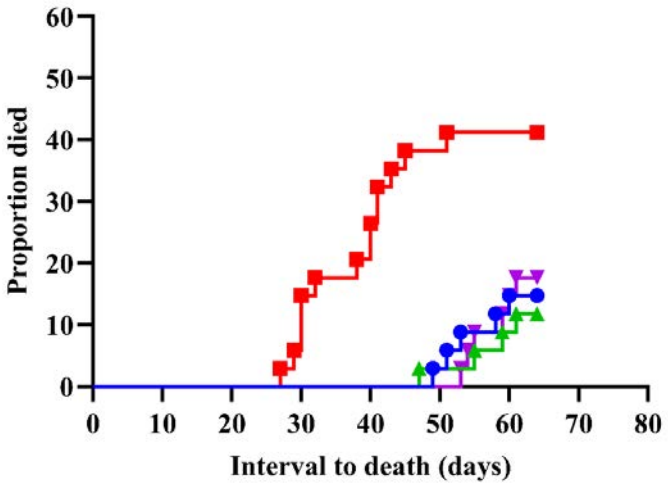


Figure 4.3. Distribution of the proportion of crayfish that died during the study after treatment with crayfish saline (control, ●), MF (■), 5-HT (▲) or naloxone (▼).

4.4.3 Spawning

Spawning success was significantly greater in 5-HT- and naloxone-treated crayfish compared to Control and MF (Table 4.1), with logistic regression models suggesting that spawning was 4.34 and 3.86 times more likely for these treatments on any given day, respectively (Table 4.2). When a multiple comparisons test was applied to spawning rates, there was no difference in spawning rate between the Control and MF treated crayfish and those treated with 5-HT and naloxone, but it was significantly greater in the 5-HT compared to the Control and MF treated crayfish and tended to be greater in the naloxone compared to the Control treated crayfish ($p=0.063$; Table 4.1). Figure 4.4 illustrates spawning in the four different treatments. The 5-HT and naloxone treated crayfish ($p<0.01$, Table 4.3) spawned earlier and to a greater degree than animals treated with MF or saline, which did not differ (Table 4.3).

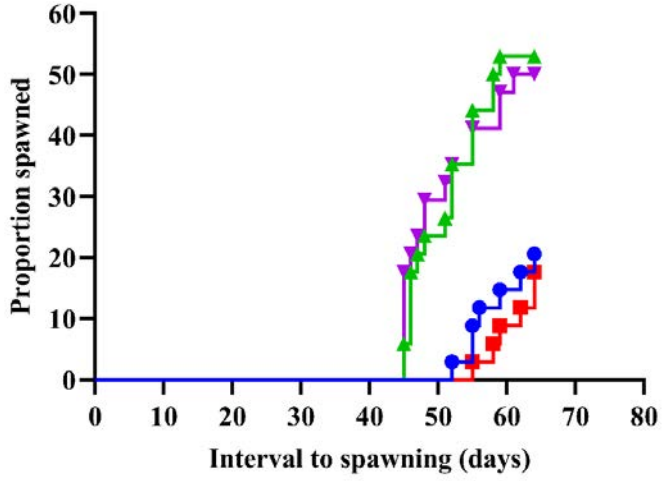


Figure 4.4. Distribution of the proportion of crayfish that spawned during the study after treatment with crayfish saline (control, ●), MF (■), 5-HT (▲) or naloxone (▼).

4.4.4 Egg Production and Fecundity

The mean number of eggs/female and fecundity were significantly greater in the animals treated with 5-HT than all other treatments, with naloxone subsequently having significantly more eggs and greater fecundity than Control and MF-treated animals (Table 4.1). An effect of BW was also observed on the mean number of eggs/female when BW was entered as a covariate (p<0.001). BW was positively associated with the mean number of eggs produced per female (Figure 4.5; p<0.001).

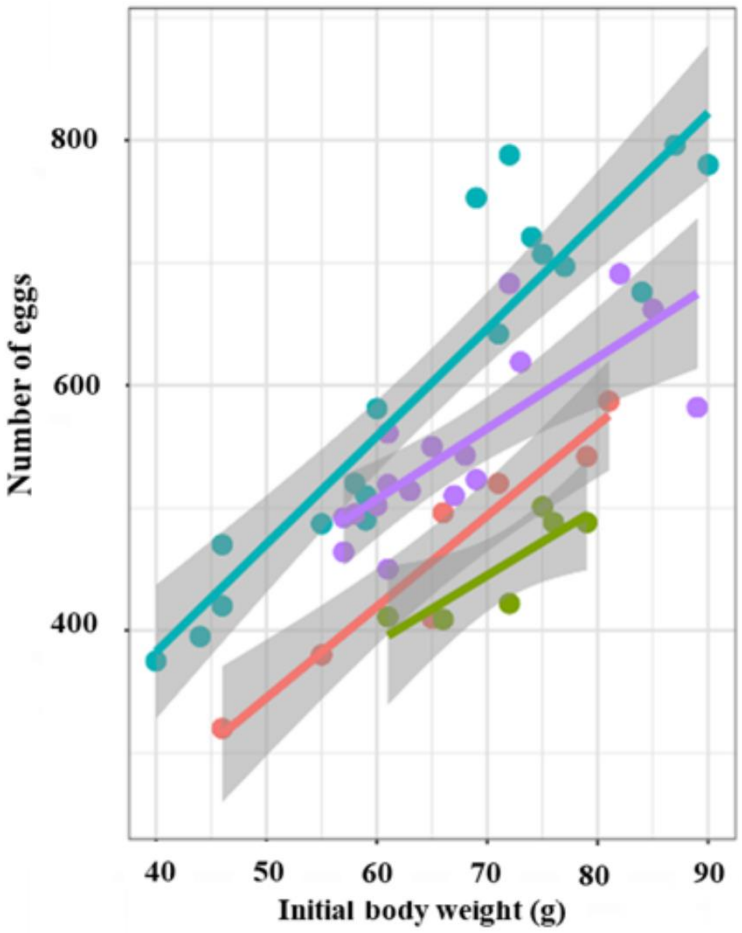


Figure 4.5. The relationship between initial body weight and the mean number of eggs produced per female separated by treatment (control,) MF ( ), 5-HT ( ) or naloxone ( ).

4.4.5 Moulting

Moulting occurred significantly more often in MF-treated crayfish than all other treatments (Table 4.1 and Table 4.2). Figure 4.6 illustrates moulting in the four treatments. The mean days to moulting were similar between the Control animals and those treated with 5-HT and naloxone, but the animals treated with MF moulted earlier than all other treatments (Table 4.1, Table 4.3 and Figure 4.6).

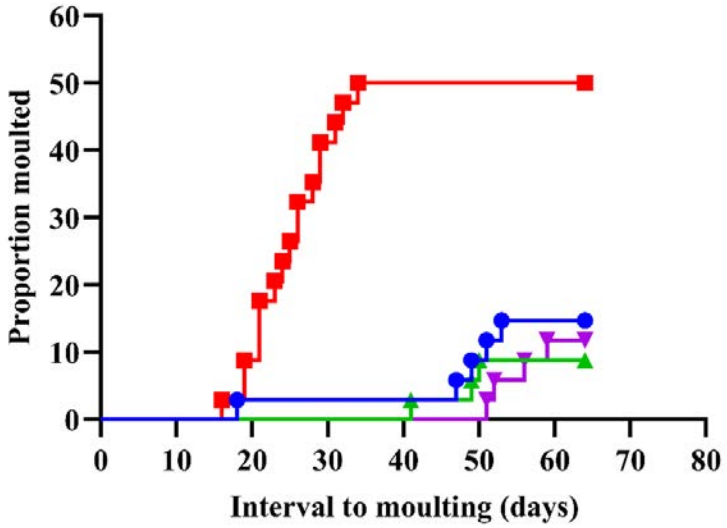


Figure 4.6. Distribution of the proportion of crayfish that moulted during the study for redclaw that were treated with crayfish saline (control, ●), MF (■), 5-HT (▲) and Naloxone (▼).

4.4.6 Hatching

The hatching rate was significantly greater in crayfish treated with 5-HT and naloxone compared to those treated with the saline and MF treatments. The hatching rate was also less in the MF-treated animals compared to Control animals (Table 4.1). A small but significant effect of treatment was also observed on the mean days to hatching, with embryos from 5-HT-treated animals taking slightly less time compared to the Control and MF-treated animals (Table 4.1). An effect of BW was also observed on the mean days to hatching when BW was entered as a covariate (p<0.001). BW was positively but weakly associated with the mean interval to hatching for 5-HT and naloxone treated crayfish (Figure 4.7; $r^2 = 0.26$, p<0.001).

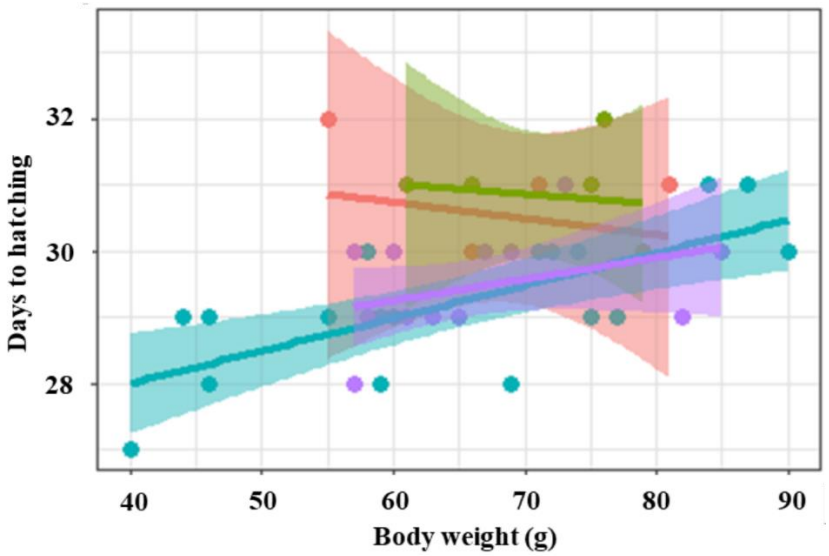


Figure 4.7. The relationship between initial body weight and the days to hatching separated by treatment (control, ●) MF (●), 5-HT (●) or Naloxone (●).

4.5 Discussion

This study evaluated the effects of IM injection of several commercially available maturation-inducing hormones on the reproductive capacity of female redclaw. 5-HT and naloxone both shortened the maturation period and significantly increased GSI, oocyte diameter, spawning (%), eggs/female, fecundity, and hatching (%) compared to the saline Control suggesting that both hormones can enhance the efficiency of juvenile production in redclaw. MF-treated redclaw moulted more frequently, but this came at the expense of reduced survival and poorer gonad development.

