School of Science
Department of Environment and Agriculture

Growth-dependent haemolymph physiology in freshwater crayfish farmed in Western Australia

Mai Van Ha

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DECLARATION

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PREAMBLE

The purpose of this research is to improve understanding of the physiology of marron, *Cherax cainii* and yabbies, *Cherax albidus*. In the broader context of the current research theme, the thesis has emphasised four different aspects that affect the physiology of the freshwater crayfish farmed in Western Australia. These aspects are moult stages, weight classes, food type, and feeding or starvation, which were pre-selected variables to investigate the impacts on the haemolymph physiology (Chapter 4, Chapter 5, Chapter 6), hepatopancreas (Chapter 4) and exoskeleton (Chapter 7) via different experiments under laboratory controlled conditions. In addition, the natural population structure of yabbies (Chapter 3) was estimated in to a purpose-built earthen pond environment.

The thesis consists of nine chapters. Chapter 1 is an introduction which briefly highlights current trends and issues in the crayfish industry in Australia, and gives an overview of the crayfish population estimation, moult stages and weight-related physiological changes in crayfish body tissue, feed and feeding ecology. This chapter also justifies and underlines the needs, aims and objectives of the current research.

Chapter 2 reviews the research into the aquaculture of crayfish, including marron and yabbies. The ecology, biology and nutritional requirements of crayfish are presented in the chapter. The feeding practice, growth and health conditions of crayfish are also summarised. The relevant investigations into haemolymph homeostasis and exoskeleton composition in relation to moult stages and body weights are reviewed as a main part of the chapter. Methods for population estimation of crustaceans and the relationship between environmental factors and crayfish population are summarised in this chapter. The information on feed and feeding practices in crustaceans and their foregut evacuation is reviewed.

Chapter 3 to Chapter 7 report the main research of the thesis and attempt to investigate seasonal changes in yabby population in relation to water and soil quality parameters, and to evaluate the effects of moult stage, weight class, short-term starvation and food type on osmolality, haemolymph constituents, hepatosomatic indices, food consumption, foregut evacuation rate and approximate composition of exoskeleton. All these chapters form an essential component of this research and can be viewed as independent experiments bound by a common theme. The individual

chapters were written to facilitate their independent publication in different journals. Therefore the reader may find some minor repetitions in the 'Introduction', 'Materials and Methods' and 'Discussion' sections. The names of journals where these chapters are or will be published are in the list of publications.

Chapter 3 aims to estimate the population size of yabbies in purpose-built ponds using the mark-recapture method, as well as investigating the impact of seasonal variations in biotic and abiotic factors on yabby population. The effects of physico-chemical and biological factors on population size comprise the main research theme of this chapter.

Chapter 4 studies the influence of moult stages and weight classes on haemolymph osmolality, intermoult increment, intramoult intervals and hepatosomatic indices in freshwater crayfish. The correlation of body weight and osmolality in every moult stage is also studied for marron and yabbies.

Chapter 5 investigates the haemolymph constituents as functions of moult stage, body weight and feeding status in marron and yabbies. Changes in osmolality over a moult cycle are monitored and compared between fed and starved crayfish. Chapter 6 details the experiment to evaluate the feeding-related parameters such as food consumption, gut evacuation rate and haemolymph osmolality for different food types and weight classes of marron and yabbies.

In Chapter 7, proximate analysis of peeled exoskeletons from different moult stages and weight classes is investigated. Changes in the levels of proximate parameters in every moult stage and weight class are discussed. Chapter 8 narrates the concept of the study in the form of a schematic diagram and discusses the effects of moult stages, weight classes, feeding status, food types and crayfish species on haemolymph constituents, hepatosomatic indices and exoskeleton compositions. The cross-linked data of osmolality from different experiments in this research are also compared and discussed to confirm the reliability of the results.

Chapter 9 highlights the key findings, which are followed by recommendations for future research.

ABSTRACT

A contribution towards the knowledge base that potentially can lead to better management through improved productivity of the two iconic freshwater crayfish species of Western Australia - marron, *Cherax cainii*, and yabbies, *Cherax albidus* - has prompted this research. Five experiments on marron and yabbies under laboratory and field conditions were conducted to (1) quantify the structure and population sizes of the existing stocked yabby populations in outdoor ponds and the effects of abiotic and biotic factors associated with their seasonality, (2) evaluate the feed intake and foregut evacuation rates of crayfish in relation to various food types and body weight classes, and (3) understand the underlying mechanism of the weight-dependent moult stages in haemolymph physiology and exoskeleton composition.

The location of the pond, as well as biotic and abiotic factors, influenced the size and structure of yabby populations. Male yabbies were 2.5-3.0 times more abundant than females irrespective of the pond location and the trapping season. During the autumn-summer season, most of the studied nutrients from the pond bottom soil and water column were correlated with the abundance of yabbies. Dissolved inorganic nitrogen, soluble reactive phosphorus and the density of zooplankton had a higher correlation with the yabby population irrespective of the spatial and temporal variations in biotic and abiotic factors. The water pH and hardness were negatively related to the yabby population size.

Osmoregulatory capacity (OC), hepatosomatic indices and intra-moult intervals were significantly higher during intermoult stage (C), while the growth and moisture content of hepatopancreas were the highest in postmoult stage (AB). However, these differences between the species were evident only in crayfish larger than 15 g. The haemolymph constituents were dependent on the moult stage, body weights and feeding status. Variations in osmolality and haemolymph constituents (protein, glucose, K^+ and Cl^-) were highest in intermoult stage (C) and lowest in postmoult stage (AB). Apart from K^+ and Cl^-, which had the same concentrations, other haemolymph components differed between the fed and starved crayfish. Maximum haemolymph osmolality was recorded at 7-8 hours after feeding in both crayfish species.

Food consumption (FC) increased with the increase of crayfish weight, while food consumption index (FCI) was higher in smaller crayfish. The FC and foregut evacuation (FGE) of crayfish were food type and weight dependent; lower FC and FGE were observed in smaller crayfish and shrimp flesh. Irrespective of the weight classes and food types used, a significant 16.3% reduction in FGE in both marron and yabbies occurred 4-7 hours post-feeding at, compared to 7.7% at 1-4 hours post-feeding and 3.2% at 7-10 hours post-feeding. The foreguts from all weight classes was empty 10 hours after a single feeding event.

The exoskeleton compositions varied with various moult stages in both marron and yabbies. The ash content (44.7 ± 2.7%), chitin (35.4 ± 2.6%) and gross energy (18.8 ± 2.6 kJ g^{-1}) were highest during intermoult stage (C) and early premoult stage (D0), while the maximum level of protein (21.7 ± 1.8%) was found during postmoult stage (AB) and carbohydrate contents (34.7%) were highest at late premoult (D_2) in both crayfish species. A constant lipid level was found over all moult stages of a moult cycle. Protein, chitin, lipid and gross energy were weight class independent, while ash and carbohydrate were significantly higher in the larger crayfish. The percentage of dry exoskeleton to wet body weight of intermoult staged crayfish was both moult stage- and weight class-dependent, while it was species-independent.

The current research reveals that the structure of yabby populations from purpose-built ponds is defined by combined effects of water and bottom soil quality while biotic factors play a significant role. In conclusion, the moult stages, body weights, food types and feeding status are important variables that have an impact on the physiology of marron and yabbies. In addition, the selection of a suitable feeding strategy is based on food type, food consumption, gut evacuation and the seasons.

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LIST OF ABBREVIATIONS

AB	Postmoult
%	Per cent
µL	Microlitre
µm	Micrometre
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
C	Intermoult
CARL	Curtin Aquatic Research Laboratory
cm	Centimetre
CP	Crude protein
D	Premoult
DIN	Dissolved inorganic nitrogen
FC	Food consumption
FCI	Food consumption index
g	Gram
FGE	Foregut evacuation
HI_{dry}	Dry hepatosomatic index
HI_{wet}	Wet hepatosomatic index
HM%	Moisture content of hepatopancreas
kJ g^{-1}	Kilojoule per gram
L	Litre
LSD	Least Significant Difference
mg L^{-1}	Milligram per litre
mL	Millilitre
mOsm kg^{-1}	Milliosmole per kilogram

OC	Osmoregularoty capacity
OM	Degree centigrade
ºC	Degree centigrade
pH	$-\log_{10}[H^+]$
R_s %	Proportion of dry matter intake remaining in the foregut
SE	Standard error
SPSS	Statistical Package for the Social Science
SRP	Soluble reactive phosphorus
TAN	Total ammonia nitrogen
TN	Total nitrogen
TP	Total phosphorus
WA	Western Australia

LIST OF PUBLICATIONS

1. Ha, M.V. and Fotedar, R. 2016. The effects of biotic and abiotic factors on the abundance and population structure of yabbies, C*herax albidus* (Clark 1936) in outdoor earthen ponds. Freshwater Crayfish, Submitted.

2. Ha, M.V. and Fotedar, R. 2016. Haemolymph constituents and osmolality as functions of moult stage, body weight, and feeding status in marron, *Cherax cainii* (Austin and Ryan, 2002) and yabbies, *Cherax albidus* (Clark, 1936). Saudi Journal of Biological Sciences, in press.

3. Ha, M.V. and Fotedar, R. 2016. Osmoregulatory capacity, health status and growth as functions of moult stages from various weight classes in marron, *Cherax cainii* (Austin and Ryan 2002) and yabbies, *Cherax albidus* (Clark 1936). Marine and Freshwater Behaviour and Physiology, Accepted.

4. Ha, M.V. and Fotedar, R. 2016. Osmolarlity, food consumption and foregut evacuation rate in marron, *Cherax cainii* (Austin and Ryan 2002) and yabbies, *Cherax albidus* (Clark 1936) fed three different food types. Saudi Journal of Biological Sciences, Submitted.

5. Ha, M.V. and Fotedar, R. 2016. Exoskeleton composition as a function of the moult stages and body weight of marron, C*herax cainii* (Austin and Ryan, 2002) and yabbies, C*herax albidus* (Clark, 1936). Aquaculture International, Submitted.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND INFORMATION

Currently, the Australian freshwater crayfish aquaculture industry is based on three parastacid species: yabbies, *Cherax albidus* (Clark 1936), marron, *Cherax cainii* (Austin and Ryan 2002) and red claw, *Cherax quadricarinatus* (von Martens). The commercial potential of these species is well recognised internationally, and these species have been exported from Australia for market evaluation, aquaculture research and commercial trials (Holdich 1993; Medley et al. 1994; Austin 1995; Alonso 2010).

Marron and yabby are potential species for aquaculture in Western Australia (WA) (Piper 2000; Sang and Fotedar 2008) and have been targeted as candidate species for the development of inland aquaculture under the National Aquaculture Strategy (World Aquaculture Adelaide on 8 June 2014). Marron production is based on semi-intensive aquaculture in purpose-built ponds, while commercial yabby production is currently derived from harvesting of farm dams, especially in WA (Piper 2000). Recently, the production of marron and yabbies in WA has not significantly improved (Alonso 2010; ABARES 2014). Besides the lack of technical knowledge, the main reason for the stagnant production is the lack of unified farming management practices and the inherent nature of the industry, which is driven by 'lifestyle choices' of the growers rather than commercial production.

Currently, there is inadequate fundamental research on the underlying mechanism of the physiological changes occurring in the haemolymph and other tissues/organs of farmed freshwater crayfish and their influence from various moult stages (Sang 2010). This lack of knowledge is one of the restrictions not only for the advancement of knowledge in crayfish physiology but also for improving management protocols of crayfish aquaculture in Australia. The constraint has created a growing need for the WA crayfish industry to cope with understanding physiological responses from different tissues and organs in relation to dependent variables such as moult stage, body weight and nutritional condition.

The change in physiological and biochemical parameters as health indications of crustaceans (Charmantier et al. 1989; Lin et al. 1991; Young-Lai et al. 1991; Mayer

et al. 1992; Bambang et al. 1995a; Bambang et al. 1995b; Lignot et al. 1997; Lignot et al. 1998) is a function of multiple factors (Vargas-Albores and Ochoa 1982; Skinner 1985; Lignot et al. 1999; Lignot et al. 2000; Pratoomchat et al. 2002b; Pascual et al. 2006; Marcy et al. 2009). Osmoregulatory capacity (OC: difference between the osmotic pressures of the haemolymph and of the external medium), hepatosomatic indices, the moisture content of the hepatopancreas and growth rate have been commonly used to depict the physiological state of an animal and thus allow prediction of stressors such as moult stage, body weight, feed and feed regime (Lignot et al. 2000).

Unlike marron with semi-intensive farming systems of a single population cohort, yabbies naturally are farmed in farm dams (Piper 2000) with a multiple cohort population. Aquaculturists and fishers in Western Australia (WA) have noticed considerable changes in the seasonal yield of yabbies, water quality, recreational fishing practices and natural mortality among farm dams (Lawrence et al. 1998). However, there is limited published information available to quantify the relationship between productivity and biotic and abiotic factors in farm dam production systems for yabbies, except for the studies by Lawrence et al. (1998) and De Graaf et al. (2010). Both studies have focused on the effects of physio-chemical parameters, including seasonality, water quality and physical properties on the yield, returns and density of yabbies and the population structure of yabbies and marron in a number of farm dams in WA. No studies have been conducted on the relationship between crayfish population size and biological factors.

Moulting has significant implications for the growth and development of crayfish. A moult cycle is a highly complex process, the success of which requires precise coordination (Chang 1995). In crayfish, moult cycle consists of five distinctively defined stages known as postmoult (AB), intermoult (C), and premoult (D_0, D_1, D_2) (Ha and Fotedar, unpublished). Moult stages, together with body weights and nutritional conditions determine periodic changes in physical and biochemical compositions of important organs/tissues of a crustacean such as haemolymph and hepatopancreas (Heath and Barnes 1970; Richard 1980; Vargas-Albores and Ochoa 1982; Lignot et al. 1999; Cheng et al. 2002; Pascual et al. 2006; Marcy et al. 2009; Durliat and Vranckx 1982), exoskeleton (Drach and Lafon 1942; Welinder 1974; Ravichandran et al. 2009; Ekpenyong et al. 2013), and muscle (Huner et al. 1990;

Bilal et al. 2013). In return, changes in these constituents could be used as an indication for moult staging in crustacean (Chang and O'Connor 1983; Skinner 1985).

Crustacean has a hard exoskeleton which serves to protect their soft body parts from enemies and diseases (Nagasawa 2012). The main components of the exoskeleton are proteins, chitin, and some lipids (Willis 1987) which are responsible for the construction of complex structures of protein and chitin (Glynn 1968). Different layers of the exoskeleton have different content levels of protein and chitin (Vigh and Dendinger 1982; Roer and Dillaman 1984). Most of the previous studies on the composition of the exoskeleton have focused on investigating the composition of body parts in intermoult stage (Huner et al. 1996; Ekpenyong et al. 2013), hardened and the unhardened exoskeleton (Drach and Lafon 1942; Welinder 1974; Ravichandran et al. 2009; Ekpenyong et al. 2013), and shed exoskeleton (also known as exuviae) (Lasker 1966; Paranjape 1967; Ikeda and Dixon 1982; Segawa et al. 1983; Nicol et al. 1992). Compositions of crustacean exoskeleton have also been investigated for their uses in food processing for animals and humans and for improving soil quality (Lovell et al. 1968; Meyers and Rutledge 1971; Huner et al. 1996). Research on the biochemical compositions of crustacean exoskeleton in all the stages of a moult cycle is limited. Except for Chandumpai et al. (1991) worked on the biochemical compositions of the exoskeleton in the five moult stages of the tiger prawn and Pratoomchat et al. (2002a) worked on the mud crab in their eight moult stages, none has been done on yabbies and marron.

Achieving optimal growth with lowest feed requirements in cultured crayfish requires careful feed and feeding management (Jory 1995; Jory et al. 2001). Feeding strategy in aquacultured freshwater crayfish is determined by the consumption rate and foregut evacuation efficiencies, which also influence feed selection, time, rate, and frequency (Jory 1995). Food consumption and foregut evacuation are themselves influenced by a number of internal and external factors such as water quality, body weight, moult stage (Sedgwick 1979; Hill and Wassenberg 1992; Maguire and Allan 1992; Nunes and Parsons 2000; Wasielesky et al. 2003; Soares et al. 2005), food composition (Gonzalez-Pena et al. 2002), and food availability (Loya-Javellana et al. 1995). The rate of foregut clearance in Australian red claw and red swamp crayfish,

Procambarus clarkii has been reported to be useful for estimating daily feeding rates and food conversion ratio (Loya-Javellana et al. 1995; Simon and Jeffs 2008). At present, there is a lack of research related to maximum feeding rate as determined by foregut evacuation in marron and yabbies in a single feeding event under the controlled laboratory conditions, despite their relevance for the formulation of an optimal diet and the development of effective feeding strategies (Simon and Jeffs 2008).

Despite the importance of physiological knowledge in improving the development of the freshwater crayfish industry in Australia, relatively limited information is available, which specifically addresses: (1) yabby population structure in outdoor ponds and the effects of water and soil abiotic and biotic factors on yabby populations, (2) feed consumption and foregut evacuation of crayfish in relation to various food types and body weight classes, (3) dependence of moult stage and body weight of crayfish on physiological indications and compositions in crayfish tissues such as the haemolymph, hepatopancreas and exoskeleton.

1.2 AIM

To understand the moult stage- and body weight-related physiology of freshwater crayfish farmed in Western Australia.

1.3 OBJECTIVES

The above aim can be achieved by fulfilling the following specific objectives:

1. To estimate yabby population size and structure over a season and year in three outdoor earthen ponds in Western Australia.
2. To investigate the effect of physicochemical and biological factors on yabby populations.
3. To study the effect of moult stage and body weight on osmolality, hepatosomatic indices, body weight increment, moult of marron and yabbies under controlled laboratory conditions.
4. To evaluate the concentration of haemolymph constituents of marron and yabbies in different moult stages, weight classes and nutritional conditions.

5. To assess the food consumption rate of marron and yabbies of various weight classes fed with different food types.

6. To determine the evacuation rate of ingested food from the foreguts of marron and yabbies in different body weight classes fed different food types and food types at various times post-feeding.

7. To evaluate the effect of food type on the concentration of haemolymph osmolality in marron and yabbies.

8. To quantify biochemical compositions of exoskeletons in different moult stages and body weight classes of marron and yabbies.

9. To provide recommendations for further research based on the outcomes of the current research with the aim of understanding physiological variations during a moult cycle for better management and development of crayfish aquaculture in Australia.

1.4 SIGNIFICANCES

The significant outcomes of the research are highlighted below:

1. The study will assist in understanding the mechanisms of the role that moult stage and feeding physiology play in freshwater crayfish aquaculture.

2. The present study will contribute to understanding the relationship between haematological physiology and growth governed by various moult stages.

3. The research has established a matrix and quantified the intricate relationships among crayfish haemolymph physiology, moult stages, feed types, nutrient deprivation, gut evacuation rates, exoskeleton and weight classes of yabbies and marron.

4. The research will add to the selection and further use of crayfish exoskeleton as a source of nutrients.

5. The research has widened the use of mark-recapture methods in man-made crayfish farming systems in order to relate the population structure to biotic and abiotic factors.

6. The findings of this study may be used as references for other related studies in other commercial decapod crustacean aquacultures.

CHAPTER 2: LITERATURE REVIEW

2.1 CRAYFISH BIOLOGY

2.1.1 Brief overview of marron and yabbies

The two main types of crayfish studied in the current project are marron, *Cherax cainii* and yabbies, *Cherax albidus/destructor*. Structurally, marron has five keels on their head surface and two small spines on the telson, which makes them readily distinguishable from other Cherax species. By contrast, yabbies, another *Cherax* species common in Victoria, have four keels along the head and no spines on the telson, but they have long and large chelipeds (Bryant and Papas 2007).

The following describes the systematic classifications of marron and yabbies.

"Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Pleocyemata

Infraorder: Astacidea

Family: Parastacidae

Genus: Cherax"

Species: *C. cainii* (Austin and Ryan 2002) and *C. albidus* (Clark 1936)

There are two discrete forms of marron, a hairy form and a smooth form, which used to be considered as subspecies (Austin and Knott 1996) up till 2002 when they were proved to be distinct species by the allozyme evidence (Austin and Ryan 2002). The hairy form is scientifically named *C. tenuimanus*, while the smooth form is known as *C. cainii*. The hairy form is found exclusively in the Margaret River in WA, while the smooth form are widely distributed (Austin and Ryan 2002) due to their farming potential.

Marron is the world's third largest freshwater crayfish native to Australia and could grow up to 2 kg in weight (Morrissy 2000). Due to its attractive attributes, such as rapid growth (40-120 g $year^{-1}$), delicate flavour, a non-aggressive and non-burrowing behaviour, a simple life cycle, and ability to be transported alive in international markets (Morrissy 1976; O'Sullivan 1988; Lawrence 2005), marron has been recognised as a potential aquaculture species (Rouse and Kartamulia 1992).

The common habitats for marron are naturally clear, deep running water with shelters (Merrick and Lambert 1991; Mosig 1998; Wingfield 1998; Molony et al. 2004). Burrowing is not characteristic of marron (Clunie et al. 2002) even though it has been reported that in areas where refuge habitat is limiting, marron may burrow in the banks of dams (Mosig 1998). Marron farming has been particularly successful in aquaculture ponds and clay bottomed farm dams (Morrissy 1976; Morrissy et al. 1990; Merrick and Lambert 1991; Lawrence 1998; Mosig 1998; Wingfield 1998), where materials such as tyres, rope fibre, pipes, etc. are often added to the dams to provide substrate for refuge and shelter (Mosig 1998).

According to Mosig (1998) and Tay et al. (2007), the common predators for marron are cormorants, water rats, tortoises and fish, but they have also been reported to be cannibalised by larger marron, especially during moulting (Merrick and Lambert 1991). It is thus important that refuge habitat and shelters are made available for marron during the early stage of the development cycle. The common food for marron includes decaying and dead organic materials, but marron may also feed on invertebrates, aquatic vegetation, and even fish (Bryant and Papas 2007).

Another Australian farmed freshwater crayfish, yabby is the most popular species in Australia (Merrick and Lambert 1991). Their distribution can extend over 2 million km^{-2} (Sokol 1988), encompassing various climate zones, from the dry climates of the outback to the cool climates of the south. Yabbies are found in wider habitats. They are commonly found in lakes, billabongs, swamps, irrigation canals, farm dams, and bore drains (mostly still, warm waters), but they can also be found in slow, muddy rivers and creeks. Yabbies are especially resilient and can survive years of drought by burrowing. Yabby density in farm dams can be as high as 5 m^{-2} and standing stocks could be up to 340 kg ha^{-1}.

Promoted in the mid-1970s by Dr. Noel Morrissy (Lawrence, 1998b), marron farming in Australia has increased significantly since the 1980s (O'Sullivan, 1988). For example, by 2014, in Western Australia alone, there were around 35 marron farms, producing 60 tonnes (Alonso 2010; ABARES 2014). Due to its popularity and increasing international interests, marron was recently introduced into Zimbabwe, South Africa, USA, Japan, the Caribbean and China as a commercial aquaculture species and has been distributed widely (Bryant and Papas 2007). Marron biology along and farming practices in Western Australia have been review by Fotedar et al. (2016).

Feeding classification of freshwater crayfish has been varied. For example, D'Abramo and Robinson (1989), Momot et al. (1978), Lorman and Magnuson (1978), Sokol (1988) and Tacon (1993) classify them as polytrophic omnivores, since their diet has been found to contain a significant amount of detritus (Woodland 1967; Mason 1975; Momot et al. 1978; Wiernicki 1984; Goddard 1988; Growns and Richardson 1988; McClain et al. 1992; O'Brien 1995). Momot (1995), while leaning toward the classification of freshwater crayfish as polytrophic omnivores, also proposed that they can be described appropriately as obligate carnivores because freshwater crayfish consumes detritus and plant- based matter to make up for their deficiency in animal protein. O'Brien (1995) on the other hand, argues that marron is most appropriately described as "microphage detritivores" with the possibility of being opportunistic omnivores.

2.1.2 Life cycle and reproduction

Unlike the other species of decapod crustaceans, crayfish do not have a larval stage in the life cycle (Holdich 1993). Prawns and shrimps typically go through 11-12 larval stages from the juveniles to adults, each requiring different diets. After crayfish hatch out of the egg, they already resemble crayfish and are equipped for survival. By the time they leave the mother, they can feed on most plant and animal foods, thus making culturing of crayfish much easier than culturing of prawns and shrimps.

Fertility of crayfish, however, is quite low compared to shrimp and prawn. For example, while shrimps can produce between 200,000 and 1,000.000 eggs and giant

river prawns about 80,000 eggs, cultivated crayfish may only produce between 100 and 1,000 eggs, depending on the species (Lee and Wickins 1992).

According to Holdich and Reeve (1988), crayfish usually mate in autumn. The male deposits a spermatophore on the female, which is then partially dissolved by the female abdomen fluid. The fertilised eggs, which are attached to the female's abdominal appendages, stay there throughout winter. When the juveniles hatch out of the eggs in spring, they are still attached to the mother. They become active after one moult and after a few days, they leave the mother to become independent, although due to a pheromonal bond they are often attracted back to the mother (Holdich 1993). However, when this bond wears off, the mother treats the juveniles as food. It is thus crucial that the juveniles are separated from the mother in hatcheries.

Spawning of Western Australia's marron occurs between August and November. The highest percentage of berried females occurs in late August and September, up to 96%, compared to 50% in September and 11% in December (Morrissy 2000). Juveniles' release from berried females occurs in late November and early December. After spawning, female gonads recover quickly. In 81% of mature females, their gonads reach maturity in March.

Breeding season for yabbies is spring when the water temperature reaches 15^{o}C to 16^{o}C and may continue into autumn if the temperature remains high enough. In warmer waters, however, yabbies breed throughout the year. Depending on the water temperature, the eggs take from 3 to 10 weeks to hatch. In summer, when the water temperature is high, the eggs take only 3 to 4 weeks to hatch, but the first batch of eggs in spring takes 8 to 10 weeks to hatch. The female normally is ready to breed again three weeks after hatching, once the young have left (Mosig 1998; Morrissy 2000).

2.1.3 Environmental tolerances

Tolerance data is needed to accurately predict the possible success of crayfish production at a particular site, especially for man-made water bodies. However, tolerance data is a limited tool to predict habitat suitability over a broader spatial scale "due to the variability in physicochemical parameters between different water bodies and reaches, even within the same region" (Bryant and Papas 2007).

According to Morrissy (1978), marron has a greater tolerance to salinity than yabbies due to the comparatively high natural salinity concentrations in Western Australia. Marron's favourite habitat is in flowing water, which typically contains higher oxygen levels. This may account for the fact that, of the three species, marron tolerate the lowest level of low dissolved oxygen concentration. Marron prefers cooler climate with an optimal temperature of 17.5°C to 24.5°C. However, Morrissy et al. (1990) found the highest distribution of marron in the temperature extremes of 8^{o}C and 26^{o}C. Yabbies, by comparison, display a greater tolerance range, accounting for the success of yabby production in aquaculture across Australia. Refer to Table 2.1 for the summary of the environmental tolerances for marron (Shipway 1951; Morrissy et al. 1990; Merrick and Lambert 1991; Lawrence 1998; Mosig 1998) and yabbies (Mills and McCloud 1982).

Table 2.1: Summary of the environmental tolerances for marron and yabbies, adapted and modified from Bryant and Papas (2007)

Species	DO (mg L^{-1})			pH		Water temperature (^{o}C)				Salinity (%)		
	Optimal	distress	Death	Low	High	Optimal	Min	Max	Stress	Growth Impaired	Max	Death
Marron	>6.0	2.5	0.9	6.8	8.0	17-24	8.0	28.6	-	0.7	1.7	-
Yabby	5.0	2.0	-	7.0	8.9	22-27	1.0	35.5	34	0.6	1.5	2.1

The tolerance data presented above is for aquaculture conditions. The data may not accurately represent tolerances in the natural environment. It has not been possible to find tolerance data under specific conditions in natural waters.

2.2 NUTRITIONAL REQUIREMENTS

In general, previous studies have found that for optimal growth rates in crayfish, their diets should contain at least 20% protein, ideally 30%. Tsvetnenko et al. (1995), for example, observed that marron reached the highest growth rates when fed with 20% or 30% protein diets. Similarly, Hubbart et al. (1986) found that freshwater crayfish achieved the best growth when their diets contained a protein level of 30%.

Improvement in the growth of yabbies was also achieved when the pellet contained 30% protein, whereas a pellet containing 15% protein resulted in nutritional stress and slower growth (Jones et al. 1997). Apart from being a source of energy, protein supplies essential amino acids such as histidine, arginine, isoleucine lysine,, leucine, phenylalanine, methionine, tryptophane, threonine, and valine (Claybrook 1983; D'Abramo and Robinson 1989). In the artificial diets, the sources of protein normally come from blends of fish meals, soybean meals, and shrimp meals (Tarshis 1978; D'Abramo et al. 1985).