In the present study, treatment with 5-HT and naloxone resulted in greater ovarian maturation as evidenced by greater GSI and oocyte diameter while treatment with MF did not significantly affect any of the reproductive variables assessed compared to the Control treated crayfish and reduced the survival of crayfish over the course of the study. In common with the results found in this study, treatment with 5-HT or naloxone have been shown to increase the ovarian index in other decapod crustaceans including freshwater crayfish, prawns and crabs (Cahansky et al., 2008; Kulkarni et al., 1992; Meeratana et al., 2006; Prasad et al., 2014). In freshwater crayfish *P. clarkii*, parenteral administration of 5-HT at a dose rate of 15 µg/g BW resulted in a significant increase in the ovarian index (30.5%) and oocyte diameter (34.0%) compared to concurrent controls (Kulkarni et al., 1992). When the freshwater prawn *Macrobrachium rosenbergii*, was injected with a lower dose of 5-HT (1 µg/g BW) than was used in this study, they exhibited a significant increase in mean ± SEM ovarian index of 5.8 ± 0.1% compared to 1.6 ± 0.3% among control animals (Meeratana et al., 2006). In that study, the ovaries of 80% of 5-HT-treated females had synchronous development of mature oocytes, while the ovaries of 90% of controls had contained only pre-vitellogenic or early vitellogenic oocytes. In the same species, IM injections of 5-HT alone or in combination with spiperone every 4 days significantly increased the GSI and diameter of oocytes on days 14, 25 and 49 compared to the vehicle injected or untreated control animals (Tinikul et al., 2009). In Indian prawn *Fenneropenaeus indicus,* a 2.5×10^{-6} mol/g BW injection of 5-HT stimulated a mean increase in ovarian index of 15.9% and oocyte diameter of 23.3% compared to control-treated animals (Santhoshi et al., 2009). In this study, redclaw injected with 1.3 µg/g 5-HT exhibited an increase of 28.9% in GSI and 87.4% in oocyte diameter indicating that 5-HT plays a stimulatory role in enhancing ovarian maturation.

In the current study, 5-HT or naloxone treated crayfish exhibited oocytes with mean diameters of 1800 µm and 1900 µm, respectively, which is close to the diameter reported for oocytes classified as reaching the mature stage of development (Abdu et al., 2000). The histological appearance was also consistent with mature-stage oocytes with irregular nuclei and indistinct nuclear membrane which occurs in crayfish that are ready to spawn synchronously (Abdu et al.,

2000). The stimulatory effect of treatment with naloxone on oocyte maturation observed in this study is consistent with effects observed when naloxone treated pellets were fed twice a week at 1×10^{-9} mol/g BW to redclaw for 13 weeks throughout the post-reproductive period which resulted in significantly larger oocytes at the near-mature secondary vitellogenic stage compared to control treatments (Cahansky et al., 2008). In *P. clarkii*, injections of naloxone at doses of 1×10^{-8}, 1×10^{-7} and 1×10^{-6} mol per crayfish on the Day l, 5 and 10 progressively increased the ovarian index in a dose dependent manner compared to controls treated with physiological saline (Sarojini et al., 1996). In the freshwater crab, *B. guerini*, injection of either 5-HT or naloxone alone at doses of either 1×10^{-10}, 1×10^{-9} or 1×10^{-8} mol in a 100 µL volume per crab on Day 1, 5, 10 and 15 significantly increased the ovarian index and oocyte diameter in a dose dependent manner compared to the control animals (Prasad et al., 2014). In the present investigation, redclaw that received injections of 6.7×10^{-1} µg/g naloxone showed an increase in GSI of 26.0% and an increase in oocyte diameter of 98.2%, demonstrating that naloxone serves as a stimulant in promoting ovarian maturation.

In the present study, in keeping with the stimulatory effect observed on ovarian development and maturation, 5-HT or naloxone treatment resulted in significantly greater spawning rates and shorter intervals to spawning compared to MF and saline-treated females. Similarly, spawning rates were increased in shrimp following treatment with 5-HT. For example, in Pacific white shrimp *Penaeus vannamei*, administration of 50 µg/g and 15 µg/g BW of 5-HT induced significantly greater spawning rates (mean ± standard error) of 35.4 ± 1.8% and 19.5 ± 14.6%, respectively compared to 6.1 ± 10.5% in control treated shrimps (Vaca and Alfaro, 2000). In the wild *Litopenaeus stylirostris* and the pond-grown *Litopenaeus vannamei*, combined injection of 5-HT at 25 µg/g BW plus spiperone either at 1.5 or 5 µg/g BW induced ovarian maturation and spawning at rates similar to those obtained with eyestalk ablation (Alfaro et al., 2004). Spawning was also induced in *Penaeus semisulcatus* by injecting 50 and 100 µg/g of 5-HT (Aktaş and Kumlu, 2005).

The results of the current study indicated that unlike 5-HT or naloxone, administration of MF to adult redclaw females did not appear to improve GSI or rates of spawning. Several studies documented the antagonistic role of MF on reproduction. For example, when MF was administered to females of different species of decapod crustaceans such as *C. quadricarinatus*, *Litopenaeus stylirostris* and *Litopenaeus vannamei* no significant effects on ovarian maturation were observed (Abdu et al., 2001; Alfaro et al., 2004; Marsden et al., 2008). Oral administration of MF inhibited ovarian development at later stages and reduced fecundity in the black tiger prawn, *Penaeus monodon* (Marsden et al., 2008). In juvenile tadpole shrimp, *Triops longicaudatus*, MF significantly decreased the number of growing oocytes when administered with diet repeatedly (Tsukimura et al., 2006). In the current study, MF significantly increased

the moulting, mortality rates and shortened the interval to moulting. In the haemolymph of *M. rosenbergii*, the concentrations of MF rose at the premoult stage and declined after moulting which mimics changes in concentrations of ecdysteroids known to influence moulting in insects (Laufer et al., 2005; Wilder et al., 1995). Injection of MF into female crabs, *Oziotelphusa senex senex* stimulated up to 80% of the crabs to moult (Reddy et al., 2004). The potential role of MF in stimulating moulting has also been reported previously in redclaw, where immersion in 4.5 µg or 9 µg/female/week of MF resulted in a moulting rate of 100%, but a survival rate of only 46.7% at the highest dose (Abdu et al., 2001). These reports suggest that moulting is not necessarily linked to increased maturation and spawning success (Nagaraju, 2007).

Research on the signalling pathways of MF on regulating reproduction and moulting in crustaceans is elusive. After being released from the MO, MF is delivered to target tissues by MF-binding proteins via haemolymph (Takáč et al., 1998). Unlike insects, crustaceans lack haemolymph esterases that breakdown MF, and the half-life of MF in haemolymph seems to be relatively short (approx. 45 min), which is likely due to its adsorption by cellular membranes that might affect the concentration of MF required to stimulate ovarian maturation (Borst, 2003). Furthermore, it is possible that the inhibition of ovarian maturation in our study occurred via the stimulation of ecdysis as demonstrated by greater moulting rates. This effect appears similar to the action of ecdysteroids that are secreted from the Y organ and influence moulting in many decapod crustaceans including *Callinectes sapidus*, *Cancer magister*, *Carcinus maenas*, *Homarus americanus*, *Hyas araneus*, *Metopograpsus messor*, and *P. clarkii* (Nagaraju et al., 2006; Reddy et al., 2004; Sainath and Reddy, 2010; Techa and Chung, 2015). For instance, *in vitro* incubation of the Y-organ of the crab, *C. magister* along in MF for 24 h caused significantly greater secretion of ecdysteroids in the medium, compared to controls and other treatments, with secretion increased at increasing MF concentration (Tamone and Chang, 1993). This finding suggest that the MF might play a role in triggering a premature moult by stimulating the *in vivo* secretion of ecdysteroids from the Y-organ. Additionally, injection of homogenates of the MO from *C. sapidus* into the white shrimp, *Penaeus setiferus*, resulted in accelerated moulting (Yudin et al., 1980). A significant increase in moulting rate and a reduction in the interval to moulting observed in this study may be the result of an MF-induced increase in ecdysteroids secretion, which led to frequent moulting in crayfish within a shorter period. Moreover, MF induced increased mortality in Kuruma prawn, *Marsupenaeus japonicus* (Toyota et al., 2020) and redclaw *C. quadricarinatus* (Abdu et al., 2001). Considering a greater mortality rate in the current study it could be concluded that, administration of MF at the dose administered could have lethal effects on reproductive females. It may be that MF either has its own signalling pathway or it might share the signalling pathway with ecdysone in stimulating

moulting, although further investigation will be needed to elaborate its mechanism of action in redclaw.

In the current study, the quality of spawns was evaluated in terms of the number of eggs per female, fecundity (number of eggs/g BW of female) and hatching rate. Crayfish treated with 5-HT or naloxone produced a significantly greater number of eggs/female and fecundity was greater compared to the MF and Control treated animals. However, 5-HT appeared to be more effective than naloxone in enhancing reproduction as it produced significantly more eggs and had higher fecundity. While 5-HT exerts its stimulatory role directly on ovarian maturation and development of oocytes, naloxone functions by reversing the effects of enkephalins after binding with it (Fingerman, 1997; Prasad et al., 2014). Moreover, 5-HT might play a positive role in ovarian muscle contraction that contributes to ovulation and eventually greater spawning in redclaw. In American lobster *H. americanus* ovaries, 5-HT induced muscle contraction that contributed to extrude oocytes during spawning (Howard and Talbot, 1992). Therefore, the mode of action and discreet timing of 5-HT on ovarian muscle contraction requires further elucidation. Additionally, a combined study of 5-HT and naloxone could be conducted to relate how both interact in the ovarian maturation pathway to putatively enhance follicle recruitment, synchronise ovulation and timing of egg release in redclaw for selective breeding. Moreover, synergistic investigation could also reveal whether naloxone can stimulate the secretion of endogenous 5-HT or gonad stimulating hormone and can further contribute to the ovarian maturation and spawning.

In this study, BW was positively correlated with the number of eggs produced. Given that dosage was adjusted per unit of BW it may indicate a more advanced stage of sexual development and/or more body reserves being available for reproduction with increasing BW. In decapod crustaceans such as redclaw, *C. quadricarinatus*, blue swimming crab, *Portunus pelagicus*, freshwater caridean shrimp, *Neocaridina davidi*, total number of eggs produced were observed to increase with increasing female BW (Masser and Rouse, 1997; Tropea et al., 2019; Zairion et al., 2015). However, the results of the present study are limited because of the limited data and narrow range of BWs of crayfish assessed. To justify the appropriate association between BW and the number of eggs produced effects of treatment on a greater number of animals with a broader range of BWs should be evaluated.