Freshwater crayfish tolerate low levels of lipids in their diets (D'Abramo et al. 1985; Goddard 1988; Fotedar et al. 1997). Lipid levels exceeding 8% have been found to inhibit their growth (Andrews et al. 1972; Foster and Beard 1973; D'Abramo 1979; Davis and Robinson 1983). The adequate level of lipid content for crayfish is 0.1 to 3% if it contains fatty acids as linoleic (18:2 n-6), cholesterol (0.4% dry weight), docosahexaenoic (22:6 n-3), and eicosapentaenoic (20:5 n-3) (Zandee 1966; D'Abramo et al. 1985; Lee and Wickins 1992). The crustacean gets their common sources of lipids from lecithin and marine or plant oils (Tarshis 1978; Huner and Lindqvist 1984).

In addition to protein and lipid profiles, the protein-to-energy (P: E) ratio (mg protein per kcal) for optimal growth also needs to be taken into account (Lee and Wickins 1992). Lee and Wickins (1992) suggested a P:E ratio of 63 to 117 at 25-35% protein level and 3-4 kcal g^{-1} energy level, whereas D'Abramo and Robinson (1989) proposed that the P:E ratio in the crustacean diet should be close to 120 (30% protein and 2,500 kcal kg^{-1}).

Freshwater crayfish also requires carbohydrates in their diets, even though the requirements are somewhat unclear. According to D'Abramo and Robinson (1989), the natural crayfish diets readily contain high levels of carbohydrates, while the commercial diets normally contain 25% or more carbohydrate. Carbohydrates in crayfish diets are commonly obtained from soluble polysaccharides and starch (Hubbart et al. 1986).

2.2.1 Natural food for crayfish

Crayfish are able to derive nourishment from many organic substances naturally found in the aquaculture systems, such as aquatic plants, aquatic organisms, pellets, planktons, benthic animal, algae, etc. According to Mitchell et al. (1995), O'Brien (1995), and Tidwell et al. (1996), these natural foods form an important supplemental source of nutrients for crayfish. In the semi-intensive farming systems, crayfish can derive up to 50% of its nutrition from these sources (Apud et al. 1983; Lee and Wickins 1992).

Not all crayfish species have the same dietary preferences and the selection of natural foods, or a combination of foods has implication on the production of crayfish. For example, Avault and Brunson (1990) found that red swamp crayfish, *Procambarus clarkii* production increased substantially when fed with a combination of rice forage and pellets (2,016 kg ha^{-1}), compared with a production of just 881 kg ha^{-1} when fed on pellets only and 1,274 kg ha^{-1} when fed on rice forage only. In extensive or semi-intensive pond culture systems, the nutrition is derived from detritus-based ecosystems created by growing plants in the ponds, with the addition of alfafa or hay pellets to provide the basis for the production of plant detritus (Mills et al. 1994). Plant detritus is also used as the basis of nutrition for marron in the farms (Morrissy 1992b). In Europe, crayfish culture is based on the production of a variety of aquatic organisms such as plankton, benthic animal, algae, plant, etc., which are naturally found in the ponds, with the occasional substitution of fish pellets, fish meat, or vegetable matter (Ackefors and Lindqvist 1994)

In other studies, Jones et al. (1995) and Verhoef et al. (1998) reported that crayfish achieved fastest growth rates when their diets consisted mainly of zooplankton either in live or frozen form. Similarly, marron and yabbies of different body weight classes prefers live zooplankton as *Daphnia* spp. to commercial pellet if both food types are presented (Meakin et al. 2008; Meakin et al. 2009).

2.2.2 Feed and feeding for crayfish

It makes economic sense to obtain optimal growth in cultured crayfish with the lowest feed requirements. In order to achieve this, feed and feeding management are (Jory 1995; Jory et al. 2001). Marron and yabbies are polytrophic omnivores

(Lorman and Magnuson 1978; Momot et al. 1978; Sokol 1988; D'Abramo and Robinson 1989; Tacon 1993), thus they can efficiently consume formulated feeds that contain varied levels of protein from diverse sources (Jones 1997), such as dietary manipulations (Hai et al. 2009; Ambas et al. 2015b) and/or dietary supplementation of feed additives (Sang et al. 2011; Nugroho and Fotedar 2013). These have been used to improve the growth performances of marron and yabbies. However, effective feed management protocols in crayfish aquaculture have not been established due to the lack of research on size- dependant feeding.

In the consideration of feeding and ingestion rates of crayfish, food consumption (FC) and foregut evacuation (FGE), the two essential parameters, need to be frequently measured (Loya-Javellana et al. 1995), since their efficiencies affect the feed management ingredients including the selection of feed types and quantity, and feeding time, methods and frequency, as well as allow the estimation of the feeding strategy for freshwater crayfish aquaculture (Jory 1995; Loya-Javellana et al. 1995; Simon and Jeffs 2008). It is thus crucial to study the relationship between body weight and food type with FC and FGE in order to achieve an efficient feed management, while avoiding overfeeding and sub-optimal growth in crayfish (Simon and Jeffs 2008).

Food consumption differs between crayfish species and is influenced by many internal and external factors, including moult stages, body weight and water quality (Hill and Wassenberg 1992; Maguire and Allan 1992; Nunes and Parsons 2000; Gonzalez-Pena et al. 2002; Wasielesky et al. 2003; Soares et al. 2005; Simon and Jeffs 2008). Although some studies have found a relation between FC and body weight of crustaceans (Sedgwick 1979; Nunes and Parsons 2000; Simon and Jeffs 2008), others have found conflicting results among species and experimental conditions, whereby body weight increases have been linked with both declining and increasing feeding rates (Sedgwick 1979; Hunter et al. 1987; Nunes and Parsons 2000).

Despite the vast studies on FC and FGE for many crustacean species, there are a few studies for freshwater crayfish species. These are limited to Australian red claw crayfish (Loya-Javellana et al. 1995), red swamp crayfish under controlled laboratory

conditions (Huner and Meyers 1979), and yabbies under outdoor pond environment (Jones 1997).

Several factors need to be considered when it comes to artificial diets for crayfish, of which leaching is the most important factor and has a direct implication on nutrients in pelleted diets (Farmanfarmaian et al. 1982; Cuzon et al. 1994). It is important to minimise and control the bleaching, which is often done in industrialised food production by the use of binders or ingredients with binding agents (Goldblatt et al. 1980; Heinen 1981; D'Abramo and Robinson 1989; Meyers 1991; Gadient and Schain 1994). According to Lee and Wickins (1992), it is possible to bind the diets without costly ingredients. Cheap sources for protein such as soybean meal and cottonseed should be avoided since they contain anti- nutritional factors (Jones et al. 1997). Pellet quality and stability are needed to enhance the growth of crustaceans (Meyers 1991; Chen and Yenn 1992). Sterols also need be added to artificial diets of crayfish to provide the necessary external source of cholesterol (D'Abramo and Conklin 1985; Howell and Matthews 1991).

Anson and Rouse (1996) suggested the combination of diets to provide adequate nutrition for optimal growth after observing the fastest growth rates in red claw fed with combinations of Artemia and commercial shrimp or catfish diets. Anson and Rouse (1996) also cautioned the danger of suboptimal nutrition in using single commercial diets, which could lead to deaths at moult, described by Bowser and Rosemark (1981) as “moult-death syndrome”. This moult-death syndrome has also been observed in Homarid species and shrimp by D'Abramo and Conklin (1985). Anson and Rouse (1996) emphasised adding bacteria, zooplankton or detritus to diets for juvenile crayfish, particularly for those diets that contain only yolk sac derived substituting nutrition.

2.3 GROWTH IN CRAYFISH

2.3.1 Moult cycle and weight gain at moult

Moulting (also known as ecdysis) is a highly complex process. It requires precise coordination of various physiological events (Chang 1995) and is influenced by a number of environmental and endogenous factors including temperature (Hughes et al. 1972; Chittleborough 1975), food supply (Chittleborough 1975), various stressors

(Weis et al. 1992), photoperiod (Quackenbush and Herrnkind 1983), space (Cheng and Chang 1994), water condition, developmental stages, reproductive maturity, and status of limb regeneration (Skinner 1985; Cheng and Chang 1994). Moulting regulates physiological and biochemical changes of various tissues such as haemolymph and hepatopancreas (Heath and Barnes 1970; Richard 1980; Durliat and Vranckx 1982).

The moult cycle consists of a complex series of physiological and morphological stages. Despite the various terminologies proposed to describe these stages, in general common moult stage categories are often known as A, B, C and D. Many studies, however, describe crayfish moult cycle in five stages, known as A, B, C, D, and E, with several sub-stages within them (Drach 1944; Travis 1965; van Herp and Bellon-Humbert 1978; Lowery 1988; Aiken and Waddy 1992).

According to Drach (1944), stage A begins once a crayfish has shed off the exuvium and are already moving. This stage takes only 2% of the moult cycle. During this stage, crayfish absorb a substantial amount of water, which expands their volume up to 50%; the tips of their cheliped also emerged, and the cutting edges of the mandibles and maxillipeds are hardened.

The next stage (B), which is sometimes not a clear separation from stage A, occupies 8% of the moult cycle, during which crayfish are immobile. This stage is characterised by the deposition of endocuticle layer (van Herp and Bellon-Humbert 1978). Stage B ends when the chemical changes in the epicuticle, exocuticle (i.e. the preexuvial layers) are completed (Drach 1939).

Stage C is the intermoult period, the longest moult stage, which takes up to 65% of the moult cycle and has four sub-stages in itself (C_{1-4}). In this stage, crayfish shell is hardened, and the exoskeleton is mineralized and becomes rigid. Stage D (the premoult) occupies roughly 24% of the moult cycle, during which crayfish sever pore canals, accumulate reserves and synthesise the two pre-exuvial layers of the new cuticle. Stage D consists of five sub-stages (D_{0-4}).

Stage E is the moult itself (also known as ecdysis), typically divided into active and passive phases. The passive phase is indicated by decalcification of epidermal sutures, water absorbance and redistribution, and the beginning of the bulging of the

membrane as the pressure increases the thoracoabdominal. If conditions are not suitable for moulting, crayfish may lengthen the passive phase. The rupture of the membrane marks the commencement of the active phase, after which the moult cycle is completed, and crayfish enter stage A.

Crayfish continue to grow in both length and weight throughout different stages of the moult cycle, and the growth is not equal in different stages. The weight gain is the most significant during the moult itself, with crayfish gaining 30-60% of their premoult weight due to extra water stored in the crayfish tissues, thus leading to increases not just in weight but also in size and volume. Weight gain during intermoult, on the other hand, is typically less than 5% of the immediate postmoult weight, since at this stage crayfish turns water content into tissue growth and reduce the water content of haemolymph (Sardà and Cros 1984). It has been noted, though, that during intermoult period the resources for growth are gathered (Lowery 1988; Aiken and Waddy 1992).

2.3.2 Environment and growth

An environmental factor that bears the most significant implication on the growth rate and development rate of crayfish is temperature (Lowery 1988; Gydemo 1989) due partly to the changes in food consumption (Söderbäck et al. 1987; Seals et al. 1997). Crayfish obtain the highest growth rate when the temperature is within the optimal range (Jones 1990; Jones 1995; Austin 1995), typically from 17°C to 27°C for different strains of marron. At this temperature range the growth reaches over 50% of the maximum growth (Morrissy 1990). The optimal temperature for the fastest growth rates of noble crayfish and signal crayfish ranges from 20°C to 23°C (Huner and Lindqvist 1984; Järvenpää et al. 1996), even though the optimal temperature for production could be lower (17°C-21°C). Temperatures below the optimal range would inhibit growth, whereas temperatures above the optimal range would result in stress and increased mortality (Morrissy 1976; Kartamulia and Rouse 1992; Jussila 1995; Järvenpää et al. 1996).

Population density is another factor affecting freshwater crayfish growth (Morrissy 1975; Lowery 1988; Gydemo 1989; Morrissy 1992a; McClain 1995b; Morrissy et al.

1995a; Morrissy et al. 1995b; Whisson 1995). Higher population density has been found to result in slower growth (Morrissy 1975).

Photoperiod has also been found to have some effect on growth rates of crayfish, even though the effect is not significant (Sáez-Royuela et al. 1996). Several studies have found a co-relation between an increase in the photoperiod and an improvement in both growth and survival (Westman 1973; Mason 1979; Taugbøl and Skurdal 1992; Farhadi and Jensen 2015).

Other environmental factors that need to be considered for maximisation of growth in crayfish include the level of dissolved nitrites, ammonia, pH, and dissolved oxygen. To maximise growth in crayfish, the level of dissolved nitrites has been suggested to be kept close to the minimum detectable levels (Rouse et al. 1995; Liu et al. 1995), while ammonia concentrations is kept at less than 0.01 mg L^{-1} (Lourey and Mitchell (1995). pH should not be kept low, since low pH inhibits calcium uptake which inhibits growth (Wheatly 1996). According to Chittleborough (1975) dissolved oxygen saturation should be over 60% for optimal growth. Dissolved oxygen saturation below 40% would lead to an increase in mortality, while DO of between 40-60% inhibits the growth.

2.4 HEPATOPANCREAS AND CRAYFISH CONDITION

The hepatopancreas is a large organ located in the cephalothorax of the crayfish, above the midgut (Holdich and Reeve 1988). It has two lobes in the front and the third to the back of the carapace (Huner and Barr 1991). Hepatopancreas plays a significant role in the metabolism of the crustaceans (McLaughlin 1983), as further demonstrated in Section 2.4.1. Changes in the weight and composition of the hepatopancreas have been found to be co-related with ontogenetic development, growth, reproduction, moult stages, and nutrient intake (Jones 1997).

2.4.1 Role of hepatopancreas in crustacean metabolism

The hepatopancreas plays an important role in digesting, absorbing, and synthesizing digestive enzymes and carbohydrate (McLaughlin 1983). It functions as the main digestive gland, responsible for absorbing nutrients from the digestive track and excreting fluids that are needed to break down the nutrients (Goddard 1988; Holdich

and Reeve 1988). Also being the largest digestive organ, it also absorbs and stores lipids derived from food (Passano 1960; Adiyodi 1969). The hepatopancreas is also the main energy reserve in crayfish (Suprunovich et al. 1983; Huner and Lindqvist 1985). Hepatopancreas energy content affects the annual cycle, growth, and moulting in freshwater crayfish (Speck and Urich 1969; Huner and Romaire 1990), while its wet and dry weight and moisture content provide an indication of crustacean condition (Mannonen and Henttonen 1995; McClain 1995a, b). In noble crayfish, the hepatopancreas wet and dry weight have been found to be influenced by moult stages (Lindqvist and Louekari (1975).

2.4.2 Hepatopancreas response to moult

The hepatopancreas experiences different changes during the moult cycle. Its contents such as protein, lipid, and glycogen (known as organic reserves) vary during moult (Passano 1960), which leads to significant changes in the hepatosomatic indices (HI) and moisture content (Magalhães et al. 2012; Tian et al. 2012; Sugumar et al. 2013; Durliat and Vranckx 1982). The significant variation in the HI has been observed in bristled river shrimp, *Macrobrachium olfersii* (Magalhães et al. 2012). In crustaceans, the value of HI has been found to peak during the intermoult stage due to an increase in the reserves and decreases at the premoult stage due to a decrease in the reserves (Passano 1960). The lipid content has been shown to decrease at premoult, whereby fat is converted into sugar, and increase during the postmoult stage when the reserves are again accumulated (Passano 1960). The transfer of the reserves of the hepatopancreas during the moult cycle has been supported by other studies (Adiyodi 1969; Adiyodi and Adiyodi 1972; Kyomo 1988; Yamaguchi 2001; Marcolin et al. 2008).

Various changes in the contents of lipid, protein, and carbohydrate of the hepatopancreas during the moult cycle have also been observed in crabs (Heath and Barnes 1970) and other strains of crayfish (Durliat and Vranckx 1982). The content of free amino acids of the hepatopancreas have also been observed to fluctuate in the premoult stage (Richard 1980).

2.4.3 Hepatopancreatic indices as indicators of crustacean condition

Hepatopancreatic indices such as hepatopancreas moisture content (HM) and HI are good indicators of crustaceans' health condition (Huner and Lindqvist 1985; Huner and Romaire 1990; Evans et al. 1992; Villagran 1993; McClain 1995a, b; Mannonen and Henttonen 1995; Lignot et al. 2000; Magalhães et al. 2012). Cockcroft (1997), for example, successfully estimated the differences in the growth rates among wild West Coast rock lobster populations through examining their HI and HM. Similarly, Musgrove (1997) was able to measure the differences in the condition of southern rock lobster by measuring their HI and HM under different feeding treatments. Mannonen and Henttonen (1995) also found that HM, together with energy content, could be used to evaluate crayfish condition. However, they cautioned that other environmental parameters, such as food resources, population density, and water quality would have to be considered when interpreting the results.

HM in crayfish has been found to be directly affected by diet and feeding rate (McClain 1995a, b). The water content of crayfish has been found to be negatively co-related with the feeding rate. Gu et al. (1996), for instance, found that starvation increased the water content of the whole body in juvenile red claw crayfish. Evans et al. (1992), on the other hand, found a positive relationship between prolonged starvation and decrease in HI in marron. They also reported that the HI of farmed marron was higher than that of wild marron, which may be accounted for by the fact that farmed marron was fed more intensively than wild marron (Jarboe and Romaire 1995). This higher feeding rate was also found to result in higher storage of energy reserves in the hepatopancreas of farmed crayfish than that of wild crayfish in (Jarboe and Romaire 1995; McClain 1995b). Starvation has also been shown to decrease crustacean hepatopancreas mean weight. For example, Stewart et al. (1967) found that hepatopancreas mean weight of lobsters after 140 days of starvation decreased from 5.2% of to 2.6%, while the hepatopancreas mean weight of their fed counterparts was 5.0%.

The content of HI and HM and energy reserve is also a good indication of the reproductive maturity of female crayfish (Lindqvist and Louekari 1975; Huner and Lindqvist 1985). According to Huner and Romaire (1990), HM in reproductively active females is higher (80%) than that in reproductively inactive females (60-70%).

In another study, Lindqvist and Louekari (1975) found that the wet hepatosomatic index of reproductively mature female crayfish were lower than those of smaller sized immature ones.

2.5 HAEMOLYMPH OSMOLALITY OF FRESH WATER CRUSTACEAN

2.5.1 Osmoregulatory capacity in crustacean

Osmoregulatory capacity (OC) is defined as "the difference between the osmotic pressures of the haemolymph and the external medium at a given salinity" (Charmantier et al. 1989). The measurement of haemolymph osmolality allows for the evaluation of OC and this has been carried out successfully in numerous crustaceans, such as the small isopod and gammarid species (Charmantier 1975; Einarson 1993), peneid shrimps (Williams 1960; Pannikar 1968; Bursey and Lane 1971; Castille and Lawrence 1981b, a; Dall 1981; Ferraris et al. 1987; Lin et al. 1991; Bambang et al. 1995a; Bambang et al. 1995b; Lignot et al. 1997; Lignot et al. 1998; Lignot et al. 1999), crabs (Warburg et al. 1987; Diamond et al. 1989) , and homarid lobsters (Dall 1970; Thuet et al. 1988; Young-Lai et al. 1991). The osmolality of haemolymph in crustacean varies with moult cycle and body weight (Vargas-Albores and Ochoa 1982; Lignot et al. 1999; Pascual et al. 2006; Marcy et al. 2009). It tends to increase in premoult and decrease in postmoult (Baumberger and Olmsted 1928; Travis 1955; Robertson 1960; Glynn 1968; Bursey and Lane 1971; Mantel and Farmer 1983; Towle and Mangum 1985; Ferraris et al. 1987; Chen and Cheng 1993; Chen and Chia 1997; Lignot et al. 1999; Cheng et al. 2001; Cheng et al. 2002; Galindo et al. 2009).

OC is an important bio-indicator. It predicts crustaceans' stress levels and culture environment pollutants as well as defines moult stages in many crustaceans (Chang and O'Connor 1983; Skinner 1985). Many studies have examined the OC changes in number of decapod crustaceans under various experimental conditions and recorded its relationship with various elements such as heavy metals (Bambang et al. 1995a; Bambang et al. 1995b), oxygen depletion (Charmantier and Soyez 1994), and ammonia levels (Lin et al. 1991; Young-Lai et al. 1991). However, no studies have been conducted on the variations of OC during moult cycle and its relationship with health conditions in marron and yabbies.

2.5.2 Haemolymph constituents in different stages of crayfish

The haemolymph is the largest tissue in a decapod. It contains many different constituents, whose quantity and concentration change in different stages of the moult cycle. According to Hagerman (1983) and Mangum et al. (1985), the concentration of hemocyanin, a major component of the haemolymph, decreases in postmoult and rises towards premoult. Similarly, haemolymph lipid level also increases significantly during premoult in comparison with postmoult (Spindler-Barth 1976). Observation on the concentration of haemolymph glucose during premoult and postmoult has been mixed. While Galindo et al. (2009) reported a lower concentration of haemolymph glucose during intermoult and premoult stages, Telford (1968) observed that haemolymph glucose concentration was generally higher during premoult than postmoult. The postmoult decrease in glucose concentration was also observed by Meenakshi and Scheer (1961) and Hornung and Stevenson (1971).

Other constituents of the haemolymph that have been found to change during the moult cycle include protein concentration (Cheng et al. 2002) and electrolyte levels (Towle and Mangum 1985; Mercaldo-Allen 1991; Sugumar et al. 2013) in giant river prawn, *Macrobrachium rosenbergii* and blue crab, *Callinectes sapidus* and Cl^-, Na^+, K^+, Ca^{2+} and Mg^{2+} in mud crab, *Scylla serrata* (Chen and Chia 1997).

Changes in haemolymph constituents during the moult cycle provide good indication of health status in a number of crustaceans (Charmantier and Soyez 1994; Lignot et al. 1999; Lignot et al. 2000; Charmantier-Daures and Vernet 2004), such as their body weight and nutritional status (Vargas-Albores and Ochoa 1982; Skinner 1985; Lignot et al. 1999; Pratoomchat et al. 2002b; Pascual et al. 2006; Marcy et al. 2009). The biochemical changes during moulting, as well as feeding and starvation, indicate the nutritional requirements in crustaceans and provide an important basis for determining suitable diets (Sugumar et al. 2013). It is thus important to monitor and understand the physiological and biochemical changes during moult stages and their relations with body weight and nutritional status to manage aquaculture crayfish more efficiently, especially in marron and yabbies, where studies on their physiological and biochemical changes during moult stages and their relations with body weight and nutritional status are still rare.

2.6 CRUSTACEAN EXOSKELETON

2.6.1 Physical structure of exoskeleton

According to Skinner (1985), the crustacean exoskeleton has two main layers, the exterior layer known as epicuticle and the interior layer called procuticle. The procuticle consists of three sub-layers, exocuticle, endocuticle, and membranous. The epicuticle and exocuticle are combined before ecdysis to form the so-called preecdysial layer, while the endocuticle and the membranous layer are synthesized after ecdysis to form the so- called post-ecdysial layer.

The cuticle includes both inorganic and organic materials in a 60:40 ratio. The inorganic materials include calcium, copper, manganese, chloride, phosphorus, magnesium, sulfur, and potassium (Roer and Dillaman 1984; Mangum 1992; Compere et al. 1993; Pierce et al. 2001; Pratoomchat et al. 2002b; Wang et al. 2003). The organic materials include protein, glycosaminoglycans, carbohydrate, glycoprotein, mucopolysaccharides, lipid, and chitin (Glynn 1968; Vigh and Dendinger 1982; Roer and Dillaman 1984; Wheeler and Sikes 1984; Marlowe et al. 1994; Andersen 1999; Roer et al. 2001; Pratoomchat et al. 2002a).

2.6.2 Proximate composition of exoskeleton over a moult cycle

The main components of the cuticle of crustaceans are chitin, proteins, and lipids (Willis 1987). The content of these components varies over the moult cycle. The protein content increases significantly during postmoult (Pratoomchat et al. 2002b), when the cuticle is unhardened (Drach and Lafon 1942), decreases during promoult, and drops to the lowest level during late premoult (O'Brien et al. 1991). During late premoult, the contents of chitin, protein and Ca salts of the cuticle also decrease due to the degradation of the two innermost layers, which occurs because minerals and organic portions are resorbed to build up the two outer layers of new cuticle (Drach 1939; Travis 1965; Roer 1980).

Ash content of the cuticle is lower during late premoult and postmoult, when the content of calcium carbonate decreases to increase the crustacean’s flexibility. Ash content rises during intermoult and early premoult due to an increase in the deposition of calcium salt (Paul and Sharpe 1916; Travis 1963; Travis and Friberg

1963; Nagasawa 2012). Chitin, another component of the cuticle, is broken down at various degrees during the moult cycle. The breakdown of chitin is partial during the premoult stage (Nagasawa 2012) and peaks at late premoult when chitin resorption is maximised (Buchholz and Buchholz 1989). Up to half of the broken down chitin during one moult cycle is used to form new chitin in the next (Gwinn and Stevenson 1973). Chitin content in the exoskeleton of decapod brachyoures is lower during premoult (40-45%) and higher in postmoult stage (71-72%) (Drach and Lafon 1942).

2.7 ESTIMATE ANIMAL POPULATION WITH MARK-RECAPTURE

2.7.1 Mark-recapture definition

Estimation of crayfish population is essential in crayfish culture, since it governs the management regime of the population (Thompson et al. 1998). An estimation method most widely used is the mark-recapture methods, which are based on a model developed by Petersen (1896) for estimating the size of fish populations in inland waters (Seber 1982; Seber 1986; Gatz and Loar 1988; Pollock et al. 1990). This model involves a number of assumptions, including population closure (no birth, death, emigration, or immigration during the study), zero tag loss, equal catchability (i.e. all animals having the same probability of being caught), and random distribution of tagged and untagged animals. When these assumptions are approximately met, useful indication about the status of a population deductions can be derived (Ricker 1975; Pollock 1991). According to Oosthuizen et al. (2010), population closure can be achieved by installing movement barriers, while tag loss can be prevented by the use of permanent and multiple markers that pose no danger to the survival of the animals. Random distribution and equal catchability of tagged and untagged animals can be achieved by giving the animals sufficient time to recover (Mesa and Schreck 1989). Conventionally, tagging or marking is used for unique identification of individuals in recapture samples. The information on the recapture rate is then used in the mark-recapture models to estimate population size (Seber 1982).

Estimation of population parameters such as survival, recruitment, population growth rate, and size by mark-recapture methods has been widely applied in studies of human (Seber 1982) and decapod crustaceans (Guan 1997; Guan and Wiles 1999;

Lettink and Armstrong 2003; Bolat et al. 2011; Coignet et al. 2012; Kuparinen et al. 2012; De Azevedo Carvalho et al. 2013; Johnsen et al. 2013; Jugovic et al. 2015; Kordjazi et al. 2015; Oka et al. 2015). According to Nowicki et al. (2008), mark-recapture can be a convenient tool for estimating the size of crayfish populations and understanding their population trends.

There are different mark- recapture models, each with their inherent assumptions (Chao et al. 2001). The choice of a model is determined by the characteristics of the sample data and how they match the assumptions of the chosen model. The choice of a model has a crucial influence on population estimation, especially for populations that cannot be sampled using randomised or predetermined sampling methods or those that are sampled in a more opportunistic manner, which may result in more animals being captured in some locations and times than in others. This would lead to heterogeneity in capture probabilities, which would violate the assumptions of the mark- recapture models (Seber 1982; Hammond 1986). Heterogeneity is more likely to occur in wide-ranging populations such as cetacean due to the animals moving beyond the range of single study areas (Hammond 1986). When it is impractical to recapture a substantial proportion of the population, it may lead to a more fundamental problem of negative population estimation biases (Hammond 1986; Whitehead et al. 1986; Hammond et al. 1990).

Apart from estimating the population, mark-recapture analysis can also be used for estimating other population parameters such as recruitment, population growth rate, and survival if the factors that might affect these parameters, such as age or sex, seasonal changes, and impacts of management actions can be assessed, which can be done in a well-designed study. The estimation of these parameters is important for assessing the viability of the population over time, evaluating the impacts of different threats, and predicting the population's response to different management strategies (Donkers et al. 2011).

The simplest case of closed population mark-recapture studies involves two capture sessions. In the first one, a group of animals is caught, marked, and released. In the second one, the same population is re-sampled. The danger of having only two capture sessions is that the equal catchability may not be met since individuals have a different inherent probability of being captured. Also, the animals may change their

behaviour after initial capture, becoming trap happy or trap shy. These could lead to overestimation or underestimation of the population (Lettink and Armstrong 2003) (see Table 2.2 for more details). For this reason, it is more desirable to use multiple capture sessions in closed population mark-recapture studies (Lettink and Armstrong 2003).

Table 2.2: Causes and effect of unequal capture probabilities in an estimation of population (N), modified from Lettink and Armstrong (2003)

Source of bias	Possible reason(s)	Significance	N
Capture heterogeneity	Some animals less likely to be caught	Marked animals have higher capture probabilities	Under-estimated
Capture heterogeneity	Inappropriate trapping (not enough traps used)	Precludes some individuals from capture if trap already occupied	Under-estimated
Capture heterogeneity	Inappropriate trap placement (trapping position: edge or middle of water body)	Animals less likely to be captured, hence fewer animals marked	Under-estimated
Trap response	Trap-happiness (bait and trap disign) and trap-shyness (animals learn to avoid traps in fixed places)	Animals caught once are more likely to be caught again	Over-estimated

2.7.2 Techniques for marking crayfish

There are a number of marking methods used in crayfish abundance research, from invasive techniques such as injecting ink (Black 1963) and injecting visible implant elastomers (Jerry et al. 2001; Arce et al. 2003; Brown et al. 2003; Clark and Kershner 2006; Mazlum 2007) to visual marks such as painting with fluorescent paint (Brandt and Schreck 1975), external plastic tags (Gherardi et al. 2000), clipping or punching holes in the telson or uropods (Guan 1997; Guan and Wiles 1999; Toyota et al. 2003; Nowicki et al. 2008), nail enamel and permanent waterproof marker (O'Neill et al. 1993; Ramalho et al. 2010), and branding with a soldering iron (Abrahamsson 1965; Buřič et al. 2008; Kuhlmann et al. 2008).

More advanced marking techniques, which require the use of additional equipment to detect the tag's position and read the tag's information, have also been used. Examples include radio transmitters (Gherardi et al. 2000; Robinson et al. 2000; Bubb et al. 2002a; Bubb et al. 2004; Aquiloni et al. 2005), coded wire tags (Isely and Eversole 1998; Kneib and Huggler 2001; Graaf 2007), and the use of microchips-Passive Integrated Transponders (Wiles and Guan 1993; Bubb et al. 2002b). There are also internal tags that are suitable for use with crustaceans. Internal tags are typically inserted into the musculature of the tail.

According to Weingartner (1982), regardless of what marking/tagging technique is chosen, it needs to meet the following important requirements:

i. It should not inhibit growth.
ii. It should remain in place throughout the moulting process.
iii. It should be suitable for use during early developmental stages.
iv. It should allow for dependable recognition without the need to sacrifice the animal.

2.7.3 Effect of environmental factors on the population of crayfish

Population of crayfish has been found to be affected by various biotic and abiotic factors, such as the underlying geology and water quality parameters (Lawrence et al. 1998; Lyons and Kelly-Quinn 2003) and physical structure of bottom soil (Niemi 1977; Bohl 1987; Laurent 1988; Foster 1995; Byrne et al. 1999; Lyons and Kelly-Quinn 2003). Water quality and biological productivity are determined by the size of water body, morphometric and structural variability of the pond bottom (Bohl 1987). Pond bottom soil is important because it stores important dissolved substances beneficial for the pond ecosystem (Boyd 1995). Water quality and aquaculture production are influenced by the chemical and biological processes that occur in the layers of water directly above the pond soil (Boyd 1995).

Most of the studies that investigated the influence of the biotic and abiotic factors on the population of crayfish focused on the population of crayfish in natural environments such as river's headwaters and farm dams (Lawrence et al. 1998; Lyons and Kelly-Quinn 2003). Studies on the effect of biotic and abiotic factors on

the population dynamics of marron and yabbies in a man-made aquatic ecosystem are still limited.

The availability of crayfish has also been found to be influenced by the sex ratio. A number of studies have found that male crayfish are significantly more likely to be trapped (or caught) than females (Brown and Bowler 1977; Abrahamsson 1983; Lawrence 1998; Lawrence et al. 1998) as crayfish males are more active than females since males reach sexual maturity earlier than females (Alikunhi 1966; Hume et al. 1983). Thus, a crayfish population with a larger proportion of males is more likely to diminish more quickly over time, compared with a population with a larger proportion of females.

Zooplanktons have been shown to be an excellent source of dietary nutrients for freshwater crayfish (Hessen 1989; Jones 1989; Shelley and Pearce 1990; Kondos 1990; Brown et al. 1992; Meakin et al. 2009). Jones (1997) found that yabbies fed with zooplankton had a greater survival rate than those feeding on pellets containing 15% and 30% protein diets, while marron demonstrated a preference for feeding on live zooplankton over a pellet by spending more time feeding on Daphnia spp. when both food types were presented. Dissolved nutrients such as nitrogen and soluble reactive phosphorus, on the other hand, have been linked to low primary productivity in pond water (Schindler 1977; Elser et al. 1990).

In conclusion, marron and yabby have been targeted as candidate species for the development of inland aquaculture. A number of studies have been conducted on these two species covering a wide range of research themes such as population dynamic (Morrissy and Caputi 1981; Lawrence et al. 1998; De Graaf et al. 2010), physiology (Morrissy et al. 1984; Jussila 1997; Sang and Fotedar 2008; Ambas et al. 2013), farming practices and management (Lawrence 1998; Lawrence et al. 1998; Lawrence 2007; Fotedar et al. 2016), feed and feeding (Jones 1997; Verhoef et al. 1998; Meakin et al. 2008; Sang et al. 2009; Meakin et al. 2009; Sang and Fotedar 2010, 2011; Nugroho and Fotedar 2013; Ambas et al. 2015a), growth performance (Jones et al. 2002; Graaf 2007), post-harvest and storage (Botondi et al. 2009), diseases (Langdon and Thorne 1992; Jones and Lawrence 2001), genetic and heredity (Campbell et al. 1994; Lawrence et al. 2000; Lawrence and Morrissy 2000; Walker et al. 2000; Austin and Ryan 2002; Nguyen et al. 2002), marketing and

tourism (Merrick and Lambert 1991; Alonso 2010). However, an inadequate fundamental research to understand the underlying mechanisms in the changes occurring in the physiology of haemolymph the moult cycle are lacking.

CHAPTER 3: THE EFFECTS OF BIOTIC AND ABIOTIC FACTORS ON THE ABUNDANCE AND POPULATION STRUCTURE OF YABBIES, *CHERAX ALBIDUS* (CLARK 1936) IN OUTDOOR EARTHEN PONDS

(Freshwater Crayfish, Submitted)

3.1 INTRODUCTION

Crayfish are regarded as useful indicators of water quality and freshwater biodiversity (Nowicki et al. 2008). The most abundant crayfish in Australia, the yabby, *Cherax albidus,* is widely distributed in temperate and sub-tropical south-eastern and central parts of Australia where they are the long-standing targets for the recreational fishery and are also one of the most important aquaculture species (Lake and Sokol 1986). Yabbies have also been introduced into farm dams and purpose-built earthen ponds in south-western Australia for recreational fishing and aquaculture purposes (Austin et al. 1997) and have now been established as a prevalent species in a wide range of water bodies including dams, natural water sheds and purpose-built ponds.

Estimation of population parameters such as size, survival, recruitment, and population growth rate by mark-recapture methods has been widely applied in studies of humans (Seber 1982) and decapod crustaceans (Guan 1997; Guan and Wiles 1999; Lettink and Armstrong 2003; Bolat et al. 2011; Coignet et al. 2012; Kuparinen et al. 2012; De Azevedo Carvalho et al. 2013; Johnsen et al. 2013; Jugovic et al. 2015; Kordjazi et al. 2015; Oka et al. 2015). Nowicki et al. (2008) suggested that mark-recapture can serve as a convenient tool for estimating the size of crayfish populations and understanding their temporal population trends.

The growth rates, development and survival of organisms are strongly influenced by varied abiotic and biotic factors (Limongi et al. 2015). The variation of crayfish population is a function of the water quality parameters (Lawrence et al. 1998; Lyons and Kelly-Quinn 2003) and physical structure of bottom soil (Niemi 1977; Bohl 1987; Laurent 1988; Foster 1995; Byrne et al. 1999; Lyons and Kelly-Quinn 2003). Pond bottom soil quality, although having been paid less attention to than water quality, is important for bottom dwellers such as crayfish, and can be considered a storehouse for many trace elements in a pond ecosystem (Boyd 1995). Nevertheless,

the chemical and biological processes occurring in surface layers of water directly above the pond soil also influence the water quality and aquatic productivity (Boyd 1995). An understanding of the dynamics involved in soil nutrient compositions in relation to water nutrient variations and yabby production can be useful tools in the management and assessment of yabbies population. Therefore, the current challenge is to include and identify the effects of bottom soil and water quality parameters on size and structure of yabby populations in any anthropogenic ecosystem, meaning that these factors can ultimately be manipulated to stimulate the sustainable population densities of yabbies.

There is a limited published information available on the biotic and abiotic causes responsible for the population dynamics of yabbies in a man-made aquatic ecosystem. Lyons and Kelly-Quinn (2003) and Lawrence et al. (1998) investigated the relationship between environmental factors and crayfish production in the river's headwaters and farm dams. Their research defined the impact of only abiotic factors including seasonality, water chemistry and physical properties such as height and size of the dam, wind speed and direction, the age of dam, catchment height, colour of the soil, and harvesting frequency on the yield, economic returns and density of yabbies. However, phytoplankton and zooplankton as biological indicators for water quality assessment (Purushothama et al. 2011) and key positions in the food chains of an aquatic ecosystem (Dede and Deshmukh 2015) have not been studied in yabby ponds.

This study aims to investigate the effect of biotic and abiotic factors over a period of two years, on the size and structure of yabbies population in purpose-built ponds of Western Australia.

3.2 MATERIALS AND METHODS

The study site consisted of three outdoor earthen ponds stocked with yabbies and located in the same aquaculture facility at Gingin, Western Australia (110 km from North Perth city with longitude 31°21'15.05" and latitude S 115°48'54.99" E). No supplementary food was provided during the study period. The physical structure bottom was mainly cobble-stone and sandy that was similar to all three water bodies. The physical description of these ponds is shown in Table 3.1.

Table 3.1: The description of selected outdoor earthen ponds stocked with yabbies used for the research

Features	Pond 1	Pond 2	Pond 3
Water surface area (m^2)	6500	7500	6500
Maximum water depth (m)	2.8	3.0	2.0

During summer, the water level of the ponds was maintained daily by topping up of the water at the rate of 2000 L $minute^{-1}$ and for 10 hours from a bore well. Water and bottom soil quality samples of the ponds were analysed fortnightly. The estimation of yabby population was conducted monthly over 24 months from March 2012 to February 2014.

Water quality analysis

Water temperature (°C), pH, and dissolved oxygen (DO) (mg L^{-1}) were measured on site using pH meter (HQ11d Portable pH/ORP Metre), and oxygen meter (WTW, Model Oxi 340i), respectively.

Five litres of water from 20 cm below the water surface was collected and mixed from 5 different random locations in a pond. Finally, one litre of water from this mixture was used for analysis of total alkalinity, calcium, hardness, organic matter (OM), total nitrogen (TN), total ammonium nitrogen (TAN), nitrate (NO_3^-), nitrite (NO_2^-), soluble reactive phosphorous (SRP), and total phosphorus (TP) using the procedures described in APHA (1998). Dissolved inorganic nitrogen (DIN) was analysed as a sum of TAN, NO_3^- and NO_2^-.

A representative pond bottom soil sample from each pond was prepared from a mixture of 5 sub-samples collected from 5 random sampling points using a soil core sampler (cylinder shape with a diameter of 7 cm and depth of 10 cm). The soil samples were dried at 70°C for 24 hours before being manually spread on a board covered with thick paper and rolled with a wooden rolling pin to break up any lumps. Stony gravels were discarded from the pulverised mixture and then passed through a sieve with circular perforations of 2 mm in diameter. If there were any coarse

particles, they were put in a mortar and rubbed with a rubber-tipped pestle to remove the adhering, fine particles. This operation was repeated until the coarse particles were clean. When all of the fine particles were passed through the sieve, the sample was thoroughly mixed before the portion was taken out for the analysis of TN (Kjeldahl method), TP (Bowman 1988), and OM (Walkley-Black method).

Zooplankton density and phytoplankton chlorophyll-a

One hundred litres of pond water from 5 random sampling points was filtered through a bolting silk (20 μm aperture) conical shape plankton net between 4 and 5 p.m. The collected samples were preserved in 4% formaldehyde for the analysis of zooplankton in the laboratory (APHA 1998). A 'Sedgwick-Rafter Counting Cell' was used. All of the zooplankton in the counting chamber were observed and counted under the microscope (Leica Microsystem DM 2500- German at x 100 magnification) using the keys and monographs published by Shiel (1995). The results are expressed as number of organisms per litre. Chlorophyll-a from phytoplankton was analysed using one litre of pond water filtered through Whatman glass fibre paper (GF/C type 0.45 μm pore size) after APHA (1998).

Sampling of yabbies

The mark-recapture technique for closed populations was employed to estimate the population size and density of yabbies from March 2012-February 2014 using 15 net traps with metal frames (50 cm length x 30 cm height x 30 cm width), and covered with 15 mm mesh to trap only yabbies with a total body length more than 3.0 cm. To meet the assumption of a closed population by Peterson-Lincoln estimation (Seber 1982), ponds were isolated during the experiment by installing a plastic fence with a height of 50 cm approximately 20 cm from the water edge. Captured yabbies were marked with the Dykem marker (Ramalho et al. 2010). The Dykem marker was applied directly to the wet carapace. Different shapes and colours were selected and applied to the dorsal side of the carapace of each captured yabby for 24 mark-recaptures/occasions over a two year experiment. The marked yabbies were released within the same area from which they were initially captured.

The mark-recaptured procedure was conducted monthly by using the traps baited with the scad fish, *Caranx kurra*, which were set along the banks of the ponds. Five

traps were set up during the late afternoon in each pond at a water depth of approximately 1 metre with 20 metre intervals. The positions of traps remained unchanged during the two-year sampling. The traps were left overnight and lifted up early in the morning.

The number of total yabbies per trap and the number of marked yabbies caught were recorded. Body weight of each captured yabby were measured to the nearest 0.1 g using field balance, 200 x 0.1 g capacity, HL-200I-Hach-USA.

The Lincoln-Petersen method (also known as the Lincoln index) was used to estimate the population size in the ponds. The Chapman (1951) modification of the Lincoln-Peterson formula was applied to estimate the population size:

$$N = CM/R \text{ if } R >= 7, \text{ OR } N = [(M + C)(C+1) / (R + 1)] - 1 \text{ if } R < 7$$

In which:

N = estimator of population size at the time of marking

C = total number of individuals captured in 2nd sample

M = total marked individuals in the first sampling

R = number of individuals in 2^{nd} sample that are marked

Density was found by the equation: D (individual m^{-2}) = N/A, where N is the population size in numbers and A, is the area occupied by the crayfish population. (Seber 1982).

Statistical analysis

SPSS 18.0, 2014 was used to analyse the data. Results were presented as mean ± SE. The normality of data was assessed by the Shapiro-Wilk test (Winer 1991) and the homogeneity of variance was assessed by the Levene test (Winer 1991) before analysis. One-way ANOVA (analysis of variance) and LSD (least significant difference) post hoc tests were used to determine significant differences between ponds and seasons, while the T-test was used to compare between years. To satisfy the assumptions of normality and homogeneity of variance, data were transformed to $\log_{10}(x + 1)$. To avoid type I error when a large number of statistical analyses were performed on the same data set, the significance level was adjusted.

To select predictor variables for multiple linear regressions, a matrix of correlation between predictor variables and the response (population size) with each predictor variable (water biochemical variables) was constructed to show the relationships. If any pair of predictor variables had a correlation coefficient of more than 0.7, either was selected for a multiple linear regression; otherwise, all predictor variables were used for multiple linear regression analysis.

Multiple linear regression analyses were completed to determine the relationship between yabbies population with the water and soil physicochemical and biological factors. Estimated regression coefficients were used to weigh the importance of the predictor variable in relation to the response if the significance level $P < 0.05$. Using multiple linear regressions, it was assumed that each predictor variable is approximately normally distributed.

3.3 RESULTS

Table 3.2: Population size, total body weight per trapping occasion, sex ratio and density of yabby for different ponds and years (season-pooled data)

Factor	Pond	Year 1	Year 2
Estimated population	1	$_1735 \pm 21^a$	$_1895 \pm 25^b$
	2	$_2562 \pm 32^a$	$_2681 \pm 27^b$
	3	$_3315 \pm 19^a$	$_3417 \pm 23^b$
Total body weight (g)	1	$_1592 \pm 13^a$	$_1606 \pm 19^a$
	2	$_2515 \pm 21^a$	$_2523 \pm 23^a$
	3	$_3385 \pm 19^a$	$_3402 \pm 27^a$
Sex ratio (male/female)	1	$_12.6 \pm 0.2^a$	$_12.5 \pm 0.1^a$
	2	$_12.5 \pm 0.1^a$	$_12.6 \pm 0.2^a$
	3	$_12.7 \pm 0.3^a$	$_12.7 \pm 0.2^a$
Estimated density (individual m^{-2})	1	$_10.131 \pm 0.004^a$	$_10.162 \pm 0.003^b$
	2	$_10.122 \pm 0.003^a$	$_10.154 \pm 0.002^b$
	3	$_20.050 \pm 0.002^a$	$_20.060 \pm 0.001^a$

Same alphabetical superscripts (a, b) in the same row (comparisons between years) and numerical subscripts (1, 2, 3) in the same column of each parameter are not significantly different at the P = 0.05 level. Year 1 = March 2012-February 2013; Year 2 = March 2013-February 2014. n = 12, trapping occasion.

The sex ratio of yabbies, the total weight of captured yabbies from 5 traps per trapping occasion were pond- and sampling year-independent (Table 3.2). Irrespective of the year and the pond location, the males were trapped 2.5-3.0 times more frequently than females. Estimated yabby population and density were significantly higher in the second year.

Table 3.3: Total body weight, population size and sex ratio in different seasons and ponds (year-pooled data)

Factor	Pond	Spring	Summer	Autumn	Winter
Estimated population	1	$_{1}713 \pm 18^{a}$	$_{1}744 \pm 35^{a}$	$_{1}745 \pm 25^{a}$	$_{1}665 \pm 21^{a}$
	2	$_{2}451 \pm 25^{a}$	$_{2}422 \pm 18^{a}$	$_{2}425 \pm 20^{a}$	$_{2}421 \pm 51^{a}$
	3	$_{3}317 \pm 21^{a}$	$_{3}308 \pm 15^{a}$	$_{3}321 \pm 17^{a}$	$_{3}286 \pm 32^{a}$
Total body weight (g)	1	$_{1}628 \pm 15^{a}$	$_{1}635 \pm 18^{a}$	$_{1}619 \pm 28^{a}$	$_{1}492 \pm 21^{b}$
	2	$_{2}527 \pm 25^{a}$	$_{2}520 \pm 14^{a}$	$_{2}526 \pm 27^{a}$	$_{2}414 \pm 15^{b}$
	3	$_{3}391 \pm 16^{a}$	$_{3}421 \pm 22^{a}$	$_{3}406 \pm 31^{a}$	$_{3}353 \pm 17^{b}$
Sex ratio (male/female)	1	$_{1}2.6 \pm 0.2^{a}$	$_{1}3.0 \pm 0.3^{a}$	$_{1}2.7 \pm 0.1^{a}$	$_{1}2.6 \pm 0.2^{a}$
	2	$_{1}2.5 \pm 0.2^{a}$	$_{1}3.1 \pm 0.2^{a}$	$_{1}2.6 \pm 0.2^{a}$	$_{1}2.5 \pm 0.2^{a}$
	3	$_{1}2.6 \pm 0.1^{a}$	$_{1}2.9 \pm 0.3^{a}$	$_{1}2.5 \pm 0.2^{a}$	$_{1}2.6 \pm 0.1^{a}$

Same alphabetical superscripts (a, b) in the same row (comparisons among seasons) and numerical subscripts (1, 2, 3) in the same column of each parameter (comparisons among ponds) are not significantly different at the $\alpha = 0.05$ level. n = 6, trapping occasion.

Sex ratios of captured yabbies were pond- and season-independent (Table 3.3). Estimated population size and total body weight per trapping occasion significantly differed between ponds, while season had no effect on estimated population.

Table 3.4: The level of physicochemical water quality parameters in different seasons and ponds (year-pooled data) during two years of sampling

Factor	Pond	Spring	Summer	Autumn	Winter
Water temp. (t^oC)	1	$_1$18.1 ± 1.2^a	$_1$23.4 ± 1.6^b	$_1$16.6 ± 2.1^a	$_1$12.3 ± 1.0^c
	2	$_1$19.5 ± 1.6^a	$_1$22.9 ± 1.1^b	$_1$15.1 ± 1.7^a	$_1$12.5 ± 1.2^c
	3	$_1$19.3 ± 1.8^a	$_1$22.6 ± 1.7^b	$_1$17.7 ± 1.1^a	$_1$11.8 ± 0.9^c
pH	1	$_1$6.9 ± 1.2^a	$_1$7.2 ± 1.3^a	$_1$7.6 ± 0.9^a	$_1$6.7 ± 1.0^a
	2	$_1$7.2 ± 0.8^a	$_1$7.5 ± 1.2^a	$_1$8.1 ± 0.7^a	$_1$7.1 ± 1.2^a
	3	$_1$6.6 ± 1.0^a	$_1$6.8 ± 0.7^a	$_1$6.5 ± 0.8^a	$_1$6.8 ± 1.5^a
DO ($mg\ L^{-1}$)	1	$_1$7.2 ± 0.3^a	$_1$7.2 ± 0.5^a	$_1$7.4 ± 0.2^a	$_1$8.2 ± 0.3^b
	2	$_1$7.0 ± 0.4^a	$_1$7.3 ± 0.4^a	$_1$7.1 ± 0.5^a	$_1$8.2 ± 0.2^b
	3	$_2$6.4 ± 0.3^a	$_2$6.1 ± 0.3^a	$_2$6.3 ± 0.3^a	$_2$7.4 ± 0.4^b
Total alkalinity ($mg\ L^{-1}$)	1	$_1$97.2 ± 6.4^a	$_1$102.3 ± 6.9^a	$_1$98.2 ± 7.5^a	$_1$94.2 ± 5.5^b
	2	$_1$95.5 ± 5.4^a	$_1$99.6 ± .8^a	$_1$98.6 ± 6.3^a	$_1$92.4 ± 5.9^a
	3	$_1$90.4 ± 8.6^a	$_1$92.7 ± 7.2^a	$_1$92.7 ± 5.4^a	$_1$93.6 ± 6.3^a
Hardness ($mg\ L^{-1}$)	1	$_1$45.0 ± 5.4^a	$_1$47.8 ± 5.4^a	$_1$39.6 ± 3.9^a	$_1$38.7 ± 4.1^a
	2	$_1$46.6 ± 6.2^a	$_1$46.3 ± 6.1^a	$_1$40.1 ± 4.7^a	$_1$40.6 ± 3.2^a
	3	$_1$42.7 ± 3.1^a	$_1$41.8 ± 4.2^a	$_1$38.6 ± 5.1^a	$_1$38.5 ± 3.7^a
Calcium ($mg\ L^{-1}$)	1	$_1$15.2 ± 3.5^a	$_1$15.4 ± 3.1^a	$_1$13.4 ± 2.9^a	$_1$13.9 ± 3.1^a
	2	$_1$14.6 ± 2.7^a	$_1$16.1 ± 4.1^a	$_1$14.2 ± 3.2^a	$_1$15.6 ± 2.7^a
	3	$_1$11.8 ± 2.9^a	$_1$14.8 ± 3.5^a	$_1$12.9 ± 4.2^a	$_1$14.8 ± 3.5^a
OM ($mg\ L^{-1}$)	1	$_1$0.60 ± 0.09^a	$_1$0.89 ± 0.20^b	$_1$0.84 ± 0.18^b	$_1$0.35 ± 0.08^c
	2	$_1$0.61 ± 0.16^a	$_1$0.79 ± 0.16^b	$_2$0.67 ± 0.16^b	$_2$0.32 ± 0.07^c
	3	$_2$0.32 ± 0.05^a	$_2$0.63 ± 0.17^b	$_2$0.65 ± 0.13^b	$_3$0.21 ± 0.04^c
DIN	1	$_1$0.49 ± 0.07^a	$_1$0.65 ± 0.09^b	$_1$0.34 ± 0.04^c	$_1$0.31 ± 0.08^c

($mg\ L^{-1}$)	2	$_{1}0.41 \pm 0.04^{a}$	$_{1}0.74 \pm 0.05^{b}$	$_{2}0.45 \pm 0.06^{c}$	$_{2}0.20 \pm 0.06^{d}$
	3	$_{2}0.30 \pm 0.03^{a}$	$_{2}0.49 \pm 0.08^{b}$	$_{1}0.30 \pm 0.07^{c}$	$_{2}0.19 \pm 0.05^{d}$
TN ($mg\ L^{-1}$)	1	$_{1}0.46 \pm 0.10^{a}$	$_{1}0.85 \pm 0.21^{b}$	$_{1}0.72 \pm 0.14^{b}$	$_{1}0.33 \pm 0.08^{a}$
	2	$_{1}0.32 \pm 0.12^{a}$	$_{1}0.89 \pm 0.14^{b}$	$_{1}0.71 \pm 0.12^{b}$	$_{1}0.24 \pm 0.05^{a}$
	3	$_{1}0.32 \pm 0.09^{a}$	$_{1}0.71 \pm 0.13^{b}$	$_{1}0.57 \pm 0.20^{b}$	$_{1}0.20 \pm 0.07^{a}$
TP ($mg\ L^{-1}$)	1	$_{1}0.51 \pm 0.02^{a}$	$_{1}0.81 \pm 0.11^{b}$	$_{1}0.73 \pm 0.04^{b}$	$_{1}0.26 \pm 0.06^{c}$
	2	$_{1}0.41 \pm 0.03^{a}$	$_{1}0.78 \pm 0.02^{b}$	$_{1}0.62 \pm 0.07^{b}$	$_{1}0.24 \pm 0.01^{b}$
	3	$_{2}0.20 \pm 0.08^{a}$	$_{3}0.47 \pm 0.11^{b}$	$_{2}0.43 \pm 0.03^{b}$	$_{2}0.16 \pm 0.02^{c}$
SRP ($mg\ L^{-1}$)	1	$_{1}0.21 \pm 0.02^{a}$	$_{1}0.34 \pm 0.03^{b}$	$_{1}0.25 \pm 0.03^{a}$	$_{1}0.13 \pm 0.02^{c}$
	2	$_{1}0.22 \pm 0.01^{a}$	$_{1}0.31 \pm 0.02^{b}$	$_{1}0.32 \pm 0.05^{a}$	$_{1}0.11 \pm 0.03^{c}$
	3	$_{1}0.10 \pm 0.01^{a}$	$_{2}0.18 \pm 0.04^{b}$	$_{2}0.14 \pm 0.01^{a}$	$_{1}0.10 \pm 0.01^{c}$

Same alphabetical superscripts (a, b, c) in the same row (comparisons among seasons) and numerical subscripts (1, 2) in the same column of each parameter (comparisons among ponds) are not significantly different at the $\alpha = 0.05$ level. n = 12.

Water temperature, DO, OM, TN, TP, SRP and DIN fluctuated significantly between seasons and ponds (Table 3.4). Their concentrations were higher during the summer and autumn. On the contrary, pH, alkalinity, hardness and calcium were season- and pond-independent.

Table 3.5: Concentrations of biotic parameters in different seasons and ponds (year-pooled data)

Factor	Pond	Spring	Summer	Autumn	Winter
Chlorophyll-a ($mg\ m^{-3}$)	1	$_{1}4.79 \pm 0.85^{a}$	$_{1}8.30 \pm 1.21^{b}$	$_{1}7.32 \pm 0.94^{b}$	$_{1}4.9 \pm 1.01^{a}$
	2	$_{1}4.10 \pm 0.22^{a}$	$_{1}8.96 \pm 1.13^{b}$	$_{1}7.12 \pm 1.01^{b}$	$_{1}3.9 \pm 0.91^{a}$
	3	$_{2}2.23 \pm 1.02^{a}$	$_{2}5.14 \pm 1.02^{b}$	$_{2}5.00 \pm 0.46^{b}$	$_{2}1.8 \pm 0.65^{a}$
Zoopl. density (individual L^{-1})	1	$_{1}102 \pm 12^{a}$	$_{1}155 \pm 14^{b}$	$_{1}140 \pm 18^{b}$	$_{1}61 \pm 18^{c}$
	2	$_{1}88 \pm 18^{a}$	$_{1}132 \pm 11^{b}$	$_{1}120 \pm 19^{b}$	$_{1}63 \pm 16^{c}$
	3	$_{2}52 \pm 11^{a}$	$_{2}64 \pm 12^{b}$	$_{2}59 \pm 9^{b}$	$_{2}27 \pm 16^{a}$

Same alphabetical superscripts (a, b) in the same row (comparisons among seasons) and numerical subscripts (1, 2) in the same column of each parameter (comparisons among ponds) are not significantly different at the $\alpha = 0.05$ *level. n = 12.*

Pond location had an effect on zooplankton density and chlorophyll-a level (Table 3.5). The summer and autumn showed higher values of all investigated biological parameters in 3 ponds.

Table 3.6: The correlation coefficient (R^2) between physicochemical and biological parameters and yabby population in different seasons (pond-pooled data)

Parameters	Spring	Summer	Autumn	Winter
Water temperature	0.05	0.06	0.03	0.12
pH	-0.06	-0.07	-0.06	-0.07
DO	0.02	0.02	0.04	0.01
Alkalinity	0.21	0.10	0.15	0.27
Hardness	0.15	0.24	0.16	0.13
Calcium	0.19	0.32	0.17	0.28
DIN	0.66	0.74	0.61	0.53
TN	0.45	0.36	0.64	0.45
TP	0.51	0.62	0.57	0.45
SRP	0.55	0.71	0.56	0.33
Chlorophyll-a	0.55	0.70	0.58	0.58
Zooplankton density	0.64	0.65	0.69	0.63

Most values for R^2 were close to zero, ranging from -0.50 to 0.74 (Table 3.6). Among the studied parameters, DIN, SRP and zooplankton density had a stronger correlation with the yabby population size. The correlation between water quality variables on population size was similar among seasons. R^2 values in year round were negative for pH.