Hatching rate was significantly greater in 5-HT- or naloxone-treated crayfish compared to those MF and Control-treated groups. In *P. semisulcatus*, administration of 5-HT successfully increased spawning, hatching rate and nauplii production (Aktaş and Kumlu, 2005). In *P. monodon*, treatment with 50 µg/g BW of 5-HT compared to eyestalk ablation resulted in a significantly greater mean ± standard deviation hatching rate (81.7% ± 5.4 *vs*. 65.5% ± 14.5) and nauplii production (83,993 ± 8,310 vs 71,344 ± 16,621), respectively (Wongprasert et al.,

2006). There is currently no report describing hatching rate or juvenile production following naloxone treatment in crustaceans including redclaw. Mean hatching rate in MF treated animals was significantly less than all other treatments including Controls, which suggests that MF could have a negative impact on broodstock and egg quality. Synthetic analogues of juvenile hormone are used as insecticides and so perhaps this may have had a negative effect on the survival of developing embryos (Hu et al., 2019; Hu et al., 2020b). The reasons for lesser hatching rates are unknown and will require further investigation but could be due to asynchronous ovulation in response to MF treatment or with endocrine disruption, nutritional imbalance, or loss of DNA integrity in developing embryos.

In the current study, the mean interval from spawning to hatching was slightly less in crayfish treated with 5-HT compared to the Control and MF treated animals. Nevertheless, the magnitude of difference was less than one day. While BW was found to be positively associated with the mean interval to hatching fewer Control and MF treated animals spawned and the interval to hatching could only be examined across a relatively narrow range of BWs in animals in these treatments compared to 5-HT treated animals. Therefore, further study involving the effects of treatment on a broader range of BWs is required to verify this relationship. In freshwater molluscs, 5-HT in a receptor-independent signalling pathway activates intracellular mechanisms that link environmental cues acquired by an organism which can affect the rate of progeny development, hatching time, and the behavioural traits of juveniles (Voronezhskaya, 2021). Although the impact of 5-HT treatment on the interval to hatching has not been reported in decapods, the results of this study suggest that treatment with 5-HT has the greatest potential to induce early ovulation with complete oocyte maturation, perhaps in response to stimulating the release of ovary stimulating hormone (Fingerman, 1997; Kulkarni et al., 1992). This in turn increased the rate of embryonic development and shortened the period from spawning to hatching. Further studies are needed to determine the physiological cause of a shorter interval from spawning to hatching after treatment with 5-HT and whether the reduction in this interval reported in this study is consistent, will affect the growth and survival of juveniles and be economically beneficial in commercial settings.

In this study treatments were restricted to specific dose rates, treatment regimes, and IM administration of hormones. Future studies could be conducted including dose response trials and measurement of physiological concentrations of 5-HT and naloxone in crayfish haemolymph to determine optimal doses and tissue concentrations needed to enhance reproduction. Alterations to the frequency of administration and the route of administration could also be investigated to determine if there is a more optimal and practical way of administering treatments. Effects of treatments on total protein, amino acid, and fatty acid profiles in the hepatopancreas might also provide information on factors that may affect gonadal

function and egg quality. Furthermore, studies could be undertaken to examine whether 5-HT and naloxone stimulate the release of pheromones into the water which could play a role in early induction of gonadal maturation and spawning (Alfaro et al., 2004). Sex dependent effect of MF, 5-HT and naloxone could also be studied in redclaw as demonstrated by 5-HT in narrow clawed crayfish *Pontastacus leptodactylus* (Farhadi et al., 2020). The pathways through which MF, 5-HT and naloxone employ their influence on gonadal maturation remain to be fully determined. More research using RNA interference, recombinant protein technology, and gene editing may help us to better understand the endocrine regulation of reproduction in crustaceans.

4.6 Conclusions

In summary, 5-HT and naloxone treatment induced greater spawning rates and greater hatching rates in redclaw, which suggest that both hormones have potential to promote gonadal maturation in females and enhance production of higher quality eggs. However, with better egg production, fecundity and slightly shorter time to hatching, 5-HT hormone treatment appears the most promising candidate to enhance juvenile redclaw production for grow-out and contribute to further intensification of redclaw industry. By contrast, parenteral treatment with MF did not increase female spawning but induce greater rates of moulting and mortality, suggesting that it might have a lethal effect on broodstock. However, further research looking whether 5-HT + naloxone act synergistically to improve female redclaw fertility is warranted. So too are longitudinal studies examining juvenile survival and reproductive output of females over time across multiple spawning events. This will help determine if benefits of 5-HT or naloxone can be sustained progressively with and without multiple treatment episodes.

Chapter 5. Formulation of Practical Diet to Induce Spawning Condition and Improve Egg and Embryo Quality in Redclaw, *Cherax quadricarinatus*

5.1 Abstract

Redclaw is a crustacean species with vast potential as a high quality and valuable product that can be cost-effectively grown, however its production is currently limited by hatchery production volumes. Reproductive outputs may be influenced by nutritional inputs, with astaxanthin and cholesterol likely candidates for reproductive improvement. Therefore, the present study examined the effects of nutritional supplementation with either cholesterol (CHOL; 1 g/kg) and/or astaxanthin (AX; 100 mg/kg) for 75 days on ovarian maturation, the frequency of spawning and egg and embryo quality in redclaw crayfish in a 2 × 2 factorial design. The crayfish were housed in 100 L circular tanks, with 6 replicate tanks for each of the four dietary treatments, each containing 7 females (average body weight 61.5 g) in recirculating aquaculture systems. Both AX and AX + CHOL supplementation increased ovarian maturation compared to Control and CHOL supplementation alone as evidenced by elevated GSI, and lower HSI. Additionally, the interval to spawning was less (p<0.05) in crayfish supplemented with AX and AX + CHOL. Moreover, AX supplementation alone resulted in an elevated spawning rate (63.9%, 22.2%, 38.9% and 30.6%, p=0.005), the mean ± SEM fecundity (10.0 ± 0.13, 7.2 ± 0.36, 8.2 ± 0.16 and 7.7 ± 0.34 eggs/g female, p<0.001), number of eggs/female (602.0 ± 26.5, 472.4 ± 40.8, 524.1 ± 33.3 and 465.1 ± 26.9 eggs/female, p<0.001), hatching rate (82.4 ± 1.3, 76.4 ± 3.3, 74.7 ± 1.9 and 74.3 ± 1.8%, p=0.003) and number of hatched juveniles (504.1 ± 24.8, 318.6 ± 39.2, 404.6 ± 29.2 and 328.7 ± 31.9, p<0.001) compared to CHOL, AX + CHOL, and Control fed crayfish, respectively. Female crayfish supplemented with CHOL had significantly higher moulting rates (52.8%, 16.7%, 22.2% and 24.9%, p=0.003) compared to AX, AX + CHOL supplementation and Control diets, respectively. We conclude that a dietary supplementation of 100 mg/kg AX improved the reproductive characteristics in female redclaw, while CHOL supplementation increased moulting rate without improving reproductive output.

5.2 Introduction

Redclaw, *C. quadricarinatus* is a high quality and economically produced freshwater crustacean aquaculture species with global scope. However, the expansion of redclaw farming is currently limited by inconsistent and variable quality of juvenile production (Rigg et al., 2021; Valverde. et al., 2020) necessitating greater understanding of conditions that enhance quantity and quality of eggs. Enhancing reproductive performance of female redclaw is one potential strategy to overcome this impediment to further expansion. Gonadal maturation and reproduction in crustaceans is an energy and nutrient-demanding process because females transfer considerable energy and nutrient reserves to eggs in order to nourish the developing pre-hatch embryos after fertilisation (Hernández-Abad et al., 2018). Nutritional deficits can lead to poor egg quality, which can impact hatchability, larval survival rates, and overall reproductive performance. A range of dietary nutrients influence reproductive performance of aquatic animals. Among them, cholesterol and astaxanthin have been found to enhance ovarian maturation, spawning rate, and fecundity in decapods (Barım-Öz and Şahin, 2016; Niu et al., 2014).

Cholesterol, a non-polar lipid precursor of ovarian steroid hormones such as estrogens, androgens and progestins (Rimon-Dahari et al., 2016), which are involved in reproduction and moulting in crustaceans (Kumar et al., 2018; Mykles, 2011). Additionally, cholesterol strengthens cellular integrity against environmental anomalies like heat or salinity stress (Yang et al., 2016). In decapod crustaceans, dietary cholesterol is accumulated as cholesteryl esters which are transported throughout the body via lipoproteins in hemolymph. It contributes to physiological processes such as lipid absorption, steroid hormone synthesis, growth, moulting performance and reproduction (Kumar et al., 2018; Tian et al., 2020). Unlike vertebrates, crustaceans cannot synthesise cholesterol and rely on dietary sources to maintain growth and development (Kumar et al., 2018b). The optimal levels of dietary cholesterol for crustaceans range between 0.2% and 2.0%, varying with species, age, body weight and physiological state (Kumar et al., 2018b). Dietary cholesterol accumulates in developing ovaries of *Penaeus monodon* (Palacios et al., 2000) and improves GSI of Pacific white shrimp, *Litopenaeus vannamei* broodstock (Liang et al., 2023). Cholesterol has been demonstrated to improve egg production, hatching rates and larval quality on a variety of marine crustaceans, including crabs and copepods (Crockett and Hassett, 2005; Hassett, 2004; Wu et al., 2010). However, information relating to cholesterol requirements for freshwater crustaceans are limited and to our knowledge, the effect of dietary cholesterol on gonadal maturation and egg quality in redclaw has not been reported.