Table 3.7: Multiple linear regression coefficients and significant levels with population size as the response and biological factors as predictor variables in different seasons (pond and year-pooled data). R^2 is presented in brackets.

Factor	Spring (0.48)		Summer (0.71)		Autumn (0.67)		Winter (0.65)	
	Reg. slope	Sig. level	Reg. slope	Sig. level	Reg. slope	Sig. level	Reg. slope	Sig. level
Chlorophyll-a	7.2	0.003	6.5	0.008	7.5	0.004	4.0	0.001
Zoopl. density	14.5	0.005	16.6	0.002	12.8	0.007	7.8	0.003

Compared to chlorophyll-a, zooplankton density had higher regression slopes (Table 3.7) over four seasons. The highest correlation coefficient (R^2) between biotic parameters and size of yabbies population was during summer time ($R^2 > 0.7$).

Table 3.8: Multiple linear regression coefficients and significance levels with population size as the response and water chemistry as predictor variables in different seasons (pond and year-pooled data). R^2 is presented in bracket.

Factor	Spring (0.61)		Summer (0.79)		Autumn (0.69)		Winter (0.72)	
	Reg. slope	Sig. level	Reg. slope	Sig. level	Reg. slope	Sig. level	Reg. slope	Sig. level
Water temp.	3.9	0.034	3.7	0.047	2.3	0.037	1.6	0.046
Calcium	0.2	0.032	0.1	0.021	0.3	0.022	0.3	0.014
OM	2.3	0.018	1.9	0.006	0.9	0.045	1.7	0.023
DIN	6.5	0.024	6.2	0.045	4.6	0.012	4.2	0.023
TN	5.3	0.026	4.2	0.012	3.9	0.016	2.6	0.028
TP	5.0	0.045	4.2	0.032	3.6	0.026	3.2	0.013
SRP	8.5	0.037	14.6	0.025	5.2	0.024	7.1	0.031
pH	-1.2	0.061	-2.1	0.056	-0.9	0.062	-2.0	0.053
Alkalinity	0.2	0.156	0.2	0.235	0.1	0.451	0.5	0.234
Hardness	-0.1	0.084	-0.2	0.061	-0.3	0.057	-0.2	0.075

DO	0.5	0.315	0.9	0.245	1.3	0.238	0.7	0.269

SRP had a highest positive effect on the size of yabby population, whereas a negative impact was recorded only in pH and hardness (Table 3.8). Among the studied water quality parameters, the impact of pH, alkalinity, hardness and DO was not significant on yabby population. The role of physicochemical parameters is more important during the summer and winter (higher $R^2 > 0.70$).

Table 3.9: Concentrations (mg/100 g dry soil) of bottom soil parameters in different seasons and ponds (year-pooled data)

Factor	Pond	Spring	Summer	Autumn	Winter
TN	1	$_1$45.8 ± 2.5^a	$_1$71.3 ± 5.1^b	$_1$80.3 ± 3.4^b	$_1$42.9 ± 3.4^a
	2	$_1$43.1 ± 2.2^a	$_1$73.9 ± 2.3^b	$_1$82.1 ± 4.20^b	$_1$39.3 ± 4.1^a
	3	$_2$32.3 ± 3.0^a	$_2$52.4 ± 4.2^b	$_2$57.0 ± 5.6^b	$_2$28.2 ± 2.5^a
OM	1	$_1$25.1 ± 3.2^a	$_1$25.5 ± 2.4^a	$_1$24.0 ± 2.8^a	$_1$22.1 ± 2.8^a
	2	$_1$28.3 ± 1.8^a	$_1$23.2 ± 3.1^a	$_1$22.6 ± 3.4^a	$_1$23.4 ± 3.6^a
	3	$_1$22.3 ± 3.1^a	$_1$24.4 ± 2.2^a	$_1$19.8 ± 2.9^a	$_1$24.1 ± 2.6^a
TP	1	$_1$32.5 ± 3.2^a	$_1$44.3 ± 4.4^b	$_1$48.3 ± 4.2^b	$_1$32.5 ± 3.6^a
	2	$_1$34.2 ± 4.3^a	$_1$45.2 ± 5.0^b	$_1$46.7 ± 3.6^b	$_1$31.4 ± 4.3^a
	3	$_2$21.2 ± 4.1^a	$_2$33.6 ± 3.6^b	$_2$32.8 ± 4.3^b	$_1$20.6 ± 3.5^a

Same alphabetical superscripts (a, b) in the same row (comparisons among seasons) and numerical subscripts (1, 2) in the same column of each parameter (comparisons among ponds) are not significantly different at the $\alpha = 0.05$ level. n = 12.

The concentration of TN and TP in bottom soil was significantly higher during summer and autumn (Table 3.9). Pond 1 and 2 had more TN and TP in their bottom soils, whereas the level of OM stayed stable during the year and among studied ponds.

Table 3.10: Correlation coefficients and significance levels for different chemical parameters between soil and water in different seasons

Factors	Spring		Summer		Autumn		Winter	
	R^2	Sig. level	R^2	Sig. level	R^2	Sig. level	R^2	Sig. level
TN	0.75	0.002	0.79	0.003	0.65	0.016	0.61	0.035
OM	0.79	0.017	0.82	0.016	0.66	0.007	0.53	0.049
TP	0.65	0.024	0.61	0.006	0.61	0.023	0.52	0.037

The significant correlation between soil and water for a number of chemical factors varied at different seasons (Table 3.10). Stronger correlations were found during spring and summer in all parameters. A higher inter-dependence between soil and water was reported for OM and TAN.

Table 3.11: Multiple linear regression coefficient slopes and significance levels with population size as the response and soil chemistry as predictor variables in different seasons (pond and year-pooled data). R^2 is presented in brackets.

Factor	Spring (0.61)		Summer (0.72)		Autumn (0.78)		Winter (0.60)	
	Reg. slope	Sig. level	Reg. slope	Sig. level	Reg. slope	Sig. Level	Reg. slope	Sig. level
TN	0.9	0.032	1.7	0.024	1.3	0.047	0.6	0.056
OM	1.3	0.015	2.2	0.021	1.6	0.036	0.2	0.063
TP	2.5	0.047	1.6	0.015	2.2	0.044	0.3	0.051

Soil quality parameters (TN, OM, and TP) had a positive effect on the yabbies population (Table 3.11) (positive regression slopes and $P < 0.05$) during most of the year, except winter time ($P > 0.05$).

Table 3.12: Summaries of the effect of water and soil physicochemical and biological factors on the yabby population size in different seasons during the study period

Factors	Spring	Summer	Autumn	Winter
Physical factor(s) (+ effect)	t°C, DO	t°C	t°C, DO	t°C, DO
Chemical factor(s) (+ effect)	SRP, TP, DIN, calcium, alka.	DIN, TP, SRP, calcium, alka.	DIN, TP, SRP, calcium, alka.	DIN, TP, SRP, calcium, alka.
	soil TN-TP-OM	soil TN-TP-OM	soil TN-TP-OM	soil TN-TP-OM
Biological factor(s) (+ effect)	- chlorophyll-a - zoo. density	- chlorophyll-a - zoo. density	- chlorophyll-a - zoo. density	- chlorophyll-a - zoo. density
Physical factor(s) (- effect)	pH	pH	pH	pH
Chemical factor(s) (- effect)	hardness	hardness	hardness	hardness
Biological factor(s) (- effect)	nil	nil	Nil	nil

Most of studied parameters had a positive effect on the population of yabbies, except pH and hardness, which had negative effects (Table 3.12), irrespective of seasons.

3.4 DISCUSSION

Marking crayfish is widely employed in a variety of research areas, including investigations of population size (Abrahamsson 1965; Skurdal et al. 1992; Kirjavainen and Westman 1994; da Silva and de Siqueira Bueno 2005; Bolat et al. 2011), fishery restocking (Whitmore 1997; Linnane and Mercer 1998), ranging behaviour (Robinson et al. 2000; Byron and Wilson 2001; Kerby et al. 2005), and survival and growth (Pratten 1980; Guan 1997; Guan and Wiles 1999; Karplus and Barki 2004; Huber and Schubart 2005; Pöckl and Streissl 2005; Ramalho et al. 2010). The mark-recapture method based on a model developed by Petersen (1896) remains the most frequently used technique for population estimation in inland waters (Seber 1982; Seber 1986; Gatz and Loar 1988; Pollock et al. 1990) and is a powerful method for estimating abundance as long as the underlying assumptions are met (Thompson et al. 1998).

Although not many potential predators are known to have a negative effect on the abundance of crayfish (Hogger 1988), in order to prevent potential predators, escape as a result of burrowing into banks, and inhibit the migration of yabbies across study ponds, plastic fences with a height of 50 cm were installed 20 cm from the water edge during the course of experiment for 2 years. Furthermore, one mark-recapture occasion took place within 48 hours that could lead to the method meeting the assumptions for a "closed system". In the present study, it was observed that marks remained and were clearly visible for about 100 days. Moreover, the likely fluctuation in catch per unit of effort due to catchability (Brown and Brevis 1979), positioning and location of the trap (Lyons and Kelly-Quinn 2003), and movement in and out of traps (Harlioglu 1999) are ignored in this study.

There was a difference in trapability of male and female noble crayfish *Astacus astacus* (Abrahamsson 1983) and the sex ratio also varies according to season (Qvenild and Skurdal 1989). However, in the current research, the proportion of males caught was significantly higher than that of females, irrespective of the trapping seasons. It is comparable to previous studies by Lawrence et al. (1998) with yabbies from farm dams, and Brown and Bowler (1977) with British freshwater crayfish *Austropotamobius pallipes*. The skewed sex ratio towards more males could be an effect of the females being more trap shy, more careful or occupied with other things. The berried females are either less likely to enter traps or are prevented from entering the traps by already trapped larger males as they become trapped first (Lawrence et al. 1998). Furthermore, crayfish males are more active as they reach sexual maturity earlier than females (Alikunhi 1966; Hume et al. 1983).

Estimated density during this study ranged between 0.05 and 0.16 individual m^{-2}. Higher densities of yabby were found in ponds 1 and 2, where the higher concentration of nutrients such as dissolved inorganic nitrogen and soluble reactive phosphorus, as well as higher zooplankton density in the waters, was reported (Table 3.4 and Table 3.5). The result was found to be lower than the density of narrow-clawed crayfish *A. leptodactylus* (Köksal et al. 2003), British freshwater crayfish (Moriarity 1973) and noble crayfish (Skudal et al. 1992). In other mark-recapture experiments on adult noble crayfish, the density varied between 0.13 and 1.65

individuals m^{-2}, with the highest densities in rivers and shallow ponds (Cukerzis 1975; Niemi 1977).

The lower density of yabbies from the studied pond is possibly explained by a limited natural food source (phytoplankton and zooplankton) for yabbies (Jones 1997) as a result of a low concentration of major macronutrients (nitrogen and phosphorus) (Table 3.4) (Schindler 1977; Elser et al. 1990). Moreover, the water N:P ratio (by weight) of 3:1 from our study was relatively far from the recommended Redfield ratio of 7:1, which could ideally enable optimal growth of fresh water phytoplankton. The low calculated N:P ratio suggests a shortage of nitrogen in the studied ponds. In other words, nitrogen in the pond waters becomes a limiting factor for the optimal growth of phytoplankton community. In contrast, soluble reactive phosphorus is normally a limiting factor for the growth and production of phytoplankton in aquaculture ponds (Boyd et al. 1975). A low concentration of chlorophyll-a and high transparency of pond waters (80.4 cm average) were considered evidence showing the low density of phytoplankton in the pond. Low primary productivity is the result of low levels of dissolved nutrients such as nitrogen and phosphorus (Schindler 1977; Elser et al. 1990).

The availability of crayfish is considered to be largely determined by the underlying geology and water quality parameters (Lyons and Kelly-Quinn 2003). The high degree of variation in water quality parameters between seasons in this study has supported the hypothesis that some seasons are more productive than others. During summer and autumn, favourable water quality parameters including the higher density of zooplankton (Table 3.5) as a food source for yabbies (Hessen 1989; Jones 1989; Shelley and Pearce 1990; Kondos 1990; Brown et al. 1992; Jones 1997) are recorded, and the estimated yabby populations in the ponds are maximum accordingly. Irrespective of pond location and the pond's water quality, crayfish density increases temporally, indicating a continuous recruitment from fingerling-sized yabbies to the estimated population.

The size of water body, the morphometric and structural variability of the pond bottom may be co-variables that could explain the variation in yabby populations from pond to pond in the current study, as they affect water quality and biological productivity (Bohl 1987). Pond bottom soil is a key factor in aquaculture

management. Many dissolved and suspended substances in water are derived from contact with soil. Pond soil can be a sink or a source of dissolved substances for pond water (Boyd 1995). Although the physical structure of the pond bottom was not studied, there was a degree of correlation in a number of quality parameters between bottom soil and water from studied ponds. The result was explained by Boyd (1995), who stated that substances stored in the pond soil can be released into the water through the process of ion exchange, dissolution and decomposition. In the current study, a positive relationship of soil quality parameters on yabby population size was reported, which is supported by several authors (Niemi 1977; Bohl 1987; Laurent 1988; Foster 1995; Byrne et al. 1999; Lyons and Kelly-Quinn 2003). The higher rate of mixing at the soil-water interface caused by the higher density of bottom dwelling yabbies could result in higher nutrient concentrations in the pond water as nutrients become resuspended from the bottom soil layer by the activities of yabbies (Boyd et al. 2002).

For the better management of any crayfish population, the challenge is to identify the swipe of abiotic and biotic factors responsible for stimulating the growth of yabbies (Jones 1997), which in turn, could be modified to improve the productivity. In the current research, except for zooplankton densities and soluble reactive phosphorus, linear regressions between physicochemical and biological water quality variables and yabbies population size produced low values of correlation coefficient (R^2), indicating that no single physicochemical water quality variable recorded had a strong relationship with yabbies population size. However, the result from multiple linear regressions showed a significant effect on yabbies population for most of the studied soil and water quality factors, thus indicating that the study relationships are more complex.

Biological factors played a vital role as indicated by the higher regression coefficients influencing the size of yabbies population. The key parameters related to yabbies population size in the studied ponds were chlorophyll-a/phytoplankton, zooplankton density, total dissolved nitrogen, and soluble reactive phosphorous. Lawrence et al. (1998) compared the importance of physical and chemical parameters and showed that the main parameters limiting the production of yabbies

in WA farm dams were feeding and physical factors such as bank height, pond age, pond area, wind direction.

Many studies have focused on several aspects related to the enhancement of yabbies production such as growth, nutrient requirement, and culture technique (Jones et al. 1996b, a; Verhoef et al. 1998; Verhoef and Austin 1999b, a; Lawrence et al. 2001; Lawrence 2001; Duffy et al. 2015). Freshwater zooplanktons are an excellent source of dietary nutrients for freshwater crayfish (Hessen 1989; Jones 1989; Shelley and Pearce 1990; Kondos 1990; Brown et al. 1992). Along with physical factors (Lawrence et al. 1998), DIN as a starting point for development of the live food chain, and zooplankton population mainly contributed by rotifers, copepods and cladocerans are becoming the most important indications for a good production of yabbies in WA. The importance of zooplankton to yabbies population found from the current study is in agreement with previous studies. The research by Jones (1997) showed a higher growth and greater survival of yabbies when fed with zooplankton meal compared to the pellets containing 15% and 30% protein diets. Similarly, yabbies fed with live zooplankton had a higher survival and growth rate compared with artificial feed (Austin et al. 1997; Verhoef et al. 1998), while both marron and yabbies spent more time feeding on live *Daphnia* spp rather than pellet when both food types were provided (Meakin et al. 2008; Meakin et al. 2009).

In conclusion, yabbies population in the purpose-built earthen ponds varied with pond location, seasons and water quality, defined by a number of abiotic and biotic environment parameters. During summer and autumn, most of the studied nutrients from pond bottom soil and water column correlated with and significantly affected the yabby population. Dissolved inorganic nitrogen, soluble reactive phosphorus and zooplankton density in the waters had a larger positive effect on yabbies population, while pH and hardness had a negative influence on the yabbies population.

CHAPTER 4: OSMOREGULATORY CAPACITY, HEALTH STATUS AND GROWTH AS FUNCTIONS OF MOULT STAGES FROM VARIOUS WEIGHT CLASSES IN MARRON, *CHERAX CAINII* (AUSTIN AND RYAN 2002) AND YABBIES, *CHERAX ALBIDUS* (CLARK 1936)

(Marine and Freshwater Behaviour and Physiology, Accepted)

4.1 INTRODUCTION

Since the decline in wild-caught freshwater crayfish populations from the 1970s, their culture has become a serious consideration (Jones 1997). Presently, the Australian freshwater crayfish aquaculture industry is based on three parastacid species; yabbies, *Cherax albidus* (Clark 1936), marron, *Cherax cainii* (Austin and Ryan 2002), and red claw, *Cherax quadricarinatus* (von Martens). The commercial potential of these species is well recognised internationally, and these species have been exported from Australia for market evaluation, aquaculture research and commercial trials (Holdich 1993; Medley et al. 1994; Austin 1995). Over the last five years, however, the production of marron and yabbies in Western Australia has not significantly improved (ABARES 2014). Besides the lack of nutritional knowledge, the main reason for the stagnant production is the lack of unified farming management practices as farming operations are still relying on a model of cottage industry.

The change in physiological and biochemical parameters of crustaceans is a function of internal and external factors (Vargas-Albores and Ochoa 1982; Skinner 1985; Lignot et al. 1999; Lignot et al. 2000; Pratoomchat et al. 2002b; Pascual et al. 2006; Marcy et al. 2009). Physiological measurements as bio-indicators, such as osmoregulatory capacity (OC: difference between the osmotic pressures of the haemolymph and of the external medium), hepatosomatic indices, the moisture content of hepatopancreas and growth are used to understand the underlying physiological state of an animal and thus allow prediction of stressors such as moult stage, feeding status and the shifting in development period (Lignot et al. 2000). OC has already been used as a critical bio-marker in crustaceans (Charmantier et al. 1989; Lin et al. 1991; Young-Lai et al. 1991; Mayer et al. 1992; Bambang et al. 1995a; Bambang et al. 1995b; Lignot et al. 1997; Lignot et al. 1998) in detection of stressors (Lignot et al. 2000).

Moulting or ecdysis is a highly complex process that requires precise coordination of various physiological events (Chang 1995) and is a function of a number of environmental and endogenous factors such as temperature (Hughes et al. 1972; Chittleborough 1975), photoperiod (Quackenbush and Herrnkind 1983), food supply (Chittleborough 1975), space (Cheng and Chang 1994), various stressors (Weis et al. 1992), neuropeptides (Chang and Mykles 2011; Webster et al. 2012), hardness and ionic profile of water, developmental stages and reproductive maturity (Skinner 1985; Cheng and Chang 1994). All these factors can affect moult frequency and size increment during ecdysis. Moulting also regulates physiological and biochemical changes in various tissues such as haemolymph and hepatopancreas (Heath and Barnes 1970; Richard 1980; Durliat and Vranckx 1982).

Haemolymph osmolality is a commonly monitored parameter in several crustacean species and varies with moult cycle and body weight (Vargas-Albores and Ochoa 1982; Lignot et al. 1999; Pascual et al. 2006; Marcy et al. 2009). OC can also be used as a bio-indicator, not only to predict stress levels and pollutants from the culture environment, but also as a reference to define moult stages in many crustaceans (Chang and O'Connor 1983; Skinner 1985). The changes in OC in a number of decapod crustaceans have been evaluated from a variety of experimental conditions and the relationship between OC with ammonia levels (Lin et al. 1991; Young-Lai et al. 1991), oxygen depletion (Charmantier and Soyez 1994) and heavy metals (Bambang et al. 1995a; Bambang et al. 1995b) documented. So far, no information is available on any variations in OC and health conditions in marron and yabbies during the moult cycle which is accompanied by fluctuations in body weight.

The hepatopancreas has an important role in the metabolism of crustaceans, having roles in digestion and absorption, synthesis and secretion of digestive enzymes (McLaughlin 1983). Moreover, it is the largest centre of organic and inorganic reserves of decapods (Adiyodi 1969) and is also the largest organ for the absorption and storage of lipids derived from food (Garcia et al. 2002). Wet and dry weight of hepatopancreas, relative to total body weight, and hepatopancreas moisture content have been used to indicate the health condition of crustaceans (Huner and Lindqvist 1985; Huner and Romaire 1990; Evans et al. 1992; Villagran 1993; McClain 1995a; Mannonen and Henttonen 1995). Changes in the relative size and composition of the hepatopancreas

are associated with ontogenetic development, reproduction, moulting, growth and nutrient intake (Jones 1997). Lindqvist and Louekari (1975) showed that hepatosomatic indices are influenced by moult stages in the noble crayfish *Astacus astacus,* while no study has considered the effects of size on hepatosomatic indices during the moult cycle of marron and yabbies.

The aim of this research was to determine any effect of moult stages of various weight classes of marron and yabbies on the OC, hepatosomatic indices, and weight increments during the moulting process.

4.2 MATERIALS AND METHODS

A total of 305 marron (2.5-73.9 g wet body weight) and 305 yabbies (3.1-74.8 g) were purchased from commercial wholesalers in Western Australia. The animals were transported to Curtin Aquatic Research Laboratory for 3 months' acclimation. Marron and yabbies were separately stocked in round fibreglass tanks (diameter 120 cm x height 75 cm) with 800 L of filtered water and constant aeration. Each tank was equipped with a recirculating biological filtration system (Fluval 205, Askoll, Italy). The water in the tank was continuously recirculated at a rate of approximately 4 L minute^{-1}. The tanks were also provided with a sufficient number of stacked shelters in the form of PVC pipes of appropriate diameters and lengths covering up to 50% of tank base area to provide shelters and thus avoiding cannibalism during the moulting process.

During acclimation, water quality was kept at an optimum level at a set temperature (23 ± 1°C) using a submersible thermostat (Sonpar®, Model: HA-100, China), stable dissolved oxygen (8.1 ± 0.4 mg L^{-1}) and a photoperiod of 12 hours of light and dark. The crayfish were fed *ad libitum* with commercial pellets (24.0% crude protein, 6.0% lipid and 5.0% ash) on alternate days. After one hour of feeding, the uneaten feed was siphoned out from the tanks. The amount of water lost during syphoning or evaporation was topped up daily using the same water source. During the experimental period, both marron and yabbies from acclimated tanks were divided into two groups to be used for two independent experiments.

Group 1 for experiment 1: Two hundred crayfish (100 marron and 100 yabbies of 6 weight classes) were individually weighed, housed and numbered in transparent plastic containers (30 cm length x 15 cm width x 10 cm depth) with 10 mm diameter holes on

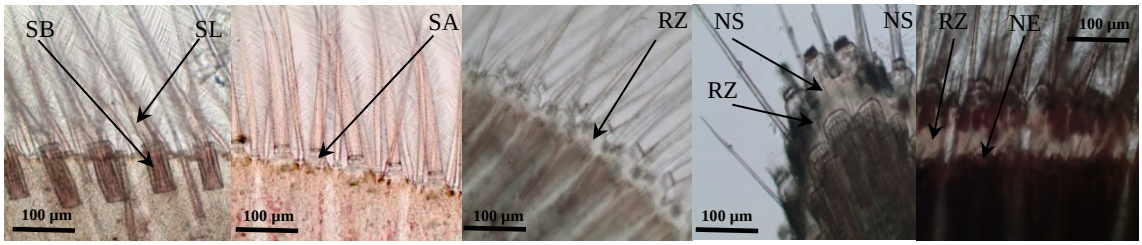
the lids and sides for water circulation and feeding. These containers were then placed in a single layer in the tanks (300 cm length x 200 cm width x 50 cm depth) holding 1800 L of water.

During three month experiment, the same water quality conditions, procedure and frequency for water level, feed, and feeding were maintained as during acclimation. Each tank was provided with two recirculating biological filtration systems (Fluval 205, Askoll, Italy) with a total flow rate of 8 L minute^{-1}. Water temperature, pH, and dissolved oxygen were measured daily using digital pH/ºC and dissolved oxygen meters (CyberScan pH 300 and CyberScan DO 300, Eutech Instruments, Singapore). Total ammonia nitrogen (TAN = NH_3 + NH_4^+) was monitored daily using ammonia (NH_3/NH_4^+) test kits (Mars Fishcare, Chalfont, PA, USA).

Group 2: Two hundred and seventy (135 marron and 135 yabbies) animals remained in the acclimation tanks for experiment 2.

Examination of moult stages

Prior to moult stage examination, individual crayfish were placed in crushed ice to render them inactive. Each animal was then placed on its back in a petri dish, the uropods flattened, covered with distilled water and a cover-slip, and setae on the apical quarter of the uropod margin were then examined under a compound microscope (Leica Microsystem DM 2500- German at x 100 magnification). Five discrete moult stages: postmoult (AB), intermoult (C), premoult (D_0, D_1, D_2) (Figure 4.1) were identified by examining setal development and changes in epidermal retraction state on the uropods according to Drach (1939) and Drach and Tchernigovtzeff (1967) and further modified by Ha and Fotedar (unpublished).



Postmoult (AB) Intermoult (C) Premoult (D_0) Premoult (D_1) Premoult (D_2)

Figure 4.1: Five moult stages of marron and yabbies based on the setae development of the uropod and changes in epidermal retraction state. SB: setal base, SL: setal lumen, SA: setal articulation, RZ: retracted zone, NS: new setae, NC: new epidermis.

The main characteristics used to define the moult stages were AB: setal base is clearly visible, thin cuticle, epidermal tissue is visible inside the setal lumen; C: thick cuticle layer at setal bases where the epidermis lies just underneath; D_0: a translucent space to form between the old cuticle and the epidermis; epidermis retraction starts from the cuticle; D_1: retraction of epidermis from is maximal, new setae appear and are clearly visible; D_2: the appearance of the new, folded cuticle layer, and the new setae are visible.

Sampling and data collection from Group 1:

Both marron and yabbies were grouped in 6 weight classes. They were examined for moult stages every 2 days. Weight was measured, and a haemolymph sample was taken when the next moulting stage was attained. The excess water was removed from crayfish using absorbent paper hand towels before weighing and haemolymph sampling.

Approximately 50 µL of haemolymph was extracted from the pericardial cavity through the intersegment membrane between the cephalothorax and the first abdominal segment using a 0.5 mL syringe (Sang and Ravi 2004). Crayfish were returned to the containers after haemolymph sampling. Haemolymph osmolality and medium osmolality (mOsm kg^{-1}) were measured at the same time by injecting a 20 µL sample into a micro-osmometer (Model 3MO plus, Advance Instruments, Norwood, MA, USA) expressed in mOsm kg^{-1}. The OC was calculated as the difference mOsm kg^{-1} of osmotic pressure between haemolymph and medium osmolality.

Moult interval and weight increment were accordingly calculated for every moult stage, using the following equations.

Moult Interval (day): $T_m = T_{n+1} - T_n$

where T_n = date of n moult stage, T_{n+1} = date of n + 1 moult stage

Weight increment (g) at a moult (W_m) (Jussila 1996): $W_m = W_a - W_b$ (Equation 1)

where: W_a = weight after moult, (g); W_b = weight before moult (g)

Hepatopancreas sampling and dry weight analysis from Group 2:

The hepatopancreas was dissected from 100 marron and 100 yabbies after the animals were weighed and had their moult stage assigned. The hepatopancreas was then placed in aluminium foil cups and weighed. The intact hepatopancreas were dried at 70°C for 24 hours and weighed. Results were expressed as wet hepatosomatic index (Equation 2), dry hepatosomatic index (Equation 3) and hepatopancreas moisture content (Equation 4).

HI_{wet} (%) = W_{wh} x 100 / Wt (Equation 2)

where: W_{wh} = weight of wet hepatopancreas (g); and W_t = total weight of crayfish (g)

HI_{dry} (%) = W_{dh} x 100 / W_t (Equation 3)

where: W_{dh} = weight of dry hepatopancreas (g); and W_t = total weight of crayfish (g)

HM (%) = (W_{wh} - W_{dh}) x 100 / W_{wh} (Equation 4)

For dry weight (%): Seventy (70) marron (23.2 ± 2.5 g) and 70 yabbies (23.7 ± 2.1 g) from Group 2 were used for dry weight (%) analysis after moult staging and weighing.

W_{dry} (%) at a moult stage = (W_{dry} x 100) / W_{wet}

where: W_{dry} = weight of dry crayfish at a moult (g)

W_{wet} = total weight of crayfish (g)

Statistical analysis

Analysis of variance (ANOVA) was performed to determine differences in measured parameters among moult stages and weight classes. Linear regression was used to analyse a correlation between body weight and OC in different moult stages. All analyses were performed using the statistical software SPSS 18.0, 2014. Results were presented as means ± SE. Significant tests were performed at $P < 0.05$ level.

For all tests, ANOVA assumptions regarding the normal distribution of residuals and homogeneity of variances were satisfied (Levene tests). To satisfy the assumptions of

normality and/or homogeneity of variance data were transformed to $\log_{10}(x + 1)$. Significant differences ($P < 0.05$) between the means were determined by post hoc comparison using Duncan's least significant difference analysis.