The reproductive performance of crustaceans may also be supported by astaxanthin, a principal carotenoid pigment found in the body and carapace of several crustaceans (Huang et al., 2008; Maoka, 2020; Paibulkichakul et al., 2008). Many crustaceans accumulate astaxanthin in

integuments, carapaces, eggs, and ovaries (Maoka, 2020). Finfish and crustaceans cannot biosynthesise carotenoids *de novo* and they must be obtained from their diet (Rajasingh et al., 2006). In addition to providing colour, astaxanthin plays several roles in aquatic animals, including as an antioxidant, enhancing cellular defences against photodynamic damage, provitamin A activity, improved growth and maturation and embryonic development (Torrissen, 1990). Due to astaxanthin's distinct chemical structure, it can prevent peroxidation of lipids and neutralise detrimental singlet oxygen and free radicals when present in excess and prevent reproductive cells or tissues from being damaged by oxidative stress or peroxidation and cell apoptosis (Gammone et al., 2015; Shastak and Pelletier, 2023). Astaxanthin's powerful antioxidant capabilities can help to improve gamete quality and may lead to more effective fertilisation, increasing the likelihood of viable embryonic development (Ahmadi et al., 2006; Hansen et al., 2016; Shastak and Pelletier, 2023). As an effective antioxidant astaxanthin can reduce lipid peroxidation in the ovarian tissue of narrow-clawed crayfish, *Astacus leptodactylus* resulting in increased ovarian egg number and size (Barım-Öz and Şahin, 2016). Dietary astaxanthin supplementation was also shown to significantly improve the number of eggs in *P. monodon* broodstock (Paibulkichakul et al., 2008). When ingested by crustaceans, astaxanthin is stored in the hepatopancreas and subsequently transported to the ovaries during the latter phases of maturation (Elbahnaswy and Elshopakey, 2023; Tizkar et al., 2013). Astaxanthin accumulates in redclaw broodstock ovaries as they mature and is linked with oocyte development and vitellin deposition (Sagi et al., 1995) although its dietary supplementation remains unstudied as a means of improving egg development and spawning. While astaxanthin has been shown to play crucial roles in enhancing reproduction among crustaceans, there has been no research to date specifically investigating the impact of dietary supplementation of astaxanthin on redclaw reproduction. This study therefore investigates whether dietary supplementation of 0.1% astaxanthin and/or 1% cholesterol can enhance reproductive development or viable egg quantity for female redclaw broodstock. Despite the ranges in supplementation rates that have been used for dietary cholesterol and astaxanthin, a beneficial effect on production and health status was observed in *P. monodon* when astaxanthin was supplemented at an inclusion rate of 0.1% with further improvements in growth and survival were noted when cholesterol was included at a supplementation rate of 1%. Thus, inclusion rates of 0.1% and 1% for astaxanthin and cholesterol, respectively, in that study appeared to synergistically improve growth and survival (Niu et al., 2014).

5.3 Materials and Methods

5.3.1 Feed Formulation

Four different diets were formulated to be isonitrogenous (39.4% CP/dry weight), isoenergetic (3.8 kcal/kg) and isolipidic (11.5% crude lipid) but varying in astaxanthin and cholesterol

supplementation (Table 5.1). All feed ingredients were purchased locally, and the experimental diets were produced at the Aquaculture Nutrition Laboratory, Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University. For preparation of the diet, all dry components excluding premixes and antioxidant were milled to <1 mm with a Rotor Beater Mill SR 300 (Retsch, Haan, Germany) before combining with premixes, antioxidant, oils and the required amount of cholesterol or astaxanthin in an A200 Planetary Mixer (Hobart, Troy, OH, USA). Sufficient water was added to facilitate pellet formation, which was achieved with a Dolly pasta machine (Imperia and Monferrina S.p.a., Moncalieri, Italy) with a 3 mm die and approximately 5 mm pellet length. After pelleting, feeds were steamed at 120 °C for 5 min, and then dried at 50 °C in a TD 700-F Premium Dryer (Thermoline, Wetherill Park, NSW) for 6 h to attain a residual moisture content of 10%. Dried pellets were vacuum sealed and stored at -20 °C, covered with aluminium foil to prevent light exposure.

5.3.2 Feeding Trial

The study was conducted at the Marine and Aquaculture Research Facility, James Cook University, in 100 L circular tanks with a flow rate of 1 L/min freshwater and gentle aeration in recirculating aquaculture systems. Each of the four dietary treatments were allocated to 6 replicate tanks, each containing 7 female redclaw (initial weight 61.5 ± 1.2 g) in a 2 × 2 factorial arrangement. Prior to stocking, the gonadal maturation of females was evaluated using a concentrated light source, following the method outlined by Jones (1990) for redclaw. All females included in the study were classified as being in the early stage of ovarian development, with no visible ovaries (Stage I - Immature). The temperature was set at 28 °C and photoperiod was maintained at 14 h light: 10 h dark. Animals were first acclimatised to the Control diet for 30 days to purge any residual nutrients before commencing feeding with the experimental diets. Animals were fed once per day at midday (12 noon) for 4 weeks, after which, eight animals from each treatment were randomly sacrificed for GSI, and histological examination of the ovary (Greco et al., 2022). Males were then introduced to each tank at a ratio of 1M:3F, ensuring a maximum size difference of 20 g within tanks to allow for breeding. Animals were checked daily for moulting and mortality, while spawning was checked every second day from the beginning of the experiment to 75 days. Days to spawning and moulting were calculated based on the average time from when the redclaw were introduced into the tank until they spawned and moulted. Water quality parameters such as temperature, dissolved oxygen, pH, ammonia, and water hardness were monitored weekly. Faeces and leftover feed were cleaned daily from the tank bottom by siphoning. Egg quality was evaluated by assessing the total no. of eggs, fecundity (number of eggs/g body weight of female), hatching rate and the total number of juveniles produced/crayfish.

Table 5.1: Dietary composition of Control and experimental diets.

Feed ingredients (g/kg)	**Diet 1 (Control)**	**Diet 2 (CHOL)**	**Diet 3 (AX)**	**Diet 4 (AX + CHOL)**
Defatted soybean meal	300.0	300.0	300.0	300.0
Wheat gluten	200.0	200.0	200.0	200.0
Lupin meal	200.0	200.0	200.0	200.0
Canola oil	40.0	40.0	40.0	40.0
Flaxseed oil[a]	40.0	40.0	40.0	40.0
Vitamin E	0.1	0.1	0.1	0.1
Monocalcium phosphate	14.0	14.0	14.0	14.0
Antioxidant[b]	1.0	1.0	1.0	1.0
Vitamin mixture[c]	5.0	5.0	5.0	5.0
Mineral mixture[d]	1.8	1.8	1.8	1.8
Whole wheat flour	198.1	188.1	197.1	187.1
Cholesterol[e]	-	10.0	0.0	10.0
Astaxanthin[f]	-	-	1.0	1.0
Total	1000	1000	1000	1000

[a]MMB NQ Pty Ltd Trading as Feed 2 Go, Bohle, QLD, Australia.

[b]Santoquin (66% ethoxyquin).

[c]Vitamin premix (g/kg): A (3,000 IU), D_3 (24 IU), K (10.0), inositol (250.0), nicotinic acid B_3 (45.0), pantothenic acid B_5 (10.0), folic acid (5.0), riboflavin B_2 (20.0), cyanocobalamin B_{12} (0.05), biotin (1.0), pyridoxine B_6 (10.0), thiamine B_1 (10.0), vitamin C (150.0), and antioxidant (15.0) and dextrose was used a carrier.

[d]Mineral premix (g/kg): Cu (6.0), Co (12.5), Mn (19.0), I (1.0), Se (0.5), Fe (125.0), and Zn (66.0).

[e]VWR Life Science, Ohio, USA.

[f]Carophyll Pink, DSM, Heerlen, Netherlands.

5.3.*3 Statistical Analyses*

The statistical software program, IBM SPSS Statistics version 27 for Windows (SPSS Inc., Armonk, NY, USA) was used to perform statistical analyses. Pearson's Chi-square test was used to compare proportional responses. The effect of treatment on the odds ratios (ORs) and 95% CIs of spawning, moulting and mortality was assessed using logistic regression. Body weight was initially included in models as a categorical variable (<60 g or ≥60 g) but was subsequently removed if not significant, while treatment was always retained in the final model. The Hosmer and Lemeshow test were used to evaluate the goodness of fit of the models. The approximate Chi-Squared distribution of the Wald statistic was used to calculate the probability values for all main effects that were present in the models. For all main effects that remained in the models, Odds ratios and 95% confidence intervals were computed. Cox regression was employed to assess if there were variations in the survival distribution of spawning moulting and mortality of crayfish as well as to assess the time to spawning, moulting and mortality in relation to treatment and initial body weight of crayfish. Data were expressed as Mean ± SEM and considered significant when p value is <0.05.

5.4 Results

5.4.1 Mortality

Treatments had no significant effects on mortality rates (Table 5.2).

Table 5.2: Reproductive characteristics of redclaw crayfish after dietary supplementation.

Parameters	**Control**	**CHOL**	**AX**	**AX + CHOL**	***p*-value**
n	34	34	34	34	-
GSI	$3.9^{b} \pm 0.24$	$3.9^{b} \pm 0.37$	$5.2^{a} \pm 0.13$	$5.1^{a} \pm 0.21$	<0.001
HSI	$7.0^{a} \pm 0.48$	$6.9^{a} \pm 0.41$	$4.0^{b} \pm 0.53$	$4.4^{b} \pm 0.31$	<0.001
Oocyte diameter (μm)	$1458.0^{b} \pm 56.9$	$1449.5^{b} \pm 51.7$	$1964.6^{a} \pm 70.4$	$1679.1^{b} \pm 77.7$	<0.001
Mortality (%)	19.4 (7)	13.9 (5)	13.9 (5)	16.7 (6)	0.906
Spawning (%)	30.6^{b} (11)	22.2^{b} (8)	63.9^{a} (23)	38.9^{ab} (14)	0.005
Interval to spawning (days)	$70.1^{a} \pm 1.1$	$67.6^{a} \pm 0.9$	$59.7^{b} \pm 1.2$	$61.6^{b} \pm 1.3$	<0.001
Eggs/female	$465.1^{b} \pm 26.9$	$472.4^{b} \pm 40.8$	$602.0^{a} \pm 26.5$	$524.1^{b} \pm 33.3$	<0.001
Fecundity (eggs/g female)	$7.7^{bc} \pm 0.34$	$7.2^{c} \pm 0.36$	$10.0^{a} \pm 0.13$	$8.2^{b} \pm 0.16$	<0.001
Moulting (%)	24.9^{b} (9)	52.8^{a} (19)	16.7^{b} (6)	22.2^{b} (8)	0.003
Interval to moulting (days)	63.0 ± 2.65	60.3 ± 1.6	64.5 ± 2.8	62.3 ± 2.4	0.575
Hatching rate (%)	$74.3^{b} \pm 1.8$	$76.4^{b} \pm 3.3$	$82.4^{a} \pm 1.3$	$74.7^{b} \pm 1.9$	0.003
No. of juveniles	$328.7^{b} \pm 31.9$	$318.6^{b} \pm 39.2$	$504.1^{a} \pm 24.8$	$404.6^{ab} \pm 29.2$	<0.001

^{abc} Numbers with different superscripts in the same row differ significantly ($p<0.05$).

Values within bracket indicate number of crayfish that died, spawned, or moulted for respective parameters.

5.4.2 GSI and HSI

The mean GSI was significantly greater and HSI was significantly lower in redclaw from AX and AX + CHOL treatments (Table 5.2; $p<0.05$). Redclaw from the AX group had visibly larger oocytes with notably more orange colour than other treatments Figure 5.1.

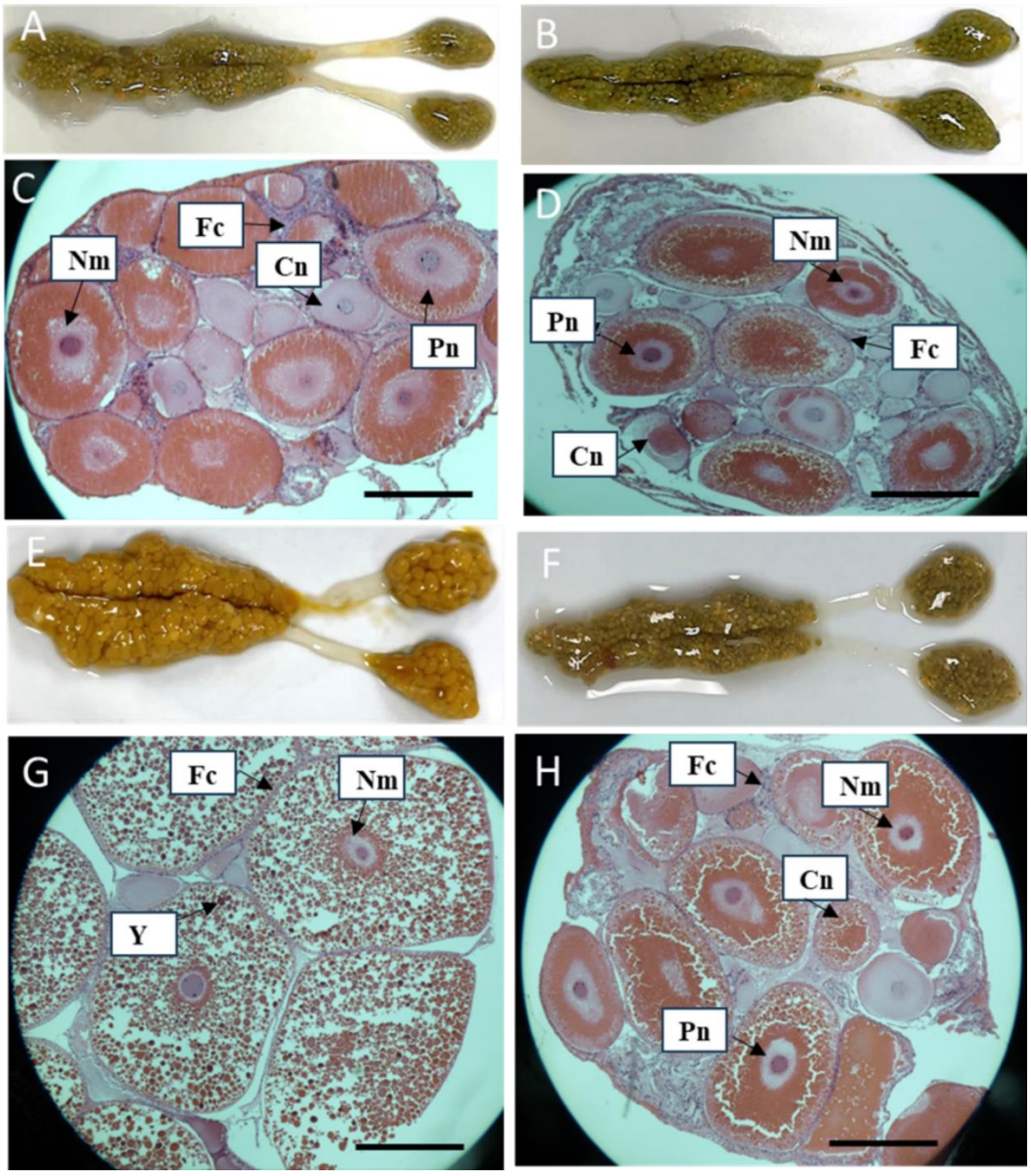


Figure 5.1. Representative images of ovaries collected from redclaw, *Cherax quadricarinatus* fed formulated diets and their respective histological ovarian sections stained with haematoxylin and eosin. A, C) Control: pre-maturation stage oocytes, B, D) CHOL: pre-maturation stage oocytes, E, G) AX: maturation stage oocytes and F, H) AX + CHOL: pre-maturation stage oocytes. Fc = follicle cell; Cn = chromatin nucleus stage oocyte; Pn = perinuclear stage oocyte; Nm = nuclear membrane; Y = yolk globule. Scale bar (▬) 0.5 mm.

5.4.3 Oocyte Diameter, Egg Number Per Female, Hatching Rate and Juvenile Number Per Female

The mean oocyte diameter, mean number of eggs/females, hatching rate and mean number of juveniles/females were significantly greater in crayfish fed diet AX compared to all other treatments ($p \leq 0.05$, Table 5.2). Body weight significantly affected the mean number of eggs and juveniles per crayfish. Crayfish with a body weight of ≥60 g compared to crayfish weighing <60 g produced significantly more eggs (620.7 ± 19.21 *vs* 440.7 ± 15.10, respectively; $p<0.001$) and juveniles (493.3 ± 20.9 *vs* 331.8 ± 19.2, respectively; $p<0.001$) per female. The mean oocyte diameter ($p<0.001$) and hatching rate ($p=0.003$) were not affected by body weight category. The histological sections of the ovaries (Figure 5.1) of AX fed crayfish showed maturation stage characterised by advanced perinuclear oocytes with irregular nuclei surrounded by an indistinct nuclear membrane. This stage features narrower follicles and larger yolk globules. However, histological sections of the ovaries of crayfish fed the Control, CHOL and AX + CHOL diet exhibited pre-maturation stage, as evidenced by the presence of chromatin nucleus and early perinuclear stage oocytes. The oocytes possess a nucleolus in the nucleus and are surrounded by a distinct clear perinuclear zone, and the single follicular layer is typically wider.

5.4.4 Fecundity

The mean fecundity was greater in the crayfish fed the AX diet compared to all the other diets. It was also greater in those fed the AX + CHOL diet compared to those fed the CHOL diet. The mean fecundity was similar between those fed the AX + CHOL diet and those fed the Control diet and those fed the Control and CHOL diets (Table 5.2). The body weight category did not significantly affect the fecundity of crayfish.

5.4.*5 Spawning Rate*

Spawning rate was significantly greater in crayfish fed the AX diet compared to crayfish fed Control and CHOL diets whereas, the mean interval to spawning was less in both the crayfish fed the AX diet and the AX + CHOL diet compared to crayfish fed the Control and CHOL diets (Table 5.2). The odds of spawning were significantly greater in crayfish fed the AX diet compared to Control group whereas, it did not differ between the other treatments and the Control group (Table 5.3).

Redclaw from the two astaxanthin supplementation treatments began spawning at day 49 (AX) and day 53 (AX + CHOL) and by the end of the experiment 40-60% of redclaw from these treatments had spawned (Figure 5.2). Those redclaw that had feeds without supplemental astaxanthin started spawning approximately 14 days later and only 20-30% of these broodstock females had spawned by the end of the experiment. The survival distributions of crayfish fed the AX diet differed significantly with the Control fed crayfish ($p<0.01$, Table 5.4) with

spawning occurring earlier and to a greater degree in the crayfish fed the AX diet. The survival distributions did not differ significantly in each of the other treatments compared to the Control fed crayfish (Table 5.4).

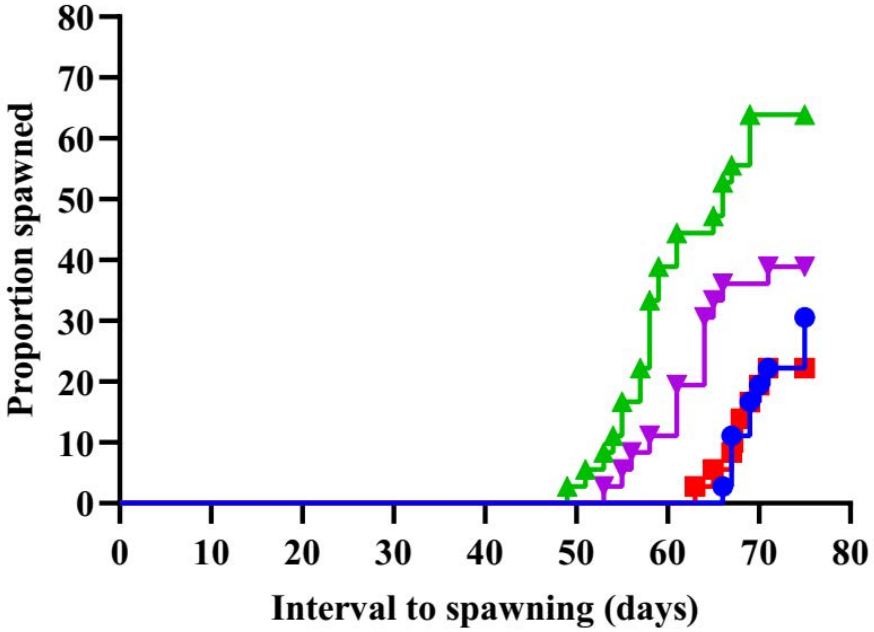


Figure 5.2. Distribution of the proportion of crayfish that spawned during the study after feeding diets that contained no additional CHOL or AX (Control,) or diets supplemented with CHOL ( ), AX ( ) or AX + CHOL ( ).

Table 5.3: Logistic regression results showing log-transformed odds ratio estimates for effects of treatment relative to control on the proportion of animals spawning, moulting, and surviving.

Parameters	**Treatment**	**B**	**SE**	**Chi-Square (Wald)**	**Df**	**Odds ratio**	**95% CI**	***P***	**Reference group**
Spawning (%)	CHOL	-0.43	0.54	0.64	1	0.65	0.23 - 1.87	0.424	Control
	AX	1.39	0.50	7.71	1	4.02	1.51 - 10.74	0.006	Control
	AX + CHOL	0.37	0.50	0.55	1	1.45	0.55 - 3.84	0.459	Control
Moulting (%)	CHOL	1.21	0.51	5.64	1	3.35	1.24 - 9.10	0.018	Control
	AX	-0.51	0.59	0.75	1	0.60	0.19 -1.91	0.387	Control
	AX + CHOL	-0.15	0.56	0.08	1	0.86	0.29 - 2.55	0.781	Control

Table 5.4: Cox regression for analysis of odds ratio estimates for effects of treatment on spawning, moulting and survival distributions.