4.3 RESULTS

The measured water quality parameters were stable during the experiment. Water temperature, pH and dissolved oxygen averaged 23.1 ± 0.8ºC, 6.8 ± 0.5, 8.12 ± 0.7 mg L^{-1} (mean ± SD), respectively. TAN was always ≤ 0.5 mg L^{-1}, while osmolality of medium was at 105 ± 4 mOsm kg^{-1}.

Table 4.1: OC (mOsm kg^{-1}) of various weight classes of and moult stages of marron and yabbies

Weight (g)	Species	AB	n	C	n	D_0	n	D_1	n	D_2	n
2-15	Marron	$_1351 \pm 8^a$	11	$_1391 \pm 10^b$	6	$_1370 \pm 9^c$	7	$_1356 \pm 14^a$	7	$_1352 \pm 12^a$	7
	Yabbies	$_1346 \pm 12^a$	10	$_1386 \pm 11^b$	5	$_1364 \pm 10^c$	6	$_1348 \pm 11^a$	7	$_1343 \pm 13^a$	8
16-30	Marron	$_2379 \pm 9^a$	9	$_2468 \pm 8^b$	6	$_2445 \pm 12^c$	5	$_2403 \pm 8^a$	9	$_2383 \pm 12^a$	8
	Yabbies	$_2368 \pm 11^a$	9	$_3449 \pm 10^b$	4	$_2444 \pm 11^b$	6	$_2402 \pm 10^a$	5	$_2377 \pm 11^a$	6
31-45	Marron	$_3393 \pm 8^a$	8	$_2469 \pm 8^b$	5	$_3459 \pm 10^b$	7	$_2407 \pm 12^a$	4	$_2389 \pm 11^a$	9
	Yabbies	$_2373 \pm 9^a$	11	$_3453 \pm 7^b$	7	$_2442 \pm 10^b$	4	$_2407 \pm 10^a$	5	$_2385 \pm 12^a$	4
46-60	Marron	$_3415 \pm 11^a$	6	$_2475 \pm 7^b$	4	$_3457 \pm 8^c$	5	$_2414 \pm 9^a$	5	$_2392 \pm 9^a$	7
	Yabbies	$_2390 \pm 11^a$	6	$_3456 \pm 9^b$	5	$_2436 \pm 9^c$	4	$_2409 \pm 12^a$	5	$_2405 \pm 9^a$	6
61-75	Marron	$_3416 \pm 12^a$	5	$_2480 \pm 11^b$	6	$_3442 \pm 8^c$	7	$_3421 \pm 11^a$	5	$_3420 \pm 8^a$	5
	Yabbies	$_3417 \pm 10^a$	5	$_3460 \pm 12^b$	5	$_2436 \pm 10^c$	5	$_3419 \pm 12^a$	4	$_3417 \pm 10^a$	5

Same alphabetical superscripts (a, b, c) in the same row (comparisons among moult stages) and numerical subscripts (1, 2, 3) in the same column (comparisons among weight classes and species) are not significantly different at the α = 0.05 level. n = number of animals used.

Moult stages in all weight classes in both crayfish fall into three OC levels based on significant differences (Table 4.1). The OC of both crayfish were significantly, however intermittently, influenced by their respective moults and weight classes. The hyper OC was at the highest in the intermoult stage (C) and then gradually decreased at early

premoult (D_0), while OC at stages D_1, D_2 and AB were similar. No difference in OC between species for all moult stages was observed in the weight class of 2-15 g.

Table 4.2: Linear regression equation, correlation coefficient and calculated probability between OC and body weight of crayfish for each moult stage of marron and yabbies

Moult stage	Species	Regression equation	n	R^2	P-value
AB	Marron	$y = 0.8x + 360.7^a$	36	0.74	< 0.05
	Yabbies	$y = 1.1x + 363.7^a$	31	0.77	< 0.05
C	Marron	$y = 1.2x + 438.3^b$	39	0.45	< 0.05
	Yabbies	$y = 1.0x + 420.1^c$	36	0.53	< 0.05
D_0	Marron	$y = 1.1x + 426.8^c$	28	0.52	< 0.05
	Yabbies	$y = 1.3x + 416.8^c$	33	0.67	< 0.05
D_1	Marron	$y = 0.9x + 412.5^c$	29	0.75	< 0.05
	Yabbies	$y = 1.0x + 402.5^d$	21	0.71	< 0.05
D_2	Marron	$y = 0.8x + 401.2^d$	31	0.49	< 0.05
	Yabbies	$y = 0.9x + 401.2^d$	23	0.66	< 0.05

Same alphabetical superscripts (a, b, c, d) are not significantly different at the $\alpha = 0.05$ level. No difference among regression slopes. n = number of animals used.

In each moult stage, the hyper-OC increased linearly with the increase in body weight in both yabbies and marron (Table 4.2). The correlation between OC and body weight in each moult stage was positive and significant ($P < 0.05$). A significant difference ($P < 0.05$) was observed among intercepts in both species. However, no difference ($P > 0.05$) could be detected among the slopes from the regression equations.

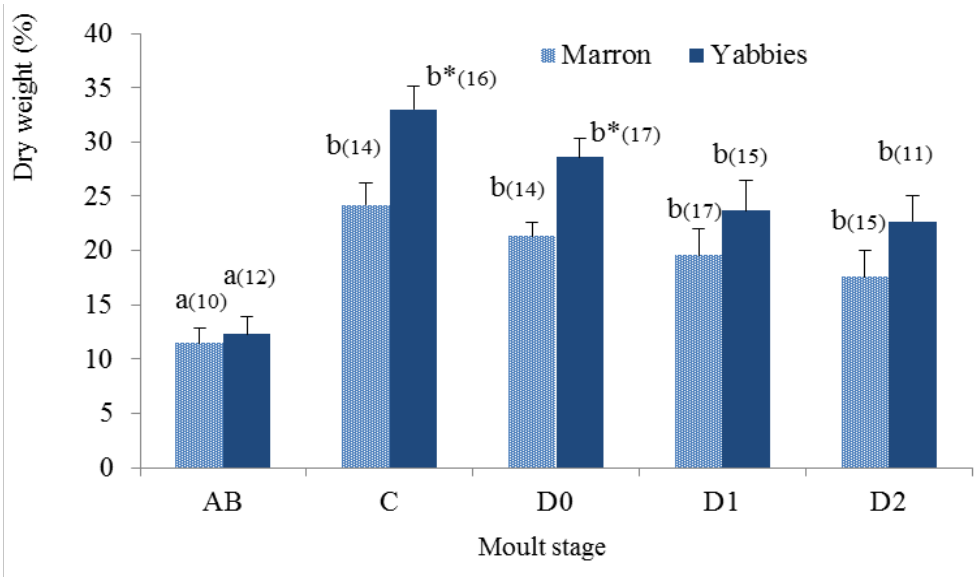


Figure 4.2: Change in dry weight (%) with moult stages at a given weight of marron (23.2 ± 2.5 g) and yabbies (23.7 ± 2.1 g). Moult stages with different letters are significantly different at $P < 0.05$. Asterisk (*) indicates a significant difference ($P < 0.05$) between species. Numbers in brackets refer to the number of observations.

For a pre-selected body wet weight of both crayfish, carcass dry weight of yabbies was significantly higher than marron at intermoult stage C and early premoult stage (D_0) (Figure 4.2). Postmoult stage (AB) had significantly lower carcass dry weight.

Table 4.3: Days to complete individual moult stages from different weight classes of marron and yabbies

Weight (g)	Species	AB	C	D_0	D_1	D_2
2-15	Marron	$_{1}2.1 \pm 0.3^{a}$ (14)	$_{1}17.8 \pm 2.1^{b}$ (12)	$_{1}7.5 \pm 1.3^{c}$ (16)	$_{1}1.9 \pm 1.1^{a}$ (10)	$_{1}1.2 \pm 1.0^{a}$ (9)
	Yabbies	$_{1}2.5 \pm 0.4^{a}$ (20)	$_{1}18.2 \pm 1.5^{b}$ (14)	$_{1}7.9 \pm 2.5^{c}$ (24)	$_{1}2.1 \pm 1.6^{a}$ (13)	$_{1}2.1 \pm 1.5^{a}$ (10)
16-30	Marron	$_{1}2.7 \pm 0.3^{a}$ (16)	$_{2}21.6 \pm 1.4^{b}$ (21)	$_{2}10.2 \pm 2.1^{c}$ (21)	$_{1}2.3 \pm 1.1^{a}$ (20)	$_{1}1.5 \pm 1.2^{a}$ (15)
	Yabbies	$_{2}4.1 \pm 1.1^{a}$ (21)	$_{3}25.4 \pm 1.5^{b}$ (16)	$_{2}11.5 \pm 1.1^{c}$ (18)	$_{2}3.5 \pm 2.1^{a}$ (17)	$_{1}2.6 \pm 1.1^{a}$ (11)
31-45	Marron	$_{2}3.8 \pm 0.6^{a}$ (19)	$_{2}23.6 \pm 2.0^{b}$ (23)	$_{2}12.6 \pm 2.2^{c}$ (13)	$_{2}4.1 \pm 2.2^{a}$ (12)	$_{2}3.4 \pm 2.1^{a}$ (9)
	Yabbies	$_{3}4.9 \pm 0.5^{a}$ (25)	$_{4}27.4 \pm 1.5^{b}$ (27)	$_{2}13.4 \pm 3.3^{c}$ (11)	$_{2}4.6 \pm 2.0^{a}$ (10)	$_{2}4.1 \pm 1.2^{a}$ (18)

46-60	Marron	$_{3}4.9 \pm 1.0^{a}$ (27)	$_{2}25.9 \pm 1.5^{b}$ (20)	$_{3}15.4 \pm 2.9^{c}$ (19)	$_{2}4.8 \pm 2.1^{a}$ (13)	$_{2}4.6 \pm 1.0^{a}$ (12)
	Yabbies	$_{4}7.6 \pm 0.5^{a}$ (16)	$_{4}29.8 \pm 1.4^{b}$ (16)	$_{3}16.9 \pm 1.6^{c}$ (24)	$_{2}4.7 \pm 1.2^{a}$ (18)	$_{2}4.9 \pm 1.1^{a}$ (10)
61-75	Marron	$_{4}7.2 \pm 0.7^{a}$ (18)	$_{3}26.9 \pm 2.0^{b}$ (12)	$_{3}19.8 \pm 2.5^{c}$ (21)	$_{3}6.8 \pm 1.1^{a}$ (21)	$_{3}6.4 \pm 0.8^{a}$ (13)
	Yabbies	$_{5}9.9 \pm 1.1^{a}$ (14)	$_{5}31.7 \pm 1.6^{b}$ (14)	$_{3}20.6 \pm 1.9^{c}$ (17)	$_{3}6.7 \pm 1.2^{a}$ (24)	$_{4}8.2 \pm 1.2^{a}$ (14)

Same alphabetical superscripts (a, b, c) in the same row (comparisons among moult stages) and numerical subscripts (1, 2, 3, 4, 5) in the same column (comparisons among weight classes and species) are not significantly different at the α = 0.05 level. Numbers in the brackets are animals used for the measurements.

Postmoult (AB) and late premoult (D_1 and D_2) stages were the shortest while intermoult (C) was the longest (Table 4.3). The intermoult stage (C) accounted for up to 55% of the entire moult cycle. Only in early premoult stage (D_0) both marron and yabbies spend the same duration, although this duration gradually increased as the crayfish grew.

Table 4.4: Total number of days to complete a moult cycle of marron and yabbies in different weight classes

Weight class (g)	Marron	n	Yabbies	n
2-15	$_{1}35.4 \pm 2.2^{a}$	15	$_{1}30.5 \pm 2.3^{a}$	9
16-30	$_{2}49.1 \pm 1.9^{a}$	12	$_{2}38.3 \pm 2.1^{b}$	10
31-45	$_{3}58.2 \pm 3.1^{a}$	11	$_{3}47.5 \pm 2.8^{b}$	13
46-60	$_{4}65.9 \pm 2.4^{a}$	10	$_{4}58.6 \pm 1.9^{b}$	14
61-75	$_{5}78.1 \pm 3.5^{a}$	16	$_{5}64.1 \pm 2.9^{b}$	10

Same alphabetical superscripts (a, b) in the same row (comparisons between species) and numerical subscripts (1, 2, 3, 4, 5) in the same column (comparisons among weight classes) are not significantly different at the α = 0.05 level. n = number of animals used.

As the weight category of both crayfish increased, it took longer for them to complete the moult cycle (Table 4.4). Except for weight class 2-15 g, both species differed from one another to complete one moult cycle.

Table 4.5: Weight increment (g) with moult stage and wet body weight

Weight (g)	Species	AB	n	C	n	D_0	n	D_1	n	D_2	N
2-15	Marron	$_1 2.8 \pm 0.5^a$	6	$_1 1.9 \pm 0.2^b$	7	$_1 1.0 \pm 0.3^c$	5	$_1 0.8 \pm 0.2^c$	8	$_1 0.7 \pm 0.3^c$	6
	Yabbies	$_1 2.5 \pm 0.4^a$	8	$_1 2.0 \pm 0.4^b$	6	$_1 1.5 \pm 0.2^b$	8	$_1 0.7 \pm 0.2^c$	6	$_1 0.8 \pm 0.3^c$	8
16-30	Marron	$_2 3.2 \pm 0.1^a$	5	$_1 1.6 \pm 0.3^b$	8	$_1 1.5 \pm 0.5^b$	7	$_1 0.9 \pm 0.1^c$	7	$_1 0.7 \pm 0.2^c$	7
	Yabbies	$_1 2.3 \pm 0.3^a$	10	$_1 1.7 \pm 0.4^b$	7	$_1 1.3 \pm 0.3^b$	6	$_1 1.1 \pm 0.2^b$	8	$_1 0.8 \pm 0.2^c$	9
31-45	Marron	$_2 3.6 \pm 0.2^a$	7	$_1 1.8 \pm 0.4^b$	5	$_1 1.7 \pm 0.3^b$	8	$_1 1.0 \pm 0.2^b$	5	$_1 0.8 \pm 0.3^c$	6
	Yabbies	$_1 2.6 \pm 0.3^a$	8	$_1 1.8 \pm 0.5^b$	6	$_1 1.5 \pm 0.4^b$	4	$_1 1.0 \pm 0.3^b$	9	$_1 0.7 \pm 0.1^c$	7
46-60	Marron	$_2 3.9 \pm 0.5^a$	6	$_1 2.2 \pm 0.2^b$	4	$_1 2.0 \pm 0.4^b$	6	$_1 1.3 \pm 0.3^b$	6	$_1 0.9 \pm 0.3^c$	6
	Yabbies	$_1 3.1 \pm 0.2^a$	5	$_1 1.9 \pm 0.4^b$	9	$_1 1.6 \pm 0.4^b$	9	$_1 1.4 \pm 0.2^b$	5	$_1 1.1 \pm 0.5^b$	7
61-75	Marron	$_2 3.7 \pm 0.2^a$	8	$_1 2.3 \pm 0.2^b$	6	$_1 2.1 \pm 0.3^b$	5	$_1 1.2 \pm 0.3^c$	7	$_1 1.1 \pm 0.3^c$	6
	Yabbies	$_1 3.0 \pm 0.3^a$	9	$_1 2.1 \pm 0.3^b$	12	$_1 1.7 \pm 0.2^b$	7	$_1 1.5 \pm 0.4^b$	5	$_1 1.3 \pm 0.3^b$	8

Same alphabetical superscripts (a, b, c) in the same row (comparisons among moult stages) and numerical subscripts (1, 2, 3) in the same column (comparisons among weight classes and species) are not significantly different at the $\alpha = 0.05$ level. n = number of animals used.

Weight increment of marron and yabbies varied significantly among moult stages (Table 4.5). It was significantly higher during postmoult (AB) than the other moult stages and was lowest during premoult stages D1 and D2. Body weight classes were found to be independent of weight increment over moult stages, notably over intermoult (C) and premoult (D_0, D_1, D_2). Only during postmoult (AB) of larger than 15 g crayfish, weight increment of marron was significantly higher than that of yabbies.

Table 4.6: Change in HM% with moult stage and wet body weight

Weight (g)	Species	AB	n	C	n	D_0	n	D_1	n	D_2	n
2-15	Marron	$_1 72.5 \pm 3.2^a$	9	$_1 66.8 \pm 3.6^b$	8	$_1 67.2 \pm 3.2^b$	7	$_1 65.3 \pm 3.4^b$	9	$_1 64.3 \pm 2.7^b$	7
	Yabbies	$_1 73.3 \pm 2.5^a$	6	$_1 67.1 \pm 2.5^b$	9	$_1 66.2 \pm 5.9^b$	6	$_1 64.7 \pm 2.1^b$	7	$_1 63.5 \pm 3.2^b$	7
16-30	Marron	$_1 77.2 \pm 3.8^a$	8	$_1 67.3 \pm 2.1^b$	11	$_1 67.5 \pm 2.9^b$	5	$_1 65.1 \pm 2.2^b$	7	$_1 63.7 \pm 3.1^b$	9
	Yabbies	$_1 72.2 \pm 2.1^a$	10	$_1 70.1 \pm 2.1^b$	12	$_1 66.3 \pm 2.5^a$	7	$_1 63.7 \pm 2.8^b$	6	$_1 64.5 \pm 3.1^b$	6

31-45	Marron	$_1$77.8 ± 3.2^a	10	$_1$66.5 ± 3.1^b	11	$_1$68.6 ± 2.7^b	5	$_1$67.7 ± 2.8^b	7	$_1$63.9 ± 3.9^b	7
	Yabbies	$_1$74.5 ± 2.6^a	8	$_1$65.2 ± 2.1^b	8	$_1$67.8 ± 2.5^b	5	$_1$65.7 ± 3.6^b	8	$_1$64.1 ± 2.5^b	6
46-60	Marron	$_1$77.3 ± 1.8^a	12	$_1$63.4 ± 3.7^b	10	$_1$66.5 ± 2.3^b	6	$_1$66.7 ± 2.9^b	6	$_1$65.5 ± 4.5^b	8
	Yabbies	$_1$71.5 ± 2.8^a	9	$_1$61.3 ± 3.2^b	9	$_1$65.8 ± 3.9^b	9	$_1$65.4 ± 2.7^b	6	$_1$64.7 ± 2.4^c	8
61-75	Marron	$_1$75.5 ± 2.6^a	11	$_1$65.3 ± 2.1^b	8	$_1$65.3 ± 2.7^b	7	$_1$64.2 ± 3.4^b	7	$_1$62.9 ± 3.9^b	9
	Yabbies	$_1$69.9 ± 2.3^a	7	$_1$63.3 ± 3.7^b	9	$_1$65.7 ± 3.1^b	8	$_1$64.3 ± 2.9^b	9	$_1$63.7 ± 2.7^b	7

Same alphabetical superscripts (a, b, c) in the same row (comparisons among moult stages) and numerical subscripts in the same column (comparisons among weight classes and species) are not significantly different at the α = 0.05 level. n = number of animals used.

There was a significant change in HM (%) over moult stages (Table 4.6). The highest value of HM (%) was observed during postmoult (AB) and the lowest was in intermoult (C) and premoult (D_0, D_1, D_2). The weight classes showed no influence on the HM (%) in all moult stages.

Table 4.7: Change in HI_{wet} with moult stage and wet body weight

Weight (g)	Species	AB	n	C	n	D_0	n	D_1	n	D_2	n
2-15	Marron	$_1$5.2 ± 0.5^a	9	$_1$6.6 ± 0.2^b	8	$_1$5.1 ± 0.2^a	7	$_1$4.3 ± 0.3^a	9	$_1$5.0 ± 0.4^a	7
	Yabbies	$_1$5.5 ± 0.2^a	6	$_1$6.7 ± 0.3^b	9	$_1$5.3 ± 0.3^a	6	$_1$4.4 ± 0.5^a	7	$_1$4.6 ± 0.3^a	7
16-30	Marron	$_1$5.0 ± 0.3^a	8	$_1$6.5 ± 0.2^b	11	$_1$5.2 ± 0.4^a	5	$_1$4.2 ± 0.5^a	7	$_1$4.7 ± 0.2^a	9
	Yabbies	$_1$5.8 ± 0.2^a	10	$_2$7.6 ± 0.3^a	12	$_1$5.6 ± 0.3^a	7	$_1$4.9 ± 0.4^a	6	$_1$4.4 ± 0.6^b	6
31-45	Marron	$_2$4.1 ± 0.3^a	10	$_1$6.2 ± 0.4^b	11	$_1$5.0 ± 0.2^a	5	$_1$5.0 ± 0.3^a	7	$_1$4.9 ± 0.7^a	7
	Yabbies	$_2$4.5 ± 0.2^a	8	$_2$7.5 ± 0.3^b	8	$_1$4.7 ± 0.3^a	5	$_1$4.6 ± 0.4^a	8	$_1$4.3 ± 0.5^a	6
46-60	Marron	$_2$4.2 ± 0.3^a	12	$_1$5.8 ± 0.3^b	10	$_2$4.1 ± 0.3^a	6	$_1$3.8 ± 0.5^a	6	$_1$3.9 ± 0.3^a	8
	Yabbies	$_2$4.6 ± 0.3^a	9	$_2$7.1 ± 0.2^b	9	$_2$4.2 ± 0.2^a	9	$_1$3.9 ± 0.4^a	6	$_1$3.7 ± 0.2^a	8
61-75	Marron	$_2$3.8 ± 0.2^a	11	$_1$5.9 ± 0.1^b	8	$_2$4.2 ± 0.5^a	7	$_1$4.5 ± 0.5^a	7	$_1$4.2 ± 0.4^a	9
	Yabbies	$_2$4.5 ± 0.2^a	7	$_2$7.5 ± 0.3^b	9	$_2$4.4 ± 0.4^a	8	$_1$4.3 ± 0.3^a	9	$_1$4.5 ± 0.2^a	7

Same alphabetical superscripts (a, b) in the same row (comparisons among moult stages) and numerical subscripts (1, 2) in the same column (comparisons among weight classes and species) are not significantly different at the α = 0.05 level. n = number of animals used.

There was a significantly higher HI_{wet} index in moult stage C in all weight classes of both marron and yabbies (Table 4.7). The lowest value of HI_{wet} was found in late premoult stage (D_2). Larger marron and yabbies of the weight range 31-75 g had a lower HI_{wet} than smaller crayfish of 2-30 g. The dependence of species on HI_{wet} was observed only in intermoult stage (C) of crayfish with a body weight of more than 15 g.

Table 4.8: Change in HI_{dry} with moult stage and wet body weight

Weight (g)	Species	AB	n	C	n	D_0	n	D_1	n	D_2	n
2-15	Marron	$_1$1.0 ± 0.1^a	9	$_1$1.6 ± 0.2^b	8	$_1$1.1 ± 0.2^a	7	$_1$1.0 ± 0.2^b	9	$_1$0.8 ± 0.3^a	7
	Yabbies	$_1$1.1 ± 0.2^a	6	$_1$1.5 ± 0.2^b	9	$_1$1.3 ± 0.2^a	6	$_1$1.2 ± 0.2^b	7	$_1$1.0 ± 0.1^a	7
16-30	Marron	$_1$1.0 ± 0.1^a	8	$_1$1.5 ± 0.2^b	11	$_1$1.2 ± 0.1^a	5	$_1$1.5 ± 0.3^a	7	$_1$1.2 ± 0.1^a	9
	Yabbies	$_1$1.3 ± 0.1^a	10	$_2$2.6 ± 0.3^b	12	$_1$1.7 ± 0.2^a	7	$_1$1.3 ± 0.1^a	6	$_1$1.3 ± 0.2^a	6
31-45	Marron	$_1$1.1 ± 0.2^a	10	$_1$1.7 ± 0.2^b	11	$_1$1.3 ± 0.2^a	5	$_1$1.2 ± 0.2^a	7	$_1$1.3 ± 0.2^a	7
	Yabbies	$_1$1.1 ± 0.1^a	8	$_2$2.9 ± 0.3^b	8	$_1$1.8 ± 0.3^a	5	$_1$1.3 ± 0.1^a	8	$_1$1.4 ± 0.2^a	6
46-60	Marron	$_1$0.9 ± 0.2^a	12	$_1$1.8 ± 0.3^b	10	$_1$1.3 ± 0.2^a	6	$_1$1.2 ± 0.3^b	6	$_1$1.3 ± 0.3^a	8
	Yabbies	$_1$1.2 ± 0.2^a	9	$_2$2.8 ± 0.2^b	9	$_1$1.6 ± 0.2^a	9	$_1$1.3 ± 0.2^a	6	$_1$1.5 ± 0.2^a	8
61-75	Marron	$_1$1.0 ± 0.2^a	11	$_1$1.6 ± 0.2^b	8	$_1$1.3 ± 0.3^a	7	$_1$1.1 ± 0.2^a	7	$_1$1.1 ± 0.2^a	9
	Yabbies	$_1$1.0 ± 0.2^a	7	$_2$2.7 ± 0.1^b	9	$_1$1.7 ± 0.1^a	8	$_1$1.2 ± 0.1^a	9	$_1$1.2 ± 0.2^a	7

Same alphabetical superscripts (a, b) in the same row (comparisons among moult stages) and numerical subscripts (1, 2) in the same column (comparisons among weight classes and species) are not significantly different at the $\alpha = 0.05$ level. n = number of animals used.

Unlike HI_{wet}, HI_{dry} was lowest during the postmoult stage (AB), while it stayed at a peak level during intermoult stage (C) (Table 4.8). Weight category did not clearly affect HI_{dry} index, whereas HI_{wet} in the intermoult stage (C) was species-dependent, except weight class of 2-15 g.

4.4 DISCUSSION

The moult cycle of crustaceans is a complex sequential series of well-defined physiological and morphological stages. The overview of the different moult stages is based on the review article by Aiken and Waddy (1992) describing the growth process

in crayfish. Various terminologies have been used to define individual moult stages in decapods with common moult stage categories such as A, B, C and D (Drach 1939; Burton and Mitchell 1987; Lignot et al. 2000; Mugnier and Justou 2004; Cesar and Yang 2007; Galindo et al. 2009; Yeh et al. 2009; Liu et al. 2010; Tian et al. 2012; Sugumar et al. 2013).

Although the moult cycle of yabbies has been documented by Burton and Mitchell (1987), there is no published description of the moult stages of marron. The purpose of the current study was not to re-define the moult stages of crayfish farmed in Western Australia but to use a simplified description of crayfish moult stages and relate them to OC and other health status parameters. Therefore, of the basis of uropod setal development and changes in epidermal retraction state, 5 simplified distinct moult stages were recognised in marron and yabbies (Figure 1), which is closely related to the terminology used by Lignot et al. (1999) and Tian et al. (2012). These distinct moult stages can be readily and practically identified with light microscopic examination: Postmoult (AB), intermoult (C) and early and late premoult (D_0, D_1 and D_2). A more refined separation of these major stages is only possible by transmission electron microscopy and thus is not practical or usually not even relevant to research into the moult process and its impact on other factors (Robertson et al., 1987). Robertson et al. (1987), Sanchez-Paz et al. (2003), Cesar and Yang (2007), Liu et al. (2004, 2010) and Yeh et al. (2009) used a similar division of the moult cycle into five moult stages.

The stress by handling and culture environment are known to be related to the change in OC, health condition and growth of crustaceans (Lignot et al. 2000). However, manipulation procedure and frequency of handling were similarly applied for every single crayfish during the experiment. Hence, handling caused- bias on data sets was same for any moult stage and weight class. Moreover, during experiment the provided stable and favourable laboratory conditions with controlled water temperature of 23.1 ± 0.8^{o}C, optimal level of DO at 8.1 ± 0.7 mg L^{-1}, TAN < 0.5 mg L^{-1}, and stable medium osmolality at 108 ± 5 mOsm kg^{-1}, which were similar to water quality parameters during acclimation should have no bearing on the changes in OC and other investigated factors. Therefore, the difference among the responses during the experiment are solely due to predictor variables of moult stage and weight class (Charmantier and Soyez 1994; Lignot et al. 1999).

The haemolymph, the largest and dynamic tissue in decapods, undergoes changes in its make-ups and quantity of constituents during a moult cycle (Chang 1995). In the current study, OC of crayfish increased as wet weight of the crayfish increased, irrespective of their moult stages, as previously confirmed on the pink shrimp, *Penaeus duorarum* (Bursey and Lane 1971), northern white shrimp, *P. setiferus* (Castille and Lawrence 1981a), the blue shrimp, and the white leg shrimp, *Litopenaeus vannamei* (Cheng et al. 2002). Lignot et al. (1999) established OC increases as animal size increases due to the adaptive need to go to seawater. However, freshwater crayfish are non- migration species or do not change habitat from freshwater to seawater, their increase in OC due to size increase could be due to the increase in volume of their haemolymph as they grow bigger.