Parameters	**Treatment**	**B**	**SE**	**Chi-Square (Wald)**	**Df**	**Odds ratio**	**95% CI**	***p***	**Reference group**
Spawning distribution	CHOL	-0.31	0.47	0.43	1	0.74	0.30 - 1.80	0.512	Control
	AX	1.25	0.37	11.50	1	3.48	1.70 - 7.20	<0.001	Control
	AX + CHOL	0.47	0.40	1.33	1	1.59	0.70 - 3.51	0.249	Control
Moulting distribution	CHOL	0.99	0.41	5.91	1	2.68	1.20 - 5.90	0.015	Control
	AX	-0.46	0.53	0.76	1	0.63	0.23 - 1.78	0.383	Control
	AX + CHOL	-0.13	0.49	0.07	1	0.88	0.34 - 2.28	0.794	Control

5.4.6 Moulting Rate

The moulting rate was significantly greater in crayfish fed CHOL compared to the Control group (Table 5.2). The mean interval to moulting did not, however, differ significantly between treatments (Table 5.2). During the acclimatisation period and before addition of males, no moulting occurred. The survival distribution differed between the crayfish fed the Control and CHOL diets with moulting occurring earlier and more frequently in crayfish fed the CHOL diet (Figure 5.3, Table 5.3 and Table 5.4). However, it was similar between crayfish fed the AX and AX + CHOL diets compared to those fed the Control diet only (Table 5.4).

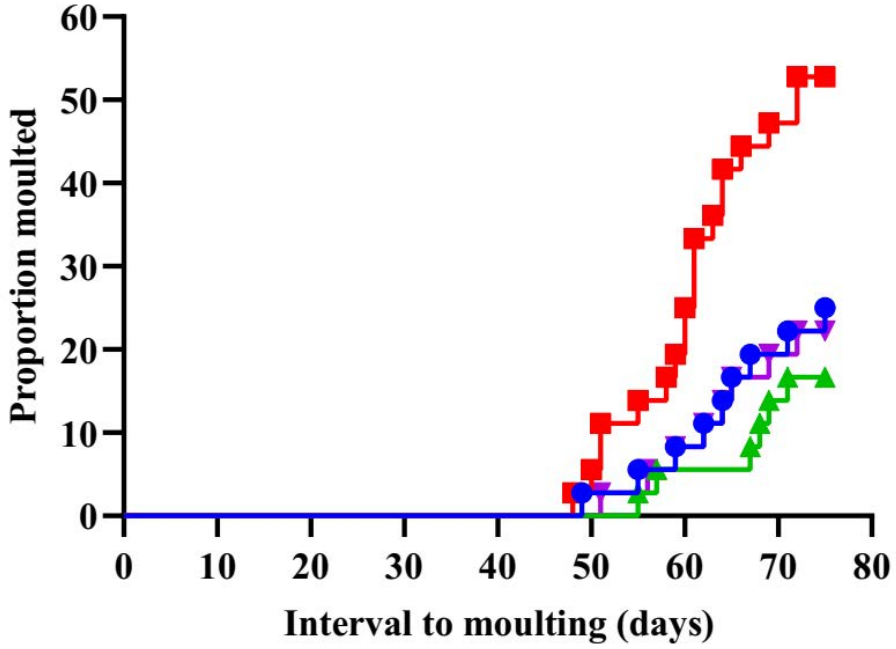


Figure 5.3. Distribution of the proportion of crayfish that moulted during the study after treatment with Control ( ), CHOL ( ), AX ( ) or AX + CHOL ( ).

5.5 Discussion

The current research focused on the development of a diet supplemented with AX and/or CHOL to stimulate ovarian maturation and spawning, while improving egg and embryo quality in redclaw. We report that cholesterol supplementation did not benefit any of the reproductive parameters measured. In contrast, astaxanthin supplementation alone significantly enhanced the reproductive outcomes of redclaw as assessed by parameters such as GSI, oocyte diameter, spawning rate, egg number, fecundity, hatching rate and juvenile number suggesting astaxanthin as a potential nutrient supplement to enhance crustaceans' reproduction.

Dietary astaxanthin has previously been demonstrated to accumulate in eggs of redclaw and stimulate ovarian development (Sagi et al., 1996b). The current research builds on this knowledge by demonstrating that dietary AX and AX + CHOL supplementation can

significantly increase GSI (33.3% and 30.8%, respectively) while also elevating oocyte diameter (34.7% and 15.2%, respectively) and lower HSI (42.9% and 37.1%, respectively) than broodstock those fed the Control diet. The positive effect of astaxanthin on crustacean ovarian development has been demonstrated across a variety of crustacean species (Jiang et al., 2022; Liñán-Cabello et al., 2004; Liñán-Cabello and Paniagua-Michel, 2004; Pangantihon-Kühlmann et al., 1998; Sagi et al., 1996b), however the current finding is novel for inducing predictable maturation of gonads leading to increased egg production and better overall yield of redclaw juveniles that is critical for the growth of the industry.

The present study revealed that crayfish fed with AX alone exhibited ovaries in the maturation stage, characterised by larger vitellogenic oocytes with irregular nuclei and an unclear nuclear membrane. This characteristic is typically observed in crayfish primed for simultaneous spawning (Abdu et al., 2000). While feeding the Control diet or a diet supplemented with CHOL alone or AX + CHOL crayfish had pre-maturation stage ovaries, containing early vitellogenic oocytes with a nucleolus in the nucleus. In this study greater GSI and lower HSI in crayfish fed the AX diet suggests a transfer of nutritional reserves from the hepatopancreas to improve gonadal development and increase oocyte diameter indicating that AX plays a stimulatory role in enhancing ovarian maturation. In *L. vannamei* females, astaxanthin was suggested to upregulate the actions of hormones such as gonad stimulating hormone, steroid hormones, and the red pigment concentrating hormone involved in the gonadal development, and ovarian maturation, leading to increased GSI (Liñán-Cabello and Paniagua-Michel, 2004).

In the current study, AX supplementation alone led to significantly greater spawning rates (108.8%, 187.8%, and 64.3%, respectively) and shorter spawning intervals (14.8%, 11.7%, and 3.1%, respectively) than when crayfish were supplemented with only the Control, CHOL, or fed AX + CHOL diet, which is consistent with the observed stimulatory effects of treatment with AX alone on ovarian growth and maturation and suggests that the presence of CHOL may inhibit this stimulatory response to some degree. In *P. monodon* (*Fabricius*) broodstock, supplementation of dietary AX significantly increased the proportion of spawns and shortened the spawning interval by 118.0% and 31.9%, respectively compared to control fed shrimps (Huang et al., 2008).

Crayfish supplemented with AX had significantly greater number of eggs/female (29.4%, 27.4%, and 14.9%, respectively), and fecundity (29.9%, 38.9%, and 22.0%, respectively) compared to those fed with Control, CHOL, and AX + CHOL diets. In decapod crustaceans, reproductive performance as measured by egg number increased after dietary supplementation of AX (Barım-Öz and Şahin, 2016; Huang et al., 2008; Paibulkichakul et al., 2008). For example, female black tiger shrimp *P. monodon* and freshwater crayfish *A. leptodactylus* fed diets supplemented with AX produced significantly greater number of eggs (22.34% and

89.69%, respectively) compared to control fed animals (Barım-Öz and Şahin,, 2016; Huang et al., 2008). While the precise mechanism by which AX supplementation enhances egg production in crustaceans remains unclear, similar results with tilapia *Oreochromis niloticus* broodstock have been explained by upregulation of genes associated with oocyte meiosis, facilitating progesterone-mediated oocyte maturation (Qiang et al., 2022) leading to enhanced egg production. Moreover, AX's ability to reduce oxidative damage to biological macromolecules may increase egg production by scavenging excess free radicals that can lead to cell apoptosis during gonadal development, thereby contributing to improved fertility. By protecting ovarian tissues from oxidative stress and inflammation, AX may provide a favourable environment for successful follicle development, ovulation and fertilisation (Qiang et al., 2022). However, specific mechanisms through which AX influences egg number in crustaceans may vary among species, and additional research is needed to fully understand the underlying processes.

Crayfish supplemented with AX had significantly greater hatching rate (10.9%, 7.9%, and 10.3%, respectively) compared to those fed with Control, CHOL, and AX + CHOL diets in the present study. For crustaceans, including redclaws, there is presently no report on the hatching rate following dietary AX supplementation. Also, the number of juveniles (55.8%, and 58.2%, respectively) were significantly greater in crayfish supplemented with AX compared to those fed with Control, and CHOL. Although the specific mechanism of the effect of astaxanthin on juvenile hatching rates requires elucidation, it has been reported to increase immune functions in crustaceans, while decreasing oxidative stress, which may explain higher juvenile survival (An et al., 2020; Chuchird et al., 2015; Kumlu et al., 1998; Young and Lowe, 2001; Zhang et al., 2023). Further mechanisms that have been suggested in fish fertility studies include elevated permeability of carotenoid-rich eggs that result in higher fertilisation rates (Tizkar et al., 2013). Overall, the positive effects of astaxanthin on the induction of ovarian maturation, spawning success as well as greater hatching rates and juvenile survival are novel findings which highlight the potential of astaxanthin for contributing to sustainable juvenile production.

Moulting is a periodic, energy-intensive process that is essential to the natural growth and development of crustaceans which is regulated by ecdysteroid, produced in two Y-organs of crustaceans (Tian et al., 2020). Supplementation with CHOL improves total lipid storage, which serves as a suitable nutritional prerequisite for the energy requirements of growth and moulting (Tao et al., 2014). Supplementation of CHOL in the current study significantly increased the moulting rate (112.0%, 216.2%, and 137.8%, respectively) in redclaw compared to Control diets and AX, AX + CHOL supplementation. The increase in moulting rate observed in crayfish supplemented with cholesterol highlights previous findings that dietary cholesterol is essential for crustaceans (Tian et al., 2020;) and that supplementation increased the ability to synthesise

the exoskeleton, thereby increasing the capacity to moult. In decapod crustaceans such as *Procambarus clarkii* and *Scylla serrata* a positive relationship between moulting performance and CHOL supplementation has been observed where moult frequency (times/day) increased significantly with the increase of dietary CHOL supplementation up to a maximum of 0.5% (Holme et al., 2006; Tao et al., 2014; Tian et al., 2020). Ecdysteroid hormones which are synthesised from CHOL are responsible for mediating the moulting process in crustaceans (Mykles, 2011). While astaxanthin has been linked to elevated steroid hormone production, and cholesterol is an essential precursor of crustacean steroid hormones, there does not appear to be a synergy between dietary astaxanthin and cholesterol supplementation at the concentrations tested in the present study. However, a dose response study and/or addition of lipid classes such as phospholipids that can facilitate the transportation of cholesterol from hepatopancreas to the ovary via haemolymph during ovarian maturation and increase their efficacy in improving reproduction could be conducted (Hou et al., 2022; Kumar et al., 2018).

Although in this study, AX + CHOL supplementation resulted in significantly greater GSI and decreased time interval to spawning compared to CHOL supplementation and Control fed diets, it did not improve any other reproductive characteristics. Indeed, cholesterol supplementation appears to reduce the efficacy of astaxanthin. Since AX is fat soluble, a plausible explanation would be that after mixing with CHOL, AX becomes dissolved and sequestered within CHOL, therefore limiting the bioavailability of AX. Factors such as dosage, duration of supplementation, could influence the outcomes of cholesterol supplementation in crustaceans. Moreover, environmental temperature may modulate cholesterol requirements, since CHOL provides membrane stability throughout a wide range of body temperatures. For example, in the copepod crustacean *Calanus finmarchicus*, both eggs and copepodites juvenile contained higher CHOL levels at the warmer acclimation temperature (Hassett and Crockett, 2009). Additionally, Sperfeld and Wacker (2009) found that at elevated temperatures, the CHOL content of *Daphnia magna* eggs increased and suggested that females set aside a greater quantity of CHOL for their progeny to support adequate development of eggs. However, further studies are required to prove whether addition of CHOL with AX can reduce or suppress the function of AX.

5.6 Conclusions

In summary, the addition of AX resulted in increased spawning rates, improved egg production, hatching rates, and number of hatched juveniles in redclaw. This suggests that AX is promising in enhancing ovarian maturation in females and boosting the production of higher-quality eggs and juveniles. In contrast, CHOL supplementation, either alone or in combination with AX, did not significantly improve reproductive characteristics in redclaw, except for an elevated ovarian index and shortened spawning intervals observed in crayfish supplemented with both AX and CHOL. However, the use of CHOL alone led to increased moulting, indicating that a substantial

amount of energy was likely redirected towards moulting rather than reproduction. This also implies that the dosage of CHOL used in the study was adequate for moulting of redclaw but was insufficient for stimulating spawning. Further research is required to assess reproductive output over time considering factors such as dosage, duration of supplementation, and environmental conditions since specific mechanisms through which CHOL and AX influences juvenile production in crustaceans may vary among species and environmental conditions. This will help verify the impact of CHOL and determine whether the benefits of AX supplementation can be consistently maintained.

Chapter 6. General Discussion and Conclusion

6.1 Significance and Major Outcomes

Redclaw, *C. quadricarinatus* are freshwater crayfish, originating from the rivers of Northern Australia and Southern Papua New Guinea, that are recognised as potential species for commercial aquaculture due to many advantageous characteristics (Jones, 1990). With growing global demand for seafood, redclaw crayfish have the potential to enter new markets, particularly in Asia where crustacean consumption is high. In recent years, China has emerged as a major producer and countries are exploring the potential of redclaw crayfish aquaculture (Haubrock et al., 2021). As the native habitat of redclaw, Australia is a leading producer and production techniques are focusing on both extensive and semi-intensive systems. However, the industry is still in its developmental stages and faces many production challenges that hinder its growth.

Traditional earthen pond-based production methods of redclaw are constrained by labour-intensive maintenance, seasonal variation (QCFA, 2013; Jones, 1998), inbreeding (QCFA, 2013; Stevenson et al., 2013), and inconsistent production. Additionally, juvenile growth and survival rates are very low (5 to 10%) due to suboptimal husbandry, inadequate protein-content of diets, and cannibalism among early juveniles (Jones, 1995; Masser and Rouse, 1997). Within hatcheries, productivity is limited by poor broodstock condition, suboptimal husbandry practices, variable female fecundity, bacterial and parasitic infestations that adversely affect egg development and survival, and asynchronous hatching. To overcome these problems improving reproductive efficiency (fecundity) of female redclaw is crucial. Studies conducted as part of this thesis identified several methods that can improve gonadal development, egg yield and enhance the number of juveniles produced within indoor hatchery settings, thereby being useful techniques for increasing the productivity of female redclaw within hatcheries.

In Chapter 2, an extensive review was undertaken to identify potential techniques currently available for intensive breeding of decapod crustaceans aiming to promote reproduction and increase juvenile production in redclaw. The techniques explored include non-invasive natural manipulations, hormonal treatments, dietary supplementation, egg and embryo quality tools and AF that can be applied or adapted to female redclaw crayfish to enhance ovarian maturation and improve the production of juveniles. In Chapter 2 the basic biology of redclaw crayfish, their anatomy, life cycle, and the stages of egg and embryonic development were described. A summary model was developed that illustrated the interconnections between the reproductive system, hormones and other parts of the body which not only orchestrates and drives the reproductive processes but also creates opportunities for manipulating and enhancing reproductive capacity. This was explored further by undertaking three studies that sought to

examine the potential for utilising techniques that stimulated natural physiological processes to improve the productivity of females.

Husbandry practices such as manipulation of photoperiod, water temperature, stocking density, sex ratios and the presence of males can induce ovarian maturation and the onset of spawning. In natural habitats, under temperatures of between 26 and 29 °C and a photoperiod of 14L:10D redclaw will generally spawn 3-5 times annually (Bugnot and López Greco, 2009; C. Jones, 1995; Jones, 1998). This review identified that a lower stocking density (20/m^2) and sex ratio (1M:4F) is preferable to improve juvenile production (Barki and Karplus, 2000). Also, the indirect presence of males was found as a potential non-invasive natural means to manipulate the reproductive behaviour of females. Separate stocking of males, while allowing chemical cues or pheromones to mix in a recirculatory system, can stimulate spawning and egg release in fin fishes (Amagai et al., 2022; Huertas et al., 2014; Kurtzman et al., 2010; Soyano et al., 2022). This strategy has potential to apply in redclaw to produce unfertilised eggs which could be utilised for cryopreservation and AF. AF is valuable for expediting the selective breeding of commercially important aquaculture species (Haldar et al., 2018). While a review of IVF techniques developed for decapod crustaceans was undertaken the opportunity to investigate some of these techniques in redclaw fell outside of the timeframe available for study. Studies which were undertaken did, however, investigate some techniques that should prove valuable in generating eggs for the purposes of AF in the future. This included the identification of potential hormones, such as serotonin, naloxone, methyl farnesoate, 17α-hydroxyprogesterone which could regulate vitellogenesis and potentially induce ovarian maturation and year-round juvenile production (Cahansky et al., 2008; Liu et al., 2014a,b; Rodríguez et al., 2002). In addition, it was identified that crustaceans require certain nutrients for enhancing egg quality and producing healthy juveniles, however, some nutrients such as some proteins, lipids, carotenoids, vitamins, and minerals cannot be synthesised *de novo* and, therefore, must be supplemented through the diet. Nutrients such as *n*-3 fatty acids, cholesterol, vitamin A, and astaxanthin were identified as potential nutrients in promoting gonadal development, spawning, higher egg quality, and increased embryo survival (Barım-Öz and Şahin, 2016; Harlioğlu et al., 2013; Kumar et al., 2018; Paibulkichakul et al., 2008).

To effectively induce gonadal maturation and boost egg production one of the lethal practices currently being used by most hatcheries is eyestalk ablation, which acts by limiting the secretion of GIH; however, eyestalk ablation raises significant ethical concerns and is invasive, causing stress and mortality in broodstock and yielding poor quality juveniles. Therefore, in Chapter 3 details of an investigation were described that examined the use of an alternative natural system that involved the use of a sex separated rearing strategy that was organised into a vertical recirculating aquaculture system. The study investigated whether pre-exposure of female

redclaw indirectly to male redclaw could stimulate spawning and enhance reproductive outputs in females. The findings of this chapter indicated that pre-exposure of females to males in a separated stocking arrangement boosted spawning rate during a dissociated period and elevated the number of eggs and juveniles produced when females were later directly associated with males. This could be due to exteroceptive stimuli, such as chemical cues in the form hormones released from males (Huertas et al., 2014) and pre-exposure to males afforded these females more time to store nutrients for yolk deposition, potentially resulting in the production of a greater number of viable eggs. This chapter also demonstrated that presence of males either directly or indirectly prevents moulting since a 50% decrease of moulting was found in the associated phase which could have improved the ability of females to divert nutrients towards reproductive processes rather than moulting (Tao et al., 2014). This study also documented the highest per unit weight fecundity (11.3 eggs per g BW) ever recorded in redclaw females weighing between 30-70 g proposing that females within this weight range may be more suitable for stocking in hatcheries, as they could maximise reproductive potential for commercial egg production. Overall, a sustainable and non-invasive method of enhancing female reproduction in redclaw crayfish was demonstrated. This aquaculture technique could successfully induce gonadal maturation avoiding the use of invasive reproductive technologies and help producing unfertilised eggs, which is a critical component for AF and cryopreservation, hence substantially facilitating the selective breeding programme in this species.

Variable fecundity and asynchronous spawning are major obstacles restricting the juvenile production and commercial expansion of redclaw. To meet the growing market demand worldwide, commercial hatcheries require predictable gonadal maturation and consistent supply of increased quantities of eggs and juveniles throughout the year, especially outside the breeding season. In Chapter 4, research focused on the effects of parenteral administration of three potential maturation-inducing hormones including MF, 5-HT, and naloxone to facilitate reproduction in redclaw in a controlled environment. The study revealed that treatment with 5-HT and naloxone resulted in greater ovarian maturation as demonstrated by greater GSI and two-fold increase in oocyte diameter of redclaw. Additionally, the histological analysis showed mature stage oocytes typically found in crayfish prepared for synchronous spawning as indicated by an irregular nucleus and indistinct nuclear membrane (Abdu et al., 2000). The results also showed injecting redclaw with 5-HT and naloxone shortened the days to spawning and significantly increased spawning (%), eggs per female, fecundity, and hatching (%) compared to the control. In contrast, redclaw treated with MF exhibited decreased reproductive features and increased mortality and moulting (%) and shortened days to moulting. Given the higher mortality rate observed in both the present study and prior research following

administration of MF (Abdu et al., 2001; Toyota et al., 2020), it can be suggested that the administration of MF, either orally or parenterally at the doses used in these studies, might have lethal effects on reproductive females. Moreover, MF as a juvenile hormone analogue, has been found its potential as an insecticide due to its ability to disrupt insect development and reproduction (Hu et al., 2019; Hu et al., 2020b). Overall, the findings of this study suggest that both 5-HT and naloxone stimulated ovarian maturation and are promising candidates to enhance spawning and egg production in redclaw. Hormonal treatment could potentially enable industries to sustain uninterrupted production cycles, resulting in greater availability of juveniles for optimal stocking densities in grow-out facilities, and may contribute to improved food security.