Irrespective of any wet weight classes, OC of the crayfish was highest in intermoult stage (C) and lowest in stages D_1, D_2 and AB. These results coincide with the previous reports by Mugnier and Justou (2004) and Lignot et al. (1999) in the white leg shrimp, by Ferraris et al. (1987) in the tiger prawn, *P. monodon* and by Charmantier and Soyez (1994) in the white leg shrimp. During the intermoult stage (C), there is a decrease in integument permeability creating an impermeable barrier between the internal and outside environment; however, at the same time, there is increased ATPase activity which induces mobilisation of ionic secretion into haemolymph (Charmantier and Soyez 1994). Moreover, an increase in permeability of the integument during late premoult and early postmoult stages allows water absorption (Ferraris et al. 1987), which results in a dilution of haemolymph and causes a reduction in the osmotic capacity of crayfish to the isosmotic stage during ecdysis (Galindo et al. 2009). The explanation was confirmed by Charmantier and Soyez (1994) in marine shrimp, suggesting that in late premoult and early postmoult stages, changes in tegument permeability and activity of Na^+/K^+-ATPase pump induced by the crustacean hyperglycaemic hormone (Lucu and Towle 2003) takes place in the posterior gills, allowing water and ions to enter tissues, resulting in an increase in body volume after moulting. In other words, the water content of haemolymph decreases during intermoult (C) (Sardà and Cros 1984).

Being an indicator of moult stage, the percentage of dry matter of whole body marron and yabbies is the highest during intermoult stage (C) and the lowest during postmoult (AB). This could be explained by decreased permeability in the integument (Ferraris et

al. 1987), which allows water intake to increase body volume in the late premoult and early postmoult stages. The dry matter (%) of yabbies is significantly higher than that of marron due to the difference in the exoskeleton structure between two species. The percentage of the dry shell to total body weight in marron is half of that in yabbies (5.8% compared to 10.2%, unpublished data). A higher shell portion in yabbies compared to marron suggests that the yabbies may require higher levels of calcium and other minerals that can come either from food, water or gastrolith (Travis 1954; Aiken 1980; Greenaway 1993) for the formation of the exoskeleton. The lower moisture content in yabbies is likely related to their burrowing habit as an adaptation for aestivation during drought.

Changes in the relative weight and composition of the hepatopancreas are associated with ontogenetic development, growth, reproduction, moult stages, nutrient intake (Jones 1997). The current research showed that the hepatosomatic indices were functions of both moult stage and body weight class, which agrees with the finding by Lindqvist and Louekari (1975) in the noble crayfish, while moisture content of hepatopancreas was only moult stage dependent that has no reference to support the result, except the claim by McClain (1995a) that the well- conditioned crayfish during intermoult stage (C) is accompanied with a low moisture content of hepatopancreas. The highest HM (%) during postmoult stage (AB) may be due to water uptake at moult (Bliss et al. 1966; Mantel and Farmer 1983; Cameron 1989; Neufeld and Cameron 1992; Chen and Chia 1997; Cheng et al. 2001; Cheng et al. 2002; Charmantier-Daures and Vernet 2004).

During the intermoult stage (C), HI_{wet} and HI_{dry} reached their maximum, similar to that seen in bristled river shrimp, *Macrobrachium olfersii* (Magalhães et al. 2012). There is a greater accumulation of reserves in the hepatopancreas during intermoult (C) to be used later in the moulting process (Passano 1960). At the premoult (D_0, D_1, D_2) in the study, a decrease of the HI_{wet} and HI_{dry} values may indicate the spending of these reserves for the formation of a new exoskeleton and growth (Magalhães et al. 2012). The transfer of the hepatopancreas' reserves during the moulting process has also been studied in the mangrove crab, *Ucides cordatus* (Marcolin et al. 2008), the fiddler crab, *Uca lacteal* (Yamaguchi 2001).

Yabbies have a larger hepatopancreas than marron, as shown by higher HI_{wet} and HI_{dry}. It suggests that yabbies are able to store more of their energy reserves than marron in the hepatopancreas as lipids which can act as a ready source of energy for mobilisation during times of need such as during gonad maturation. Yabbies undergo gonadal maturation more frequently than marron (Jones 1997). The ability of the hepatopancreas to store larger quantities of nutrients may also be advantageous for yabbies compared to marron in natural habitats where severe and possibly long-term food deprivation is a continual threat. A large hepatopancreas with a low moisture content is usually an indicator of well-conditioned animals (McClain 1995a). Yabbies can also withstand long periods of food deprivation (Lake and Sokol 1986) and a high lipid reserve in the hepatopancreas (Jones 1997) probably extends the survival time. A large hepatopancreas with a high nutrient storage capacity may also extend the range of habitats that can be occupied successfully (Jones 1997).

The length of the moult interval was dependent on the moult stages and the body weights of crayfish. In the current study, intermoult (C) and early premoult (D_0, D_1, D_2) stages take longer to complete, which is similar to many crustacean species, although the time period for the completion of a moult stage is a function of a number of environmental and endogenous factors such as temperature (Hughes et al. 1972; Chittleborough 1975), hardness of water, ionic profile of water, developmental stages, reproductive maturity, and status of limb regeneration (Skinner 1985; Cheng and Chang 1994). Morrissy et al. (1984) reported that a moult cycle in juvenile marron was between 15-40 days while in the present laboratory study, crayfish with a body weight of 2-15 g took 30-34 days to complete a moult cycle under the same condition of water quality and feeding strategy. In the current study, intermoult stage (C) accounted for up to 55% of the entire moult cycle, and crayfish with a larger body weight required more time to complete a moult stage than small ones. The mechanism is that larger crayfish has a longer intermoult (C) as the result of lower mineralisation rate during postmoult (AB) and intermoult (C) (Wheatly and Ayers 1995). A similar finding was previously confirmed by Ackefors et al. (1995) in the noble crayfish and Westman et al. (1993) in the signal crayfish, *Pacifastacus leniusculus*.

Growth in crustaceans is usually described in terms of percentage intramoult weight increment and/or moult frequency (Botsford 1985). In the current research, weight

increment of marron and yabbies varied significantly among moult stages. It was highest during postmoult (AB), which accounts up to 60% of weight increment within a moult cycle. As tissue growth occurs primarily between moults, the increases in length and volume occur immediately after ecdysis (Aiken and Waddy 1987). The AB stage is dominated by the absorption of water resulting in an expansion of crayfish body volume up to 50% (Jussila 1996). Moreover, the hardening of tips of the cheliped, cutting edges of the mandibles and maxillipeds using the calcium stored in gastroliths would attribute to the higher growth rate of crayfish during postmoult stage (AB). The weight increment of the noble crayfish during the moult was between 33% and 65% under laboratory conditions (Ackefors et al. 1995). The significant difference in weight increment between marron and yabbies was at postmoult (AB) for body weights over 15 g, denoting that the growth rate of two crayfish species during the early stage of development is similar under the same culture conditions. However, note that there was also a significant difference in weight increment between species at stage D_1 for size range 16-30 g.

At the size range of 2-15 g, moult frequency between two crayfish species was similar (Table 4.4), which would provide an explanation for similar OC, weight increment, moult interval, HM% and hepatosomatic indices between the two crayfish species of small size range 2-15 g, in all moult stages (Bryant and Papas 2007).

In conclusion, larger than 15 g marron is greater than yabbies in OC, moult interval and growth rate, while hepatosomatic indices and moult frequency is higher in yabbies. OC and health conditions of crayfish are influenced by their respective moult stages and body weights. The result helps understand underlying mechanism of crayfish physiology which would lead to improve the growth of crayfish.

CHAPTER 5: HAEMOLYMPH CONSTITUENTS AND OSMOLALITY AS FUNCTIONS OF MOULT STAGE, BODY WEIGHT, AND FEEDING STATUS IN MARRON, *CHERAX CAINII* (AUSTIN AND RYAN, 2002) AND YABBIES, *CHERAX ALBIDUS* (CLARK, 1936)

(Saudi Journal of Biological Sciences, In press)

5.1 INTRODUCTION

Marron, *Cherax cainii* (Austin and Ryan 2002) and yabbies, *Cherax albidus* (Clark 1936) are two crayfish species, indigenous to Australian fresh water habitats (Bryant and Papas 2007) and are important species for aquaculture, especially in Western Australia (Mills 1980; Johnson 1986). The growth and development of crayfish are functions of a number of intrinsic factors including moult stages, body weights and feeding status. Change in haemolymph constituents during the life cycle and culture environment are used as health status indicators in a number of crustaceans (Charmantier and Soyez 1994; Lignot et al. 1999; Lignot et al. 2000; Charmantier-Daures and Vernet 2004). The haemolymph parameters such as osmolality, protein, glucose, sodium, potassium and chloride are commonly monitored parameters in several crustacean species and their values have been determined to correlate with moult cycle, body weight and nutritional status (Vargas-Albores and Ochoa 1982; Skinner 1985; Lignot et al. 1999; Pratoomchat et al. 2002b; Pascual et al. 2006; Marcy et al. 2009).

The regulation of haemolymph protein, glucose, potassium and chloride are also important physiological mechanisms that can easily be affected by variations of culture conditions. Lignot et al. (1999) concluded that haemolymph glucose from the western blue shrimp, *Penaeus stylirostris* was dependant on the species nutritional status, while protein concentration from haemolymph of the giant river prawn, *Macrobrachium rosenbergii* was a function of weight, and moult stage (Cheng et al. 2002). Changes in haemolymph electrolyte levels have been examined in the American lobster, *Homarus americanus*, the giant river prawn and blue crab, *Callinectes sapidus* during the moult cycle (Towle and Mangum 1985; Mercaldo-Allen 1991; Sugumar et al. 2013). Increases in haemolymph osmolality of Cl^-, Na^+,

K^+, Ca^{2+} and Mg^{2+} have been observed as functions of size and moult cycle in mud crab, *Scylla serrata* (Chen and Chia 1997).

Starvation can affect crustacean haemolymph constituents due to nutrient deficiency (Lignot et al., 1999; Sugumar et al., 2013). The induced starvation of crustaceans in the intermoult stage (C) has been suggested to be a good way to understand any biochemical and physiological adaptations during the starvation mode (Barclay et al. 1983).

However, no studies are available to determine the effects of moult stage, body weight and feeding status on the concentration of protein, glucose, potassium and chloride in the haemolymph of marron and yabbies. The biochemical changes occurring in crustaceans during moulting, feeding and starvation are indicators of their nutritional requirements and are an important basis for determining suitable diets (Sugumar et al. 2013). Further, it is imperative to understand the variation in haemolymph physiology and biochemistry with moult stages, body weights, and nutritional status in order to manage them efficiently in any aquaculture situation.

The aim of this research was to describe the changes in osmolality, protein, glucose, K^+ and Cl^- concentrations in the haemolymph of marron and yabbies associated with moult stages, body weights and the feeding status.

5.2 MATERIALS AND METHODS

A total of 300 marron and 300 yabbies used in the experiments were procured from commercial wholesalers in Western Australia. The animals were transported to Curtin Aquatic Research Laboratory for 3 months' acclimation. Marron and yabbies were separately stocked in round fibreglass tanks (diameter 120 cm x height 75 cm) with 800 L of filtered water and constant aeration. Each tank was equipped with a recirculating biological filtration system (Fluval 205, Askoll, Italy). The water in the tank was continuously recirculated at a rate of approximately 4 L $minute^{-1}$. The tanks were also provided with a sufficient number of stacked shelters in the form of PVC pipes of appropriate diameters and lengths covering up to 50% of tank base area to provide shelters and thus avoiding cannibalism during the moulting process.

During acclimation, water quality was kept at an optimum level with a set temperature (24 ± 2°C) using a submersible thermostat (Sonpar®, Model: HA-100, China), stable dissolved oxygen (8.3 ± 0.7 mg L^{-1}) and a photoperiod of 12 hours of light and dark. The amount of water lost during syphoning or evaporation was topped up daily using the same water source.

The crayfish were fed *ad libitum* with commercial formulated pellets (24.5% of crude protein (CP), 6% lipid and 7.5% ash, 21.2 kJ g^{-1} gross energy, 11.5 mg CP kJ^{-1} protein/energy ratio and 8.5% moisture) on alternate days.

Examination of moult stages:

Prior to the moult stage examination, individual crayfish were placed in crushed ice to render them inactive. Each animal was then placed on its back in a petri dish, the uropods flattened, covered with distilled water and a cover slip, and setae on the apical quarter of the uropod margin were then examined under a compound microscope (Leica Microsystem DM 2500- German at x 100 magnification). Five discrete moult stages: postmoult (AB), intermoult (C), premoult (D_0, D_1, D_2) were identified by examining setal development and changes in epidermal retraction state on the uropods according to Drach (1939), Drach and Tchernigovtzeff (1967) and further modified by Ha and Fotedar (unpublished). The main characteristics used to define the moult stages were AB: setal base is clearly visible, thin cuticle, epidermal tissue is visible inside the setal lumen; C: thick cuticle layer at setal bases where the epidermis lies just underneath; D_0: a translucent space to form between the old cuticle and the epidermis; epidermis retraction from cuticle started; D_1: retraction of epidermis from is maximal, new setae appear and are clearly visible; D_2: the appearance of the new, folded cuticle layer and the new setae are visible.

Haemolymph sampling and analyses:

Approximately one hundred (100) µL of haemolymph was withdrawn from the pericardial cavity of crayfish through the inter-segment membrane between the cephalothorax and the first abdominal segment using 0.5 mL syringe. Haemolymph osmolality was measured by injecting a 20 µL sample into a micro-osmometer (Model 3MO plus, Advance Instruments, Norwood, MA, USA) and expressed in mOsm kg^{-1}. Haemolymph protein was determined using the Bio-Rad Protein Assay

Kit No. 500-0006 Bio-Rad Laboratories, Richmond, CA, USA using bovine albumin (molecular weight: 66000) as a standard, a method derived from Bradford (1976). Glucose concentration from haemolymph was analysed using reflectance photometer Glucometer 3 from Bayer Diagnostics, AMES Department and Glucofilm sticks. Haemolymph potassium was determined using Horiba K^+ meter. Chloride was quantified using Photometer LF 2400.

Crayfish were divided into 3 experiments to cover the aim of the current study:

Experiment 1: Moult stage and body weight on haemolymph constituents

Two weight classes for each species: 2-15 g (marron: 9.8 ± 2.4 g, n =75; yabbies: 9.4 ± 1.7 g, n = 65) and 60-75 g (marron: 67.8 ± 3.1 g, n = 55; yabbies: 65.9 ± 3.5 g, n = 65) were selected for the experiment. After a 24 hour starvation period, crayfish were collected from the experimental tanks in batches of 10 for haemolymph sampling. Each haemolymph sample was noted relative to moult stage (AB, C, D_0, D_1, D_2) and wet weight. Between 11 and 22 haemolymph samples were collected for each of the moult stages (Table 5.1). Osmolality and haemolymph constituents: protein, glucose, potassium and chloride were analysed after sampling.

Experiment 2: The effect of feeding and starvation on osmolality, protein, glucose, K^+, Cl^-

Marron and yabbies at intermoult stage (C) were divided into the fed (marron: 63.7 ± 2.6 g, n = 20; yabbies: 61.9 ± 3.4, n = 20) and starved (marron: 67.5 ± 3.9, n = 20; yabbies: 63.5 ± 4.9 g, n = 20) groups. Animals from both fed and staved groups were under 24 hour starvation prior to experimentation. The concentration of osmolality, protein, glucose, K^+, Cl^- was investigated 4 hours after feeding *ad libitum*.

Experiment 3: The effect of feeding and starvation on osmolality over 550 mins after feeding

Only marron and yabbies at intermoult stage (C) were used for the experiment. The crayfish were divided into the fed and starved group (marron: 63.7 ± 2.6 g, n = 130; yabbies: 61.8 ± 3.4 g, n = 130). Batches of 10 marron and 10 yabbies were collected at regular intervals (every 30 minutes) from fed and starved groups (24 hours of

starvation prior to experimentation) for sampling haemolymph. Time (minute) of each haemolymph sampling for osmolality measurement was recorded.

Statistical analyses:

SPSS 18.0, 2014 was used to analyse the data. Results were presented as mean ± SE. The normality of data was assessed by the Shapiro-Wilk test (Winer 1991) and the homogeneity of variance was assessed by Levene test (Winer 1991) prior to the analyses. One-way ANOVA (analysis of variance) and LSD (least significant difference) post hoc tests were used to determine significant differences among weight classes and moult stages. To satisfy the assumptions of normality and/or homogeneity of variance data were transformed to $\log_{10}(x + 1)$. All significant tests were at $P < 0.05$ level.

5.3 RESULTS

Experiment 1: Moult stage and body weight on osmolality and haemolymph constituents

Table 5.1: Haemolymph protein concentration (mg mL^{-1}) in different weight classes and moult stages of marron and yabbies

Weight (g)	Species	AB	C	D_0	D_1	D_2
2-15	Marron	$_1115.5 \pm 3.6^a$	$_1148.6 \pm 3.7^b$	$_1143.5 \pm 2.7^b$	$_1134.7 \pm 3.2^b$	$_1117.5 \pm 3.6^a$
		(17)	(19)	(13)	(12)	(11)
	Yabbies	$_2132.7 \pm 2.3^a$	$_2158.2 \pm 2.5^b$	$_2157.2 \pm 3.1^b$	$_1139.4 \pm 3.6^a$	$_2134.6 \pm 4.1^a$
		(18)	(21)	(15)	(14)	(13)
61-75	Marron	$_1110.2 \pm 3.6^a$	$_1144.9 \pm 2.8^b$	$_1136.4 \pm 4.2^b$	$_1128.4 \pm 3.8^b$	$_1113.5 \pm 4.2^a$
		(18)	(22)	(10)	(16)	(14)
	Yabbies	$_2125.3 \pm 3.9^a$	$_2156.6 \pm 2.2^b$	$_2152.3 \pm 2.9^b$	$_1132.1 \pm 3.8^a$	$_2129.1 \pm 3.8^a$
		(13)	(18)	(12)	(17)	(13)

Same alphabetical superscripts (a, b) in the same row (comparisons among moult stages) and numerical subscripts (1, 2) in the same column (comparisons among weight classes and species) are

not significantly different at the α = 0.05 level. Numbers in the brackets are animals used for the measurements.

Protein concentration from crayfish haemolymph was the highest at intermoult (C) (Table 5.1) and had a minimum concentration at postmoult (AB). The difference in weights of crayfish did not have any impact on the level of haemolymph protein. A significant difference in haemolymph protein between marron and yabbies in all moult stages and from each weight class occurred.

Table 5.2: Haemolymph glucose concentration (mg mL^{-1}) in different weight classes and moult stages of marron and yabbies

Weight (g)	Species	AB	C	D0	D_1	D_2
2-15	Marron	$_1 1.2 \pm 0.3^a$	$_1 3.2 \pm 0.2^b$	$_1 2.9 \pm 0.2^b$	$_1 1.7 \pm 0.2^a$	$_1 1.4 \pm 0.2^a$
	Yabbies	$_1 1.0 \pm 0.2^a$	$_1 3.8 \pm 0.1^b$	$_1 3.5 \pm 0.6^b$	$_1 1.8 \pm 0.3^a$	$_1 1.2 \pm 0.3^a$
61-75	Marron	$_1 0.7 \pm 0.2^a$	$_1 3.7 \pm 0.9^b$	$_1 3.4 \pm 0.7^b$	$_1 1.9 \pm 0.1^a$	$_1 0.9 \pm 0.2^a$
	Yabbies	$_1 0.8 \pm 0.3^a$	$_1 4.0 \pm 0.4^b$	$_1 2.7 \pm 0.1^b$	$_1 2.1 \pm 0.2^a$	$_1 1.1 \pm 0.3^a$

Same alphabetical superscripts (a, b) in the same row (comparisons among moult stages) and numerical subscripts in the same column (comparisons among weight classes and species) are not significantly different at the α = 0.05 level. Numbers of measurements (not mentioned) are same as in Table 5.1.

Glucose concentration in haemolymph varied significantly ($P < 0.05$) throughout moult cycle (Table 5.2). The highest levels of glucose were in intermoult (C) and early premoult (D_0) stages and significantly lower in the late premoult (D_2) and postmoult (AB) stages in both marron and yabbies. Glucose level was independent of weight classes and species.

Table 5.3: Haemolymph K^+ and Cl^- concentration in different weight classes and moult stages of marron and yabbies.

Factor	Weight (g)	Species	AB	C	D_0	D_1	D_2
Potassium (mmol L^{-1})	2-15	Marron	$_1 3.0 \pm 0.8^a$	$_1 4.9 \pm 0.3^b$	$_1 3.8 \pm 0.4^a$	$_1 3.0 \pm 0.4^a$	$_1 3.1 \pm 0.3^a$
		Yabbies	$_1 3.1 \pm 0.4^a$	$_1 4.3 \pm 0.2^b$	$_1 3.5 \pm 0.2^a$	$_1 3.4 \pm 0.8^a$	$_1 3.3 \pm 0.5^a$

	61-75	Marron	$_1$2.8 ± 0.3^a	$_1$4.6 ± 0.3^b	$_1$2.9 ± 0.2^a	$_1$2.9 ± 0.6^a	$_1$3.2 ± 0.3^a
		Yabbies	$_1$3.2 ± 0.6^a	$_1$5.1 ± 0.4^b	$_1$3.4 ± 0.6^a	$_1$3.7 ± 0.4^a	$_1$3.5 ± 0.4^a
Chloride	2-15	Marron	$_1$169 ± 5^a	$_1$190.9 ± 4.1^b	$_1$180 ± 3^a	$_1$174 ± 4^a	$_1$172 ± 4^a
		Yabbies	$_1$174 ± 3^a	$_1$190.8 ± 3.2^b	$_1$182 ± 3^a	$_1$175.5 ± 5^a	$_1$176 ± 4^a
(mmol L^{-1})	61-75	Marron	$_1$165 ± 4^a	$_1$185.9 ± 2.5^b	$_1$172 ± 3^a	$_1$170.6 ± 4^a	$_1$168 ± 5^a
		Yabbies	$_1$175 ± 5^a	$_1$196.4 ± 2.9^b	$_1$182 ± 3^a	$_1$175.8 ± 4^a	$_1$175 ± 4^a

Same alphabetical superscripts (a, b) in the same row (comparisons among moult stages) and numerical subscripts in the same column of each parameters (comparisons among weight classes and species) are not significantly different at the α = 0.05 level. Numbers of measurements (not mentioned) are same as in Table 5.1.

Haemolymph K^+ and Cl^- were significantly higher during intermoult stage (C) (Table 5.3). No significant difference in K^+ was recorded in both crayfish species and weight classes. Cl^- also increased significantly from postmoult (AB) to intermoult (C), and then decreased significantly ($P < 0.05$) by nearly 10% at stage D_2. Both crayfish species at different body weight classes showed similar changes in chloride concentration over the entire moult cycle.

Experiment 2: The effect of feeding and starvation on haemolymph osmolality, glucose, K^+, and Cl^-

Table 5.4: Haemolymph osmolality, protein, glucose, potassium and chloride concentrations for starved and fed (4 hours after feeding) marron and yabbies. n = number of measurements

Species	Nutritional condition	Weight	Osmolality (mOsm kg^{-1})	Protein (mg mL^{-1})	Glucose (mg L^{-1})	Potassium (mg L^{-1})	Chloride (mg L^{-1})
Marron	Starved (n = 20)	67.5 ± 3.9	476 ± 6^a	109 ± 5^a	47 ± 7.5^a	3.7 ± 0.6^a	186 ± 7^a
	Fed (n = 20)	63.7 ± 2.6	535 ± 6^b	127 ± 3^b	65 ± 18^b	3.9 ± 0.5^a	182 ± 5^a
Yabbies	Starved (n = 20)	63.5 ± 4.9	419 ± 4^c	114 ± 3.7^a	45 ± 12^a	3.4 ± 1.7^a	192 ± 8^a
	Fed (n = 20)	61.9 ± 3.4	476 ± 5^a	132 ± 6.1^b	58 ± 14^b	3.2 ± 1.1^a	194 ± 6^a

Same alphabetical superscripts (a, b, c) in the same column of each species are not significantly different at the α = 0.05 level. n = number of measurement.

The haemolymph osmolality, protein and glucose of both fed crayfish showed a significant increase after feeding (Table 5.4). No significant difference in haemolymph potassium and chloride concentrations was observed between fed and starved crayfish.

Experiment 3: Variation of osmolality during 550 mins after feeding

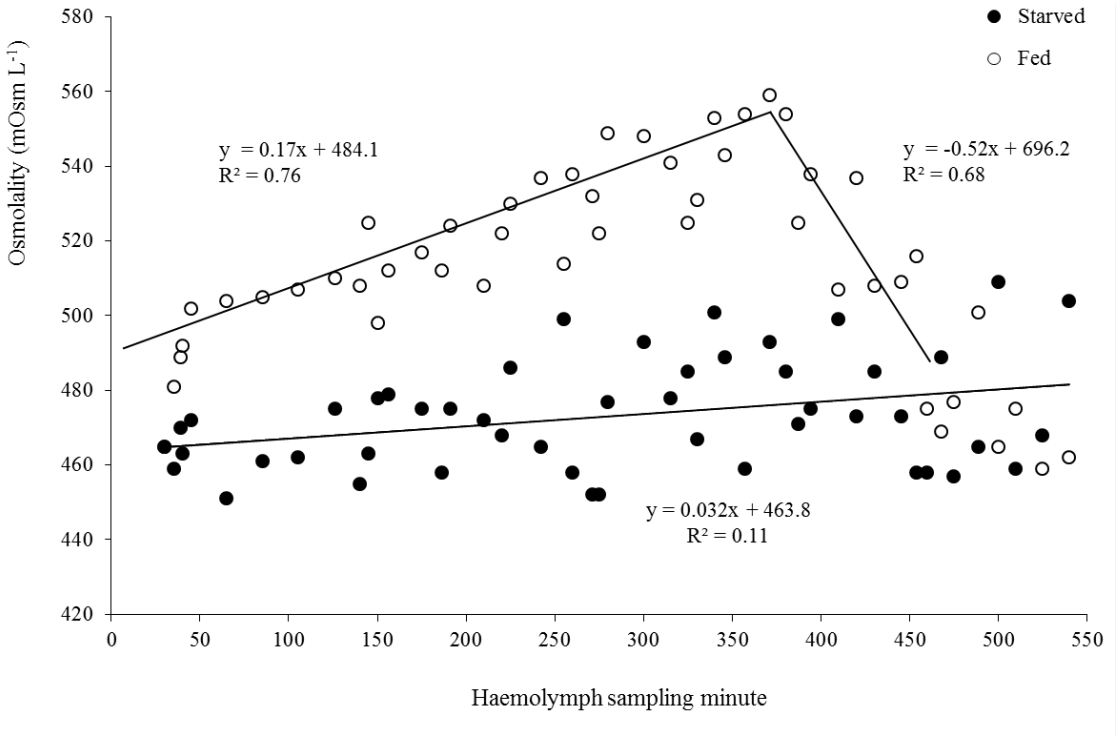


Figure 5.1: Change in osmolality of fed and starved marron with time (minute) after feeding. The lines represent the linear regressions best fitting the data.

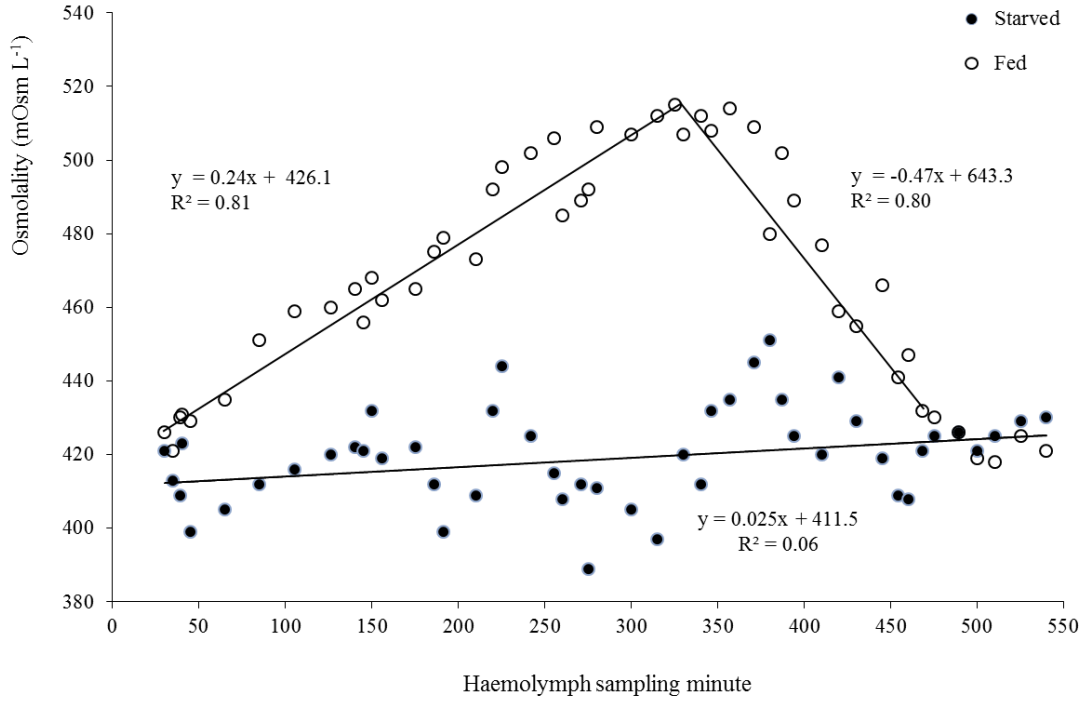


Figure 5.2: Change in osmolality of fed and starved yabbies according to time (minute) after feeding. The lines represent the linear regressions best fitting the data.