Gonadal maturation and reproduction are energy-intensive processes, and nutritional deficits can result in lower production of larvae affecting the poor hatchability and larval survival (Hernández-Abad et al., 2018; Thien and Yong, 2017). Various dietary nutrients affect the reproductive performance of crustaceans (Díaz-Jiménez et al., 2019; Harlioğlu et al., 2012; Thien and Yong, 2017). Notably, dietary supplementation with cholesterol and astaxanthin have been shown to improve ovarian maturation, spawning rates, and fecundity in decapod crustaceans (Barım-Öz and Şahin, 2016; Niu et al., 2014). Thus, in Chapter 5 an investigation of the impact of nutritional supplementation with cholesterol and astaxanthin alone or in combination on ovarian maturation and egg and embryo quality of redclaw was undertaken. Analyses of the results demonstrated that supplementation of AX and AX + CHOL in redclaw crayfish diets enhanced ovarian maturation as reflected by significantly greater GSI (33.3% and 30.8%, respectively) and shortened the spawning interval. Also, histological study revealed that AX supplemented redclaw had greater oocyte diameters compared to all other groups as evidenced by the presence of maturation stage oocytes with irregular nuclei surrounded by an indistinct nuclear membrane. Moreover, dietary AX treatment increased egg production by 29% while elevating hatching rates, yielding 56% more juveniles than broodstock without astaxanthin supplementation. In this study, no significant synergistic effects of AX + CHOL was observed; instead, CHOL supplementation appeared to impair the efficacy of astaxanthin. However, CHOL supplementation alone elevated moulting rates, implying that cholesterol supplementation shifted allocated energy from egg production towards moulting processes. This study is the first to demonstrate the potential of AX to enhance redclaw juvenile production, which is critical for the growth of the industry.

Numerous factors appear to influence reproductive function in female redclaw. The results of this thesis demonstrate that natural chemicals that are likely associated with the presence of males can enhance reproductive function in females and presents a natural way to improve fertility. Alternatively, direct administration of hormones that act directly on the reproductive

system can be utilised to manipulate reproductive function but in the case of some hormones can direct the animal down alternative paths that may antagonise reproductive activity. Likewise different dietary ingredients promoted moulting in some females and breeding in others indicating the potential for dietary cues can be used to enhance reproductive function or divert nutrients to other bodily functions, such as moulting, which could provide less available substrate for egg production. Collectively, this thesis has highlighted that there are a number of ways that reproductive function can be enhanced in female redclaw which could be utilised and/or further explored in commercial settings to improve productivity and economic returns.

6.2 Future Research Directions

The research findings described in this thesis demonstrated potential advancements in the production of unfertilised eggs which is essential for IVF and cryopreservation; an increased number of fertilised eggs via natural manipulation and hormone administration; and enhanced embryo quality and the number of juveniles through astaxanthin-enriched feed supplementation. These achievements could help address the limitations hindering the expansion of the redclaw crayfish industry. However, the thesis also highlighted specific areas for further development to advance the intensification of the redclaw aquaculture sector. These include:

- Simultaneous application of all treatments: pre-exposing females to males, administering hormones, and providing an astaxanthin-enriched diet to examine the combined effect on producing higher quality eggs and juveniles.
- Further investigation into identifying the chemicals released by males in the recirculating water, understanding the mechanisms of their transmission, and investigating the potential longevity of these chemicals, possibly through mass spectrometry-based metabolomics. Identification of chemical messengers could also generate studies in the synthetic composition of these chemicals and effects when administered within recirculating systems, determining optimum dose rates, duration and timing of treatments.
- Research could be conducted to see whether males from other related species could also be used in a dissociated, recirculating system to enhance female reproduction.
- Future investigations could encompass dose-response trials of the hormones administered in the study described in Chapter 4 and the measurement of physiological concentrations of 5-HT and naloxone in crayfish haemolymph to identify optimal doses and tissue concentrations required for enhancing reproduction. Exploring alterations in administration frequency and routes may unveil more practical and efficient treatment methods. Further studies could explore whether treatment with 5-HT and naloxone triggers the release of pheromones into the water, potentially facilitating early induction of gonadal maturation and spawning (Alfaro et al., 2004).

- The precise pathways through which MF, 5-HT, and naloxone exert their influence on gonadal maturation remain to be fully elucidated. Further research utilising RNA interference, recombinant protein technology, and gene editing may enhance the understanding of the endocrine regulation of reproduction in crustaceans.
- Further research is necessary to fully comprehend the underlying processes by which AX and CHOL influence reproduction in crustaceans. The effectiveness of AX and CHOL might vary based on dosage, duration of supplementation and environmental conditions such temperature therefore, future studies could explore a dose-response relationship and include lipid classes such as phospholipids to determine if they might enhance function of AX and CHOL during ovarian maturation, potentially improving reproductive outcomes. Studies could also be conducted to determine the proximate biochemical composition of formulated diet, gonads and hepatopancreas, haemolymph biochemical indices to determine the cholesterol and lipoprotein level as well as astaxanthin content in redclaw cephalothorax, muscle, and shell. Investigation of the nutritional composition of muscle, such as fatty acid profiles, with different dietary supplements might also have implications for human health and a means of increasing the saleable value of product if favourable outcomes linked to dietary supplementation could be demonstrated.
- Research should be carried out to optimise advanced reproductive tools. Simultaneous application of advanced cellular and traditional markers could allow rapid selection of superior quality eggs for selective breeding of redclaw. However, advanced markers for assessing egg and embryo quality have not yet been developed in decapod crustaceans including redclaws. Thus, research is needed to optimise methods that assess cell viability, mitochondrial function, nuclear maturation, and DNA integrity to evaluate egg and embryo quality in decapods more objectively. Fluorescence-based nucleic acid stains such as Hoechst 33342/propidium PI dual staining for cell viability and nuclear maturation, JC1 for the detection of functional and non-functional redclaw embryo and the TUNEL assay for DNA damage could be used for such optimisation. Moreover, validation of embryonic heart rate could serve as a valuable indicator of embryo viability, as irregular cardiac function can indicate abnormal embryonic growth.
- The availability of unfertilised eggs through a sex separated rearing strategy and the ability to harvest spermatozoa via electroejaculation in redclaw crayfish (Jerry, 2001) offer tremendous potential for the development of AF techniques such as IVF. However, the success of IVF depends on the optimisation of several factors such as degree of maturation of egg and spermatozoa, egg-sperm ratios for insemination, culture media and incubation temperature for enhanced fertilisation and hatching rates. Therefore, IVF protocols for redclaw crayfish could be studied and successful development in redclaw will provide a basis for the development of new methods for intensifying juvenile production, which might

improve fertility and revolutionise the aquaculture sector by enabling better genetic selection for reproduction and production and facilitate more consistent supply of product to markets.

- During the course of the study, a dose response trial was conducted as adverse effects of parenteral administration of 5-HT were initially observed. While a dose was identified in which adverse effects of treatment with 5-HT were no longer observed, further investigation of effects of treatments on animal behaviour and welfare would be valuable to ensure that animal well-being is not compromised by any treatments.

6.3 Limitations of the Study

The studies conducted as part of this thesis identified a number of techniques that could be used to improve the productivity of female redclaw. Some limitations of these studies, however, include:

- Each study was conducted at a specific time of the year so seasonal influences on outcomes was not determined.
- The source of animals may have limited the genetic composition or influenced the nutritional and environmental conditioning of the animals which could have introduced some bias to the results.
- A limited range of body masses were included in the studies. Perhaps different responses would have been obtained outside of the weight ranges included in these studies.
- Dose rates of hormones and dietary supplements were not always optimised prior to each study, so better optimisation may potentially improve the results that were obtained.
- Only one route of administration of hormones was investigated in Chapter 4 so it is unclear if other routes of administration such as, oral, immersion, intraperitoneal injection or topical application would also enhance reproduction.
- The Control and the MF treatments were subjected to 5 injections while the 5-HT and Naloxone only received 3 injections. Treatment with a different number of injections would have resulted in more handling stress in the Control and MF treated crayfish which could have contributed to the reduced reproductive performance obtained in animals receiving these treatments compared to the animals that were treated with 5-HT and Naloxone. In retrospect, administration of an additional 2 injections of Crayfish saline mix in the crayfish treated with 5-HT and Naloxone would have ensured that all animals were subject to the same handling stressors and better isolated the potential for the treatments alone, rather than the number of treatments, as being the causes of the differences in reproductive function that were observed.

- Longer or shorter durations of treatment may have influenced the results, and further studies could be undertaken to determine the optimum duration of treatments or if productivity were to decline with repeated episodes of treatment.
- The amount of each diet consumed in chapter 5 was not measured which could potentially affect the results if consumption or gut transit times were influenced by the dietary composition.
- The application of treatments in different environmental conditions such as with crayfish exposed to differences in water temperature and duration or intensity of light could influence the results and requires further examination in order to validate the efficacy of treatments across different environments.
- The survival of juveniles through to market size and overall yield of saleable product per treatment was not examined. Further study would help determine if there were any long-term benefits or disadvantages of the treatments that were imposed.
- The economic costs of different interventions were not examined.

6.4 Conclusions

The overarching goal of this thesis was to develop ways that might increase juvenile production in female *C. quadricarinatus* by promoting gonadal maturation and spawning while also improving egg and embryo quality. This was achieved through developing a novel sex-separated rearing strategy which documented the production of unfertilised eggs for the first time and reported greater fecundity in redclaw, by administering hormones that stimulated ovarian maturation, increased spawning rates and egg production, and by supplementing an antioxidant-rich diet that increased egg production, hatching rates and produced more redclaw juveniles. The developed methods could be adopted by the industry to increase the reproductive efficiency of female redclaw crayfish as well as improve the production of juveniles, which could contribute to the sustainability and profitability of redclaw aquaculture.

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