The osmolality of starved marron and yabbies stayed constant during the 550 minutes of feeding experiment (marron: $y = 0.032x + 463.8$, $P > 0.05$, $R^2 = 0.11$; and yabbies: $y = 0.025x + 411.5$, $P > 0.05$, $R^2 = 0.06$) (Figure 5.1 and Figure 5.2). In contrast, the osmolality of fed marron and yabbies significantly increased in the first 485 minutes after feeding (marron: $y = 0.017x + 484.1$, $P < 0.05$, $R^2 = 0.76$; and yabbies: $y = 0.24x + 426.1$, $P < 0.05$, $R^2 = 0.81$) and then significantly decreased to 540 minutes (marron: $y = -0.52x + 696.2$, $P < 0.05$, $R^2 = 0.68$; and yabbies: $y = -0.47x + 643.3$, $P < 0.05$, $R^2 = 0.80$). The osmolality of the fed crayfish was within the range of variation of the values of the starved groups. The maximum change of haemolymph osmolality was registered 480 minutes after feeding.

5.4 DISCUSSION

The stable and favourable laboratory conditions (controlled water temperature of 24^{o}C and dissolved oxygen of 8.3 mg L^{-1}) used in this experiment should have no bearing on the changes in haemolymph osmolality. Therefore, the changes in

osmolality during the experiment are solely due to the moult stages and other applied experimental variables (Charmantier and Soyez 1994; Lignot et al. 1999).

Crustaceans have to face the influence of the moult cycle on their internal environment during their entire life cycle (Passano 1960; Bliss 1985; Garcia 1988; Aiken and Waddy 1992; Franco et al. 2006). The effects of moult cycle on haemolymph protein levels have been observed in European green crab, *Carcinus maenas* (Busselen 1970), pink shrimp, *Penaeus duorarum* (Bursey and Lane 1971), American lobster (Mercaldo-Allen 1991), mud crab (Chen and Chia 1997), white leg shrimp, *Litopenaeus vannamei* (Cheng et al. 2002; Galindo et al. 2009), and in giant river prawn (Cheng et al. 2001). Similarly, the haemolymph protein level is also affected by the nutritional status in European lobster, *H. gammarus* (Hagerman 1983), European green crab (Busselen, 1970) and brown shrimp, *Crangon vulgaris* (Djangmah 1970), and in blue swimmer crab, *Portunus pelagicus* (Sugumar et al. 2013).

In the present study, haemolymph protein levels of marron and yabbies were the lowest at postmoult stage (AB), and the highest at intermoult stage (C) and early premoult (D_0). These results were similar to result from the giant river prawn (Cheng et al. 2001) and white leg shrimp (Cheng et al. 2002). However, our results were different to the protein levels observed in Japanese tiger shrimp, *P. japonicus* and pink shrimp which were highest at early postmoult stage (D_0), and lowest at postmoult stage (AB) (Bursey and Lane 1971; Chen and Cheng 1993). Shortly before ecdysis or late premoult (D_2), a peak value for haemolymph protein of brown shrimp is reached (Djangmah 1970). A three-fold dilution of haemolymph protein in European lobster at the time of ecdysis was observed by Glynn (1968). A reduction in haemolymph protein from 80-90 to 30 mg mL^{-1} in brown shrimp during ecdysis was observed by Djangmah (1970). Nicol (1967) estimated that at ecdysis of a 100 g European green crab with a premoult haemolymph volume of 37 mL, 70-80 mL of water is taken in, resulting in two to three times dilution of the haemolymph protein. A decrease in haemolymph protein level in marron and yabbies during postmoult stage (AB) is also due to water uptake following the action of Na^+/K^+-ATPase which can establish an osmotic gradient by the influx of water across epithelia (Towle and Mangum 1985).

Glucose is a molecule that has a major role in the energy metabolism of crustaceans (Galindo et al. 2009) and its variations in the haemolymph are related to the quantity and quality of carbohydrates contained in the diet (Rosas et al. 2000). We found a great variability in glucose concentrations (0.7-4.0 mg mL^{-1}), and significant differences in glucose during the moult cycles of both crayfish. In contrast, the tiger prawn, *Penaeus monodon* (Ferraris et al. 1987) and white leg shrimp (Cheng et al. 2002) maintained isosmotic conditions, showing no significant differences of glucose concentrations throughout their moult cycles. In the present study, the level of glucose in the haemolymph increased during intermoult (C) and early premoult stage (D_0), while a decline was observed during postmoult (AB), which is similar in American lobster (Telford 1968) and blue swimmer crab (Sugumar et al. 2013). The postmoult decrease in glucose may be due to its utilisation as a precursor in chitin synthesis (Meenakshi and Scheer 1961; Hornung and Stevenson 1971). In the purple shore crab, *Hemigrapsus nudus* the major portion of the required chitin is synthesized in the postmoult period by incorporating glucose carbon into chitin during the early postmoult period (Meenakshi and Scheer 1961), while the synthesis of chintin from glucose reached a peak at postmoult stage in the allegheny crayfish, *Orconectes obscurus* (Hornung and Stevenson 1971).

The variability of the haemolymphatic ionic composition throughout the moult cycle in different crustaceans has been known for a long time (Baumberger and Olmsted 1928). During the premoult (D_0, D_1 and D_2) in most of the crustaceans, the haemolymph ionic composition is affected by reabsorption and partial excretion of mineral components from the calcified cuticle (Greenaway 1993; Wheatly 1999). Most of electrolytic elements tend to be lower in postmoult (AB), due to an uptake of water at ecdysis (Bliss et al. 1966; Mantel and Farmer 1983; Cameron 1989; Neufeld and Cameron 1992; Chen and Chia 1997; Cheng et al. 2001; Cheng et al. 2002; Charmantier-Daures and Vernet 2004). The present study also showed that K^+, Cl^- levels are significantly higher at the intermoult stage (C) and early premoult (D_0). During the intermoult stage (C), there is a decrease in integument permeability creating an impermeable barrier between the internal and outside environment, however at the same time there is an increase in ATPase activity which induces mobilisation of ionic secretion into the haemolymph (Charmantier and Soyez 1994). Moreover, during late premoult (D_2) and early postmoult stages an increase in

permeability of the integument allows water absorption (Ferraris et al. 1987), that results in a dilution of ion concentration in haemolymph and causes a reduction in the osmotic capacity leading to an internal environment closer to the isosmotic stage (Galindo et al. 2009). This explanation is confirmed by Charmantier and Soyez (1994) suggesting that in late premoult and early postmoult stages, changes in integument permeability and activity of Na^+/K^+-ATPase pump induced by crustaceans hyperglycaemic hormone (Lucu and Towle 2003) takes place in the posterior gills allowing water and ions to enter tissues, resulting in an increase in body water volume after moulting. Thus, the water content of haemolymph decreases during the intermoult (C) (Sardà and Cros 1984) resulting in a higher concentration of haemolymph electrolytes.

In order to expand a new and soft exoskeleton, aquatic decapods must take up water immediately during postmoult (AB). It is therefore expected that the activity of a whole range of physiological processes related to water and ion permeability and regulation vary during the moult cycle. A number of ions have been measured in the haemolymph during the cycle and most appear to be lower in concentration during the postmoult than in premoult (Glynn 1968; Engel 1987; Ferraris et al. 1987; Mercaldo-Allen 1991; Chen and Chia 1997; Cheng et al. 2001; Cheng et al. 2002).

Feeding appeared to be another important variable controlling the osmotic regulation of the crayfish. Under the laboratory conditions, feeding of marron and yabbies was followed by an increase in haemolymph osmolality, protein, and glucose, which is similar to the fact that the haemolymph protein level decreases during starvation (Stewart et al. 1967; Adiyodi 1969; Uglow 1969; Djangmah 1970). However, no variation in the concentration of either K^+ or Cl^- was observed in fed and unfed crayfish. Therefore, part of the increase in haemolymph osmolality originates from the increased protein and glucose level and not due to ionic exchange between the haemolymph and the external medium as no changes in K^+ and Cl^- concentrations occurred after feeding in the haemolymph but are linked with rapid and massive transport of organic molecules including digestive products such as glucose from the gut to the haemolymph. Similar results have been observed by Ahearn and Maginniss (1977) in the giant river prawn, Lignot et al. (1999) in the western blue shrimp and Rosas et al. (1995) in the white shrimp *P. setiferus*. The osmolality increase in a few

hours after feeding is therefore related to storage within the hepatopancreas and/or to direct intake by organs such as muscles (Lignot et al. 1999).

In the current study, the effects of feeding on the haemolymph osmolality appeared to be time-dependent as there was an elevation of crayfish haemolymph osmolality after 7-8 hours of feeding. Whereas, an increase in haemolymph osmolality of the giant river prawn (Ahearn and Maginniss 1977) and the western blue shrimp (Lignot et al. 1999) took place 4 hours after feeding. Several studies have investigated the time required by the crustacean to return to conditions similar to those maintained before a meal (Mantel and Farmer 1983). The rate at which food clears in the digestive track after a single meal has been described in some penaeids as a reason to return to pre-fed status. In Atlantic ditch shrimp, *Palaemonetes varians*, this rate varied according to the food composition but not by the food particle size. Total clearance of food from the digestive track occurs 4-6 hours after a detritus or algal meal and 27 hours after an animal protein pelleted shrimp meal (Snow and Williams 1971). Our study showed that the time required for both marron and yabbies to compensate for the haemolymph osmotic alteration due to the pelleted feed is approximately 7-8 hours.

In conclusion, haemolymph protein, glucose and electrolyte levels differ with the moult stages of marron and yabbies with no influence by their body weight classes. Lower levels of haemolymph protein, glucose, K^+, Cl^- during the postmoult period are associated with the water uptake at moulting. Starvation results in a reduced level of haemolymph protein, glucose and osmolality, with no effects on ionic constituents. Time required for both marron and yabbies to compensate for the haemolymph osmotic alteration due to the pelleted feed is approximately 7-8 hours.

CHAPTER 6: OSMOLARLITY, FOOD CONSUMPTION AND FOREGUT EVACUATION RATE IN MARRON, *CHERAX CAINII* (AUSTIN AND RYAN 2002) AND YABBIES, *CHERAX ALBIDUS* (CLARK 1936) FED THREE DIFFERENT FOOD TYPES

(Saudi Journal of Biological Sciences, Submitted)

6.1 INTRODUCTION

Feed and feeding management are critical to attaining optimal growth with the lowest feed requirements during crayfish culture (Jory 1995; Jory et al. 2001). Crayfish such as marron, *Cherax cainii* (Austin and Ryan 2002), and yabbies, *Cherax albidus* (Clark 1936), are polytrophic omnivores (Lorman and Magnuson 1978; Momot et al. 1978; Sokol 1988; D'Abramo and Robinson 1989; Tacon 1993). As a result, they can efficiently utilise formulated feeds containing various protein levels from diverse sources (Jones 1997). Past attempts have used dietary manipulations (Hai et al. 2009; Ambas et al. 2015b) and/or dietary supplementation of feed-additives (Sang et al. 2011; Nugroho and Fotedar 2013) to improve the growth performances of marron and yabbies, but the lack of size-dependent feeding preference related research is one of the bottlenecks in establishing effective feed management protocols in crayfish aquaculture.

The food consumption (FC) and foregut evacuation (FGE) are essential parameters that need to be frequently measured when considering the feeding and ingestion rates of any crayfish species (Loya-Javellana et al. 1995). The feed management activities including the selection of the commonly available feed types, feeding time, quantity, feeding methods and the feeding frequency are dependent on the efficiencies of FC and FGE which in turn allow the estimation of the feeding strategy for freshwater crayfish aquaculture (Jory 1995; Loya-Javellana et al. 1995; Simon and Jeffs 2008). In order to achieve efficient feeding management, avoiding overfeeding leading to sub-optimal growth in marron and yabbies, it is critical to study the relationship between body weight and food types with FC and FGE (Simon and Jeffs 2008).

FC varies among crustacean species and is influenced by many internal and external factors including moult stages, body weight and water quality (Hill and Wassenberg 1992; Maguire and Allan 1992; Nunes and Parsons 2000; Gonzalez-Pena et al. 2002;

Wasielesky et al. 2003; Soares et al. 2005; Simon and Jeffs 2008). Although FC has been reported to be related to body weight of crustaceans (Sedgwick 1979; Nunes and Parsons 2000; Simon and Jeffs 2008), conflicting results among species and experimental conditions have been described with both declining and increasing feeding rates as body weight increases (Sedgwick 1979; Hunter et al. 1987; Nunes and Parsons 2000). The calculation of the feeding rates for Australian red claw crayfish, *C. quadricarinatus* has been based on the time of foregut evacuation and its measure is useful in assessing estimations of daily feeding rates (Loya-Javellana et al. 1995).

Although a large number of studies on FC and FGE have been conducted for many crustacean species, there are few studies for freshwater crayfish species, limited to Australian red claw crayfish (Loya-Javellana et al. 1995) and red swamp crayfish, *Procambarus clarkii* (Huner and Meyers 1979) under controlled laboratory conditions, and yabbies under an outdoor pond environment (Jones (1997). At present, research related to maximum ration, ingestion and FGE in a single feeding event under controlled laboratory conditions is lacking for marron and yabbies, despite their relevance to formulating the diet for optimal palatability and to develop effective feeding strategies (Simon and Jeffs 2008).

The osmolality of haemolymph in crustacean is known to vary with moult cycle, body weight, feeding status, species (Vargas-Albores and Ochoa 1982; Lignot et al. 1999; Pascual et al. 2006; Marcy et al. 2009; Ha and Fotedar 2016), and different stressors (Lignot et al. 2000). Evaluation of osmoregulatory capacities based on variation of haemolymph osmolality is used as a useful and reliable tool to monitor the physiological conditions and effect of stressors, including food types, in crustaceans (Lignot et al. 1999; Lignot et al. 2000). However, no information is available on the effect of different food types on the osmolality of decapod crustaceans such as marron and yabbies.

The aim of the present study was to evaluate feeding-related parameters such as food consumption, foregut evacuation rate and haemolymph osmolality for different food types and weight classes of marron and yabbies.

6.2 MATERIALS AND METHODS

A total of 432 crayfish (216 marron and 216 yabbies) used in the experiments were procured from commercial wholesalers in Western Australia and transported to Curtin Aquatic Research Laboratory (CARL) for acclimation. Each species was categorised into 2 weight classes:

Marron: M1 = 15.5-21.5 g (18.7 ± 1.3 g); M2 = 55-62 g (58.1 ± 2.6 g).

Yabbies: Y1 = 13.5-19.3 g (17.5 ± 1.1 g); Y2 = 56.3-63.4 g (60.4 ± 3.1 g).

The two species were separated and stocked in 10 round water tanks (diameter 120 cm x height 70 cm) containing 800 L of filtered water with constant aeration and the presence of PVC pipe of appropriate sizes (10-15 cm length and 5-10 cm diameter) to provide shelters and avoid cannibalism during moulting. Each tank was equipped with a recirculating biological filtration system (Fluval 205, Askoll, Italy). The water in the tank was continuously recirculated at a rate of approximately 4 L minute^{-1}.

During a two month acclimation, water quality was kept at an optimum level by controlling temperature (23.1 ± 1.2^{o}C) using a submersible thermostat (Sonpar®, Model: HA-100, China), stable dissolved oxygen (DO) (8.0 ± 0.9 mg L^{-1} using CyberScan DO 300, Eutech Instruments, Singapore), with a photoperiod of 12 hours of a light and dark cycle. The water lost due to siphoning and evaporation was topped up regularly using aged tap water.

Feed preparation and feeding during acclimation

The formulated feed, algae granules from *Chlorella* sp. and shrimp flesh from imported frozen white leg shrimp, *Penaeus vannamei* were used to feed marron and yabbies. The white leg shrimp was thawed and abdomen flesh was manually separated before being cut into pieces of 3-4 mm. The cut flesh was then dried at 60^{o}C for 24 hours using Panasonic MOV-212F. The three dried food types were prepared to crumbles of less than 2 mm in length and 1.0 mm in diameter (Nunes and Parsons 2000).

The proximate composition of the used foods is presented in Table 6.1. All calculations were based on dry weight. The crayfish were fed with 3 different food

types at a frequency of every 2 days. Acclimated crayfish during acclimation prior to the experiment were maintained for 48h without food to allow full evacuation of their foreguts and presumably to stimulate appetite. Dried algae granules and shrimp flesh were soaked in experiment water for 1 minute to allow an immediate and complete sinking of the food in experiment tanks.

Table 6.1: Proximate composition of the test diets

Proximate composition (mean ± SE)	Formulated feed (n = 6)	Algae granules (n = 6)	Shrimp flesh (n = 7)
Crude protein-CP (%)	24.5 ± 0.1	30.3 ± 2.2	89.2 ± 4.1
Gross energy (kJ g^{-1})	21.2 ± 4.2	18.2 ± 4.4	19.5 ± 5.5
P/E ratio (mg CP kJ^{-1})	49.2	72.3	195.6
Ash (%)	7.5 ± 0.2	5.0 ± 0.4	3.0 ± 0.3
Moisture (%)	8.0 ± 0.6	5.6 ± 0.8	76.4 ± 1.5

The analysis of crude protein, moisture, ash followed AOAC (1995); gross energy was determined using a bomb calorimeter (C2000, IKA, Staufen, Germany). Values are based on dried weight. n = number of samples analysed.

Two independent experiments were conducted to achieve the aim of the research.

Experiment 1 on food consumption

Three (3) food types and 4 weight classes (2 for each species) were tested for food consumption. Each weight class and food type was tested in separate trials with 10 individual of crayfish per weight class. In total, 360 animals (4 weight classes x 3 foods x 10 crayfish per weight class x 3 replicates) were used for the experiment.

Ten (10) pre-weighed crayfish per weight class at intermoult stage (C) were selected by examining the setae on the uropods according to Drach (1939) and Drach and Tchernigovtzeff (1967), with a complete set of functional feeding appendages, and stocked in a glass tank (60 cm length x 45 cm height x 45 cm width) with 80 litres of aged and filtered water. The water parameters were maintained similar to during the acclimation period with a set temperature (23.1 ± 0.7^{o}C), and aeration was kept at a mild rate (DO: 8.1 ± 0.5 mg L^{-1}) to minimise the loss of nutrients from foods into the water.

Three hours prior to feeding, the tanks were covered with black polyethylene sheets to simulate night conditions and also to minimise disturbance. Animals were fed at an excess rate of 3% of their body weight. After an hour of feeding, the animals were immediately removed from the tanks.

Uneaten food from the tanks was collected by siphoning with a 0.5 cm diameter hose onto a clean bucket and filtered through oven dried and pre-weighted glass filter paper with a pore size of 1.2 µm (GM/C, 47 mm circles), held in a funnel. Faeces were removed using a 10 mL pipette. The air supply was suspended for 5 minutes prior to siphoning to allow the materials to settle down to the bottom of the tanks and to optimise the recovery of uneaten food. The experiment was repeated for another batch of pre-weighed 10 crayfish of 48 hours of starvation.

For each type of food, three control aquaria (without crayfish) were used to estimate the amount of feed recovered. Control aquaria received an equal amount of food as aquaria with crayfish and food was collected after 1 hour by siphoning the remains onto the filters as described above.

Collected uneaten feed samples were then oven-dried (70^{o}C) to constant weight. Mean FC as the quantity of food eaten by 48 hour starved crayfish over a one hour period, mean FCI were calculated using the equations adapted from Nunes and Parsons (2000).

Feed consumption measurements were performed to the nearest mg using a precision analytical Sartorius BSA 224S CW balance.

Food consumption was measured as:

$FC \text{ (g dried food/crayfish/hour)} = (F_o - F_r - F_{uneaten})/N$

Where: FC = food consumption (g dried food/crayfish/hour)

N = number of individuals analysed

F_o = dry food offered (g)

F_r = dry food recovered from control aquaria (g)

$F_{uneaten}$ = dried feed recovery from experiment aquaria (g)

Mean food consumption index was calculated as: FCI = Total FC/total BW, where FCI = food consumption index (g dried feed/g BW/h) and Total BW = body wet weight.

Experiment 2 on foregut evacuation rate

All intermoult stage crayfish after experiment 1 were reused for experiment 2 with an extra 72 crayfish (36 marron and 36 yabbies from the acclimation tanks), making a total of 432 animals, represented as 4 weight classes x 12 crayfish per weight class x 3 food types x 3 replicates. The same food types and food preparation protocol used in experiment 1 were applied.

Single treatments were conducted individually with 1 weight class and 1 food type in three replicates, using 12 crayfish with a complete set of functional feeding appendages. Crayfish were stocked in an 80 L water glass tank with a mild aeration (DO: 8.3 ± 0.2 mg L^{-1}) and set temperature (22.6 ± 0.6°C) as acclimation tanks. An excess feeding rate of 3% total body weight was applied once. A maximum ingesting time of 1 hour was allowed for crayfish to reach satiation before sampling. One hour after feeding the crayfish, uneaten feed was siphoned out to maintain the animals in a food-free environment.

The proportion of dry matter intake remaining in the foregut, R_s (%), and FGE (%) were measured using a serial sampling technique (Joll 1982; Loya-Javellana et al. 1995; Nunes and Parsons 2000). Serially, a batch of 3 animals from the food-free experiment tank was sampled at 1, 4, 7 and 10 hours after food deprivation, represented by T1, T4, T7 and T10, respectively, for the determination of haemolymph osmolality, R_s (%) and FGE (%) from the foreguts of crayfish.

To determine the osmolality of haemolymph, approximately 50 µL of haemolymph was withdrawn from the pericardial cavity through the intersegment membrane between the cephalothorax and the first abdominal segment using a 0.5 mL syringe (Sang and Ravi 2004). Haemolymph osmolality expressed in mOsm kg^{-1} was measured by injecting a 20 µL sample into a micro-osmometer (Model 3MO plus, Advance Instruments, Norwood, MA, USA).

Sampled crayfish were dissected and the entire foregut contents removed, and then dried to a constant weight at 70°C for 24 hours in a forced-fan oven (Panasonic MOV-212F) to calculate the remaining dry matter of food intake in the foregut, R_s. R_s (%) was expressed as the proportion of dry matter remaining in the foregut of the initial intake of dry matter (crayfish BW x mean FCI) from corresponding weight class in the experiment 1.

FGE (%) = 100% - R_s (%). FGE rate was expressed as change in FGE (%) in 3 hour periods after 1 hour of food deprivation.

Statistical analysis

SPSS 18.0, 2014, was used to analyse the data. Results were presented as mean ± SE. The effects of size class, food type on FC and FCI were compared using one-way analysis of variance (ANOVA). FC and FCI data were $\log_{10}(x)$ transformed to satisfy ANOVA requirements for the normal distribution of residuals and homogeneity of variances (Levene's test, $P > 0.05$). If ANOVA detected a significant difference, Tukey's LSD test was used for post hoc comparisons between individual pairs of means ($P < 0.05$) (Zar 1999).

All percentage data of R_s and FGE were subjected to the arcsine transformation prior to analysis. All significant tests were at $P < 0.05$ level. One-way ANOVA analyses and post hoc comparisons were performed to compare significant difference of R_s and FGE among food types, weight classes and post-feed deprivation times.

6.4 RESULTS

Experiment 1. Crayfish weight and food type on food consumption

Table 6.2: FC (g dry feed/crayfish) and FCI (g dry feed/g BW) in deferent food types and weight classes

Factor	Species	Treatment	Formulated feed	Algae granules	Shrimp flesh
FC (n = 3)	Marron	M1	$_1$0.374 ± 0.017^a	$_1$0.318 ± 0.037^a	$_1$0.189 ± 0.023^b
		M2	$_2$0.755 ± 0.087^a	$_2$0.813 ± 0.083^a	$_2$0.465 ± 0.042^b
	Yabbies	Y1	$_1$0.387± 0.023^a	$_1$0.332 ± 0.041^a	$_1$0.194 ± 0.043^b

		Y2	$_2$0.845 ± 0.052^a	$_2$0.906 ± 0.035^a	$_2$0.534 ± 0.061^b
FCI (n = 3)	Marron	M1	$_1$0.020 ± 0.001^a	$_1$0.017 ± 0.001^a	$_1$0.010 ± 0.001^b
		M2	$_2$0.013 ± 0.002^a	$_2$0.014 ± 0.001^a	$_1$0.080 ± 0.001^b
	Yabbies	Y1	$_1$0.023 ± 0.002^a	$_1$0.019 ± 0.002^a	$_1$0.011 ± 0.002^b
		Y2	$_2$0.014 ± 0.001^a	$_2$0.015 ± 0.001^a	$_1$0.009 ± 0.001^b

Same alphabetical superscripts (a, b) in the same row (comparisons among food types) and numerical subscripts (1, 2) in the same column of each factor (comparisons among weight classes) are not significantly different at P < 0.05 level. n = number of measurements.

FC was higher in larger crayfish (M2, Y2), while FCI was higher in smaller weight classes (M1, Y1) (Table 6.2), except for shrimp flesh. Both FC and FCI were significantly higher for food types of formulated feed and algae granules.

Experiment 2. Body weight and food type on foregut evacuation rate

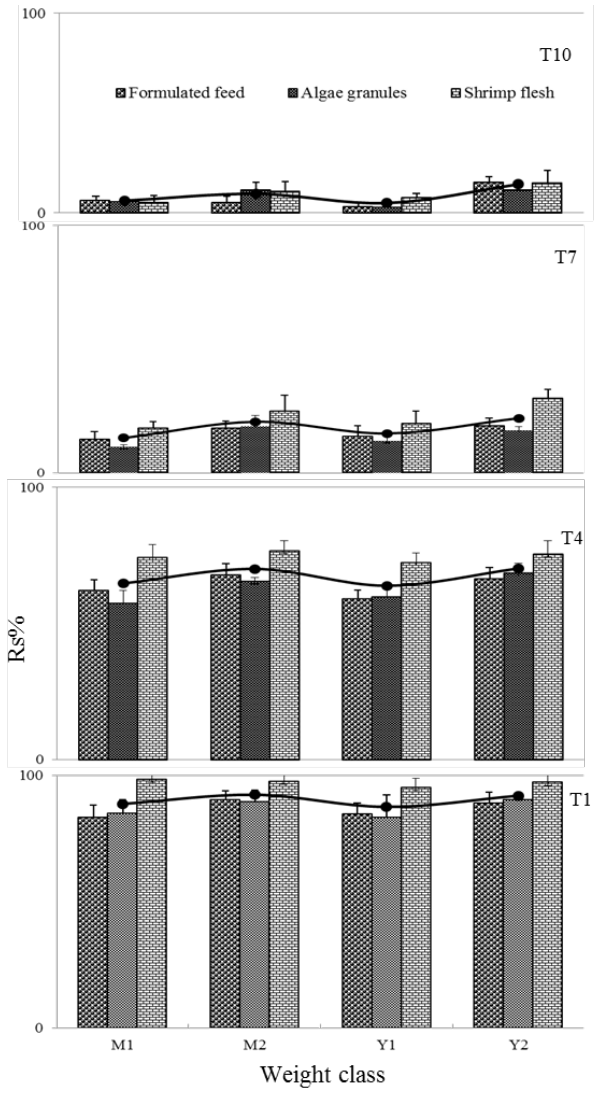


Figure 6.1: R_s (%) for 4 weight classes (M1, M2, Y1, Y2) at various post-feed deprivation times (T1 = 1 hour, T4 = 4 hours, T7 = 7 hours and T10 = 10 hours). n = 9. The line represents the overall mean of R_s (%) from food types.

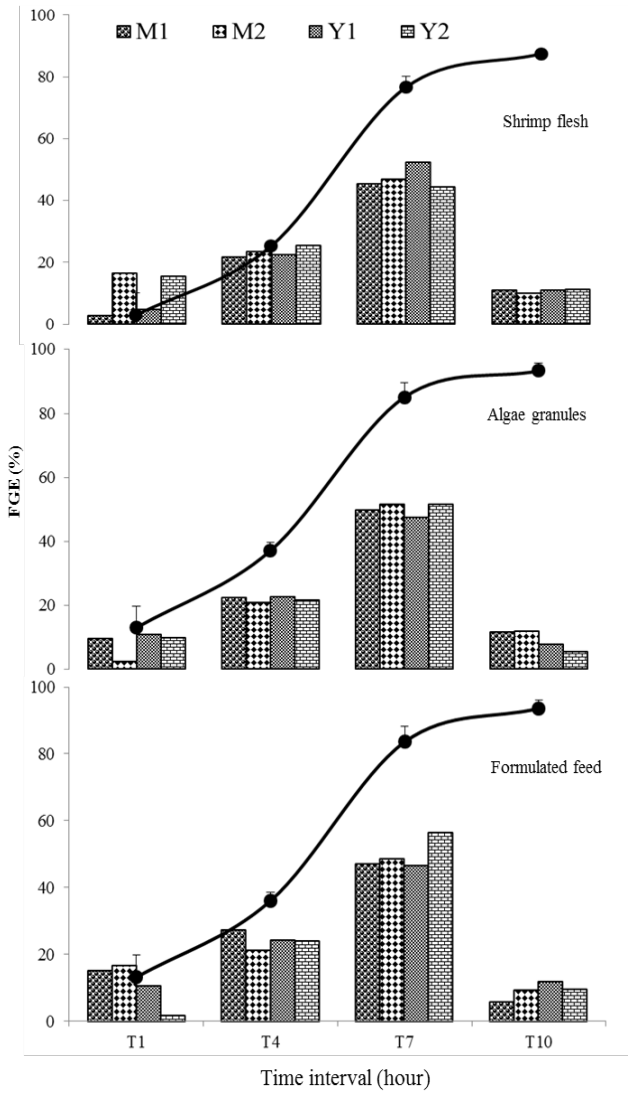


Figure 6.2: FGE (%) for 4 weight classes (M1, M2, Y1, Y2) and 3 food types at various post-feed deprivation times (T1 = 1 hour, T4 = 4 hours, T7 = 7 hours and T10 = 10 hours). n = 9. The line represents the mean accumulated FGE from weight classes.

Table 6.3: R_s (%) of marron and yabbies for different weight classes and food types at 1, 4, 7 and 10 hours after feed deprivation

Time	Food type	M1	M2	Y1	Y2
T1	Formulated feed	$_1$83.4 ± 2.4^a	$_1$90.4 ± 3.7^b	$_1$84.6 ± 2.5^a	$_1$89.1 ± 2.8^b
	Algae granules	$_1$84.9 ± 2.7^a	$_1$89.6 ± 2.1^b	$_1$83.4 ± 2.4^a	$_1$90.2 ± 0.9^b
	Shrimp flesh	$_2$98.4 ± 1.2^a	$_2$97.6 ± 3.2^a	$_2$95.1 ± 1.9^a	$_2$97.2 ± 2.3^a
T4	Formulated feed	$_1$62.2 ± 1.9^a	$_1$68.0 ± 3.0^b	$_1$59.2 ± 1.5^a	$_1$66.5 ± 1.6^b
	Algae granules	$_1$57.6 ± 2.2^a	$_1$65.5 ± 1.6^b	$_1$60.0 ± 1.7^a	$_1$68.7 ± 1.7^b
	Shrimp flesh	$_2$74.4 ± 3.2^a	$_2$76.7 ± 1.3^a	$_2$72.5 ± 3.6^a	$_2$75.5 ± 2.9^a
T7	Formulated feed	$_1$13.7 ± 1.5^a	$_1$18.1 ± 1.4^b	$_1$14.8 ± 2.1^a	$_1$19.1 ± 1.5^b
	Algae granules	$_1$10.6 ± 0.4^a	$_1$18.9 ± 2.4^b	$_1$13.0 ± 1.4^a	$_1$17.1 ± 0.7^b
	Shrimp flesh	$_2$18.1 ± 3.2^a	$_2$25.1 ± 1.5^b	$_2$20.1 ± 2.4^a	$_2$30.2 ± 2.9^b
T10	Formulated feed	$_1$4.4 ± 0.9^a	$_1$6.4 ± 1.1^b	$_1$3.5 ± 0.9^a	$_1$11.2 ± 0.3^c
	Algae granules	$_1$4.8 ± 0.8^a	$_1$7.2 ± 1.8^b	$_1$3.0 ± 1.4^a	$_1$11.7 ± 2.1^c
	Shrimp flesh	$_2$8.7 ± 1.6^a	$_2$13.2 ± 1.7^b	$_2$9.0 ± 0.9^a	$_2$19.2 ± 2.9^c

Same alphabetical superscripts (a, b, c) in the same row (comparisons among weight classes) and numerical subscripts (1, 2) in the same column of each time interval (comparisons among food types) are not significantly different at the α = 0.05 level. n = 9.

In each post-feed deprivation time (T1, T4, T7 and T10), the R_s (%) differed significantly between food types and weight classes (Table 6.3). The larger crayfish (M2, Y2), irrespective of food types, showed higher R_s (%) or lower FGE (%). The values of R_s (%) fed with formulated feed and algae granules were significantly lower than those fed with shrimp flesh.

Table 6.4: R_s (%) for different food types at various post-feed deprivation times (weight class-pooled data), overall mean of R_s (%) and FGE (%)

Food type	T1	T4	T7	T10
Formulated feed	$_1$86.8 ± 1.7^a	$_1$63.9 ± 1.1^b	$_1$16.4 ± 1.4^c	$_1$6.4 ± 1.2^d
Algae granules	$_1$87.0 ± 1.3^a	$_1$62.9 ± 2.2^b	$_1$14.9 ± 2.1^c	$_1$6.7± 1.4^d
Shrimp flesh	$_2$97.1 ± 2.1^a	$_2$74.8 ± 1.3^b	$_2$23.4 ± 1.2^c	$_2$12.5 ± 1.0^d
Overall mean of R_s (%)	90.3	67.2	18.2	8.5
Overall mean of accumulated FGE (%)	9.7	32.8	81.8	91.5

Same alphabetical superscripts (a, b, c, d) in the same row (comparisons among time intervals) and numerical subscripts (1, 2) in the same column (comparisons among food types) are not significantly different at the α = 0.05 level. n = 36.

A significantly higher R_s (%) was reported for shrimp flesh ($P < 0.05$) (Table 6.4). R_s (%) was significantly different among various post-feed deprivation times. Overall means of R_s (%) (pooled food and weight class data) were significantly different at post-feed deprivation times: values were 90.3% at T1, 67.2% at T4, 18.2% at T7 and 8.5% at T10. A significant difference in FGE (%) among food types was reported accordingly, and overall means extrapolated at 9.7% (T1), 32.8% (T4), 81.8% (T7), and 91.5% (T10).

Table 6.5: FGE (%) and overall mean of FGE for different food types (weight class-pooled data) and each 3 hour interval after feed deprivation. Number in the brackets represents hourly rate of FGE.

Food type	T1- T4	T4-T7	T7-T10
Formulated feed	22.9 ± 2.1^a	47.6 ± 1.8^b	10.1 ± 2.7^c
Algae granules	24.1 ± 1.3^a	48.1 ± 2.0^b	8.2 ± 2.1^c
Shrimp flesh	22.3 ± 2.3^a	51.4 ± 1.9^b	10.8 ±1.7^c
Overall mean of FGE (%)	23.1 (7.7)	49.0 (16.3)	9.6 (3.2)

Same alphabetical superscripts (a, b, c) in the same row (comparisons among time intervals) are not significantly different at the α = 0.05 level. n = 36.

A significant increase in FGE (%) was recorded 4-7 hours post-feed deprivation (Table 6.5). The mean cut of FGE (%) from T4 to T7 was 49.0% (hourly rate 16.3%), compared to 23.1% from T1 to T4 (hourly rate 7.7%), and 9.6% from T7 to T10 (3.2% hourly rate).

Table 6.6: FGE (%) in different weight classes at various post-feed deprivation times (food type pooled-data).

Species	Treatment	T1	T4	T7	T10
Marron	M1	$_1$11.1 ± 1.2^a	$_1$35.3 ± 1.7^b	$_1$85.9 ± 1.6^c	$_1$92.1 ± 0.2^d
	M2	$_2$7.5 ± 2.0^a	$_2$29.9 ± 2.0^b	$_2$79.9 ± 2.2^c	$_2$87.3 ± 1.3^d
Yabbies	Y1	$_1$12.7 ± 1.8^a	$_1$36.1 ± 1.6^b	$_1$84.0 ± 2.1^c	$_1$94.8± 1.5^d
	Y2	$_2$7.3 ± 1.3^a	$_2$29.8 ± 1.2^b	$_2$77.9 ± 1.3^c	$_2$85.8± 1.2^d

Same alphabetical superscripts in the same row (comparisons among time intervals) and numerical subscripts in the same column (comparisons among weight classes) are not significantly different at the α = 0.05 level. n = 27.

There was a significant increase in FGF (%) over 10 hours after feeding (Figure 6.1, Figure 6.2 and Table 6.6). Smaller crayfish (M1 and Y1) showed a higher FGE (%) than the larger crayfish (M2 and Y2).

Table 6.7: Haemolymph osmolality in different weight classes at various post-feed deprivation times (food type pooled-data)

Species	Treatment	T1	T4	T7	T10
Marron	M1	$_1$471.3 ± 3.1^a	$_1$510.7 ± 5.1^b	$_1$537.0 ± 4.2^c	$_1$438.3 ± 3.5^a
	M2	$_2$480.7 ± 4.3^a	$_2$529.7 ± 4.2^b	$_2$548.0 ± 3.7^c	$_2$450.7 ± 4.0^a
Yabbies	Y1	$_3$426.3 ± 4.1^a	$_3$460.3 ± 3.7^b	$_3$471.7 ± 4.6^c	$_3$408.0 ± 3.5^a
	Y2	$_4$437.3 ± 3.7^a	$_4$473.7 ± 2.9^b	$_4$485.0 ± 3.8^c	$_4$421.7 ± 4.3^a

Same alphabetical superscripts in the same row (comparisons among time intervals) and numerical subscripts in the same column (comparisons among weight classes) are not significantly different at the α = 0.05 level. n =27.

Osmolality was significantly higher 7 hours post-feed deprivation (T7) (Table 6.7). Larger marron and yabbies had higher haemolymph osmolality than that of smaller crayfish, irrespective of post-feed deprivation times.

Table 6.8: Haemolymph osmolality in different food types at various post-feed deprivation times (weight class pooled-data)

Treatment	T1	T4	T7	T10
Commercial pellet	$_1460.1 \pm 4.7^a$	$_1486.3 \pm 3.1^b$	$_1521.8 \pm 3.4^c$	$_1452.5 \pm 4.2^a$
Algae granules	$_1465.3 \pm 2.3^a$	$_1479.6 \pm 4.2^b$	$_1519.0 \pm 2.1^c$	$_1448.6 \pm 3.4^a$
Shrimp flesh	$_1456.9 \pm 2.6^a$	$_1469.3 \pm 5.5^b$	$_1523.6 \pm 1.2^c$	$_1445.1 \pm 4.0^a$

Same alphabetical superscripts in the same row (comparisons among time intervals) and numerical subscripts in the same column (comparisons among food types) are not significantly different at the α = 0.05 level. n = 36.

No significant difference in osmolality was found among food types, while highest haemolymph was at T7, irrespective of food types (Table 6.8).

6.4 DISCUSSION

The knowledge of weight-dependent food consumption and foregut evacuation for different food types is important to assist in the development of an efficient feeding strategy for cultured species (Jory 1995; Loya-Javellana et al. 1995; Simon and Jeffs 2008). Crayfish culture systems usually articulate the food requirement as a daily percentage of the mean body weight, which must be monitored and adjusted continuously to balance changing demands with their growth (Jory 1995).

With the aid of a relatively larger hepatopancreas, marron and yabbies are able to ingest a wide variety of diet including diverse macronutrients with wider physical properties (Jones 1997). The current study used three distinct diets with the aim of evaluating the effects of food type on food consumption and foregut evacuation in

these omnivorous species. Commercially formulated pellets are commonly used in marron and yabbies culture in Western Australia, while algae granules and shrimp flesh, although not being used, are two other diverse type of diets representing two extreme ends of the spectrum. The former represents an all-plant-based diet, commonly available in marron and yabbies' habitats and the latter represents an extreme carnivore diet.

In the current study, feed consumption was significantly higher in larger marron and yabbies, but inversely in percentage terms. These relationships are comparable to the results in the red claw crayfish (Loya-Javellana et al. 1995), the Southern brown shrimp, *P. subtilis* (Nunes and Parsons 2000), the red swamp crawfish (McClain 1995a) and the spiny lobster, *Jasus edwardsii* (Simon and Jeffs 2008). The relationship could be due to the reduction in weight-specific metabolic rate that occurs with increasing size (Musgrove 1993), although other factors such as food type may also be important (Simon and Jeffs 2008).

Results from the present study along with other investigations by McTigue and Feller (1989), Reymond and Lagardère (1990), Nunes et al. (1996) and Simon and Jeffs (2008) suggest a difference in food consumption depending on the nutrient availability and natural structure of the food (food type). In the current study, food consumption was equivalent to 0.8-2.3% of total body weight for an hour, which significantly varied depending on the food type and weight class. Food consumption rate was significantly lower in foods with higher protein content and tough structure, as in shrimp flesh (89.1% protein of dry weight), compared to formulated pellet (24.5%) and algae granules (30.3%). Much higher moisture content from foreguts of shrimp flesh-fed crayfish as a result of the hydration and expansion of the ingested shrimp flesh in the foregut could also have limited further intake (Simon and Jeffs 2008). Due to the different hydration rate of food types, the fullness of the foregut may be similar but dry ingested food could be totally different.

Nunes et al. (1996) stated that the fluctuation in water quality parameters can result in 26% of the variation in the feeding habit and rhythm of the Southern brown shrimp cultured in a semi-intensive system. However, the bias of environmental factors on feeding activity of crayfish on the experiments including variations in water quality, light intensity, and natural and artificial food availability is excluded

as the experiments were conducted under laboratory controlled conditions, and non-experimental factors were the same in all treatments and replicates. It is therefore true that all feeding-related measures (FC, FCI, and FGE) in the experiments can be merely explained by the physiology of crayfish at different weight classes and the property of food types. Reports of food consumption measures for *Penaeus* spp. vary significantly between species, weight classes, food types and experimental conditions (Nunes and Parsons 2000). The overall mean of accumulated FGE at T10 was only 91.5% (Table 6.4); this means that crayfish, regardless of food type and weight class, would require more than 10 hours to fully evacuate their foreguts at 23.1^{o}C.

The FGE results obtained in this study for both species are very similar, and are within the range of values in the literature (Table 6.9); even foregut evacuation rate is a function of a number of variables including water temperature, light intensity (Nunes and Parsons 2000), food type and body weight of animal (Simon and Jeffs 2008).

Table 6.9: Comparison of time (hour) required for full foregut evacuation in different crustacean species

Species	Time (hour) required for full FGE	Experiment conditions	Reference(s)
Cherax cainii	> 10	Controlled laboratory, 23^{o}C	This study
Cherax albidus	> 10	Controlled laboratory, 23^{o}C	This study
Jasus edwardsii	10	18°C	Simon and Jeffs (2008)
Nephrops norvegicus	10	14°C	Cristo (2001)
Cherax quadricarinatus	9	26°C	Loya-Javellana et al. (1995)
Cherax destrutor	14-16	21-22°C, field estimates	Jones (1997)
Nephrops norvegicus	12	14°C	Sarda and Valladares (1990)
Panulirus cygnus	4-6	25°C	Joll (1982)
Scylla serrata	12	18-22°C	Hill (1976)

There was a significant difference in FGE (%) between food types and weight classes. Similar results were also observed for the Australian red clawed crayfish by Loya-Javellana et al. (1995), amphipods species *Gammarus pseudolimneus* by Marchant and Hynes (1981) and the pink shrimp, *Farfantepenaeus paulensis* by Soares et al. (2005). A lower FGE (%) was recorded for the food type of shrimp flesh (Table 6.5), which had higher protein content (89.1% of dry weight) than formulated feed (24.5%) and algae granules (30.3%). The digestive enzyme-related activities would be an explanation for a faster evacuation of ingested food from foreguts. The low protein food appears to stimulate the secretion of a larger volume of digestive enzymes that would induce a faster digestion progress (Jones 1997; Simon and Jeffs 2008).

In the present study, R_s (%) at 10 hours after feed deprivation ranged between 6.0-8.92% for marron and 5.2-14.0% for yabbies. It is comparable to 5% of fullness for adult giant tiger prawn, *Penaeus monodon* after 4 hours (Marte 1980), 13% for the Southern brown shrimp after 3 hours (Nunes and Parsons 2000) and 25% for the brown tiger prawn, *P. esculentus* (18-32 g) after 1 hour without food (Hill and Wassenberg 1987). Therefore, crayfish are known to take more time for foregut evacuation than smaller decapods such as penaeid shrimp (Allan and Smith 1998; Nunes and Parsons 2000). The difference could be explained by higher food consumption as a result of the 2-5 times more spacious foregut volume of crayfish compared to penaeid species (Jones 1997) and the 1.8-2.5 times larger volume than that of the crab (Dall et al. 1990; Simon 2009).

The measurement of food intake remaining in foreguts was based on dry matter. This is to avoid overestimation of the fullness of the foreguts due to the ingestion of water in conjunction with the food due to feeding habits of marron and yabbies. The foods were ground up completely as a result of the grinding action of the mandibles during ingestion and the gastric-mill afterward, and had a paste-like consistency owing to the large amount of fluid present (Simon 2009).

In the current study, although food ingestion and foregut evacuation were dependent on food type, no effect from different food types was observed on osmolality of marron and yabbies. In all three food types used in the experiment, osmolality peaked 7 hours post-feed deprivation in both marron and yabbies. The finding was

similar to the result by Ha and Fotedar (2016) who studied osmolality in fed and starved crayfish using formulated feed. Nearly 50% of the ingested food was emptied for 3 hours from T4-T7 compared to about 30% for 6 hours from T1-T4 and T7-T10. The higher FGE during T4-T7 would likely result in higher level of osmolality due to an increased content of glucose and protein in haemolymph as critical attributions to osmolality (Ahearn and Maginniss 1977; Rosas et al. 1995; Lignot et al. 1999; Ha and Fotedar 2016).

Of the feeding rates investigated from 0.8-2.3%, depending on body weight and quality of the food, a higher feeding rate could be applied for smaller crayfish and food with lower protein content. Although different foregut evacuation rates were observed between weight classes and among food types, a fixed feeding frequency of at least 10 hours would be considered to be appropriate for all food types used in the crayfish culture, as about 90% of ingested food in foreguts was evacuated after 10 hours of feeding. The recommended feeding frequency for marron and yabbies is different from 3-6 hours for penaeids and portunids (Dall et al. 1990; Lignot et al. 1999), while it is similar to the red claw crayfish (Loya-Javellana et al. 1995) and the spiny lobster (Simon and Jeffs 2008). The explanation is related to volume of the foregut which appears to be approximately 8.5 times more spacious in marron and yabbies than the foregut of similar sized giant tiger prawn and green tiger prawn, *P. semisulcatus* (Hill and Wassenberg 1987; Jones 1997) and 1.8-2.5 times larger than the mud crab, *Scylla serrata* (Ian Knuckey, DPI, Darwin, Australia, unpublished data). Smaller foregut volume requires animals to feed several times per day to obtain sufficient food (Dall et al., 1990) for optimum growth (Allan and Smith 1995). According to Dall et al. (1990), the functional volume of foreguts is only about 60% of the maximum volume due to a restriction imposed by muscles surrounding the gastric mill and due to a space limitation in the cephalothorax. However, this is not the case for crayfish, including yabbies (Jones 1997). Food consumption in the present study provides an accurate estimation of marron and yabbies feeding levels under laboratory conditions, but effects of other factors such as water quality, light intensity, natural and artificial food availability must be considered before the recommended feeding rate can be directly applied to real production conditions (Nunes and Parsons 2000).

In conclusion, total amount of food consumed by both marron and yabbies was positively related to the body weights. However, when food consumption was expressed as an index of body weight, rates of food ingested were found to be inversely proportional to their weights. Food consumption and foregut evacuation rates of crayfish varied among food types and weight classes. The highest foregut evacuation and haemolymph osmolality of marron and yabbies occurred 7 hours after feed deprivation. Ten hours post-feed deprivation, over 90% of ingested food was evacuated from their foreguts, irrespective of weight classes and food types.

CHAPTER 7: EXOSKELETON COMPOSITION AS A FUNCTION OF THE MOULT STAGES AND BODY WEIGHT OF MARRON C*HERAX CAINII* (AUSTIN AND RYAN, 2002) AND YABBIES C*HERAX ALBIDUS* (CLARK, 1936)

(Aquaculture International, Submitted)

7.1 INTRODUCTION

The crayfish exoskeleton is crucial for maintaining species-specific shape and size, protecting the soft body parts from predators and infections (Nagasawa 2012). Protein, chitin, and lipid are the main components of exoskeleton providing the mechanical properties (Willis 1987), framework, organisation, waterproofing and prevention of infectious diseases (Kramer and Koga 1986; Willis 1987; Nagasawa 2012). The exoskeleton composition varies with the moult stages (Chandumpai et al. 1991; Pratoomchat et al. 2002b; Roer et al. 2015) and is mainly composed of protein-chitin complexes (Glynn 1968) with a different proportion of protein and chitin in various layers of the exoskeleton (Vigh and Dendinger 1982; Roer and Dillaman 1984).

Moulting is a cyclic process that occurs in all crustaceans, and is essential for growth, reproduction and metamorphosis (Kuballa et al. 2011). Moult cycle of marron, *Cherax cainii* (Austin and Ryan 2002) and yabbies, *Cherax albidus* (Clark 1936) has been subdivided into five major stages (Ha and Fotedar, unpublished): postmoult (AB), intermoult (C), and premoult (D_0, D_1 and D_2). Moult stage and weight class have been reported to affect the composition of haemolymph and the hepatopancreas of crustaceans (Vargas-Albores and Ochoa 1982; Chen and Chia 1997; Lignot et al. 1999; Cheng et al. 2002; Sugumar et al. 2013; Ha and Fotedar 2016).

The previous studies on exoskeleton compositions have focussed only on intermoult stage (Ravichandran et al. 2009; Ehigiator and Nwangwu 2011), between sexes (Abdul-Sahib and Ajeel 2005; Adeyeye et al. 2010), shed exoskeleton or exuviae (Ikeda and Dixon 1982; Nicol et al. 1992) and the hardened and unhardened exoskeleton (Ekpenyong et al. 2013). There have been a few studies on the biochemical compositions of crustacean exoskeleton during all moult stages of a

moult cycle. Chandumpai et al. (1991) investigated the total lipid content of exoskeleton over a moult cycle in brown tiger prawn, *Penaeus esculentus*. Pratoomchat et al. (2002b) examined organic and inorganic compounds in different tissues including exoskeleton from all 8 moult stages of mud crab, *Scylla serrata*. Roer et al. (2015) studied the resorption and deposition of organic and inorganic materials during the moult cycle of the red claw, *C. quadricarinatus*. However, there are no reports on the relationship of moult stage with the exoskeleton compositions of different weight classes of marron and yabbies.

The deposition of organic and inorganic materials into the exoskeleton takes place from postmoult (AB) to intermoult (C), while premoult stages (D_0, D_1, D_2) are reported to accompany the resorption of these materials from the exoskeleton (Welinder 1974; Welinder 1975; Roer 1980; Roer and Dillaman 1984; Willis 1987; O'Brien et al. 1991; Wheatly and Ayers 1995; Roer et al. 2015). The difference in proportion of dry exoskeleton to body weight of intermoult staged crayfish during the moult cycle can be used to estimate gross deposition rate (gain) and gross resorption (loss) of the exoskeleton (Wheatly and Ayers 1995; Dayal et al. 2005).

The aim of the study was to determine the relationship between moult stages of two different weight classes of marron and yabbies and their exoskeleton compositions.

7.2 MATERIALS AND METHODS

A total of 516 (265 marron and 251 yabbies) from two distinct weight classes (2-15 g and 61-75 g body weight from both species) were purchased from commercial wholesalers in Western Australia. The animals were transported to Curtin Aquatic Research Laboratory for one month acclimation. Marron and yabbies were separately stocked in round fibreglass tanks (diameter 120 cm x height 75 cm) with 800 L of filtered water and constant aeration. Each tank was equipped with a recirculating biological filtration system (Fluval 205, Askoll, Italy). The water in the tank was continuously recirculated at a rate of approximately 4 L $minute^{-1}$. The tanks were also provided with a sufficient number of stacked shelters in the form of PVC pipes of appropriate diameters and lengths covering up to 50% of tank base area to provide shelters and thus avoiding cannibalism during the moulting process.

During acclimation, water quality was kept at an optimum level at a set temperature (22.5 ± 0.8°C) using a submersible thermostat (Sonpar®, Model: HA-100, China), stable dissolved oxygen (7.8 ± 0.6 mg L^{-1}) and a photoperiod of 12 hours of light and dark. The crayfish were fed *ad libitum* with commercial pellets (24.0% crude protein, 6.0% lipid and 5.0% ash) on alternate days. The uneaten feed was siphoned out from the tanks one hour after feeding. The amount of water lost during syphoning or evaporation was topped up daily using the same water source.

Marron and yabbies were individually weighed, housed and numbered in transparent plastic containers (30 cm length x 15 cm width x 10 cm depth) with 10 mm diameter holes on the lids and sides for water circulation and feeding. These containers were then placed in a single layer in the tanks (300 cm length x 200 cm width x 50 cm depth) holding 1800 L of water. During three month experiment, the same water quality conditions, procedure and frequency for water level, feed, and feeding were maintained as during acclimation. Each tank was provided with two recirculating biological filtration systems (Fluval 205, Askoll, Italy) with a total flow rate of 8 L $minute^{-1}$.

Individual marron and yabbies were recorded for body weight at intermoult stage (C). Before moult stage examination, individual crayfish were placed in crushed ice to render them inactive. Each crayfish was then placed on its back in a petri dish, the uropods flattened, covered with distilled water and a coverslip, and setae on the apical quarter of the uropod margin were then examined under a compound microscope (Leica Microsystem DM 2500- German at x 100 magnification). Five discrete moult stages: postmoult (AB), intermoult (C), premoult (D0, D1, D2) were identified based on examining setal development and changes in epidermal retraction state on the uropods according to Drach (1939) and Drach and Tchernigovtzeff (1967).

Marron and yabbies were individually weighed and staged before dissecting for exoskeleton samples. The crayfish were frozen and dissected to separate exoskeleton from carapace and abdomen. The exoskeletons were then washed and cleaned using tap water and then distilled water to remove all of the attached tissues. The number of collected exoskeletons in each moult stage and weight class is summarised in Table 7.1.

Table 7.1: The number of exoskeletons collected from each moult stage and weight class of marron and yabbies

Species	Weight class	Moult stage				
		AB	C	D_0	D_1	D_2
Marron	2-15	28	39	29	18	13
	61-75	33	44	30	13	18
Yabby	2-15	24	45	34	16	16
	61-75	28	38	18	15	17

Each exoskeleton sample was separately dried in a drier (Panasonic MOV-212F) at 60°C for 2 hours, then the drying was continued for another 20 hours at 40°C (Ibrahim et al. 1999). Dried exoskeleton samples in each moult stage and weight class were finely ground, packed in glass bottles and stored at 4°C for further analyses.

Proximate analysis

Crude protein was analysed following the Kjeldahl method with a Kjeltec Auto 1030 analyser according to the standard methods of the Association of Official Analytical Chemists AOAC (1995). Crude protein content was calculated from the nitrogen content by multiplying by 6.25. Total lipids were determined using the Soxtec system and petroleum ether as solvent.

Ash was determined by combustion at 550°C for 24 hours in an electric furnace (Carbolite, Sheffield, UK) (AOAC 1995). Gross energy was determined using a bomb calorimeter (C2000, IKA, Staufen, Germany). The content of chitin in the exoskeletons was determined by demineralisation of 1 g of the prepared exoskeleton, which was deproteinised with 5% KOH solution for 2 hours at 100^{o}C, in 20 mL 5% HCl for 2 hours at room temperature. Chitin was separated on a coarse glass-sintered funnel, washed with water to a neutral pH and then 3 x 25 mL of acetone followed by oven drying at 105°C. Carbohydrate (%) was calculated as 100 % - (protein % + lipid % + ash % + chitin %) following Pratoomchat et al. (2002b) and Abdul-Sahib and Ajeel (2005).

Percentage of dry exoskeleton to wet body weight of intermoult staged crayfish (P_E) in each moult stage was calculated as:

P_E (%) = dry exoskeleton/wet body weight of intermoult staged crayfish x 100

The relative gross gain from the deposition of protein, chitin, ash and carbohydrate during metaecdysial was calculated as equal to P_E at intermoult stage (C) - P_E at postmoult (AB).

The relative gross loss from resorption of protein, chitin, ash and carbohydrate during proecdysial was calculated as equal P_E at intermoult stage (C) - P_E at late premoult (D_2).

Statistical analysis

SPSS 18.0, 2014 was used to analyse the data. Results were presented as mean ± SE. The normality of data was assessed by the Shapiro-Wilk test (Winer 1991) and the homogeneity of variance was assessed by Levene test (Winer 1991) prior to the analysis. Normally distributed data were subjected to analysis of variance (two-way ANOVA) and post-hoc LSD test for determination of significant different among moult stages and weight classes. All percentage data were subjected to the arcsine transformation prior to analysis. All significant tests were at $P < 0.05$ level.

7.3 RESULTS

Table 7.2: Content (% dry weight) of protein, chitin, lipid, ash, gross energy and carbohydrate in crayfish exoskeleton of different weight classes and moult stages

Factor	Size	Species	AB	C	D_0	D_1	D_2
Protein (%)	2-15	Marron	$_1 20.6 \pm 1.4^a$	$_1 16.3 \pm 1.8^b$	$_1 15.3 \pm 1.5^b$	$_1 14.6 \pm 1.3^b$	$_1 10.5 \pm 1.6^b$
		Yabbies	$_1 21.7 \pm 1.3^a$	$_1 17.0 \pm 1.3^b$	$_1 16.8 \pm 2.1^b$	$_1 16.2 \pm 2.0^b$	$_1 9.6 \pm 1.3^b$
	61-75	Marron	$_1 19.1 \pm 1.2^a$	$_1 15.9 \pm 1.6^b$	$_1 15.5 \pm 1.0^b$	$_1 14.7 \pm 1.8^b$	$_1 11.2 \pm 2.1^b$
		Yabbies	$_1 20.0 \pm 1.3^a$	$_1 16.3 \pm 2.0^b$	$_1 15.8 \pm 1.1^b$	$_1 14.6 \pm 1.3^b$	$_1 11.5 \pm 1.4^b$
Chitin (%)	2-15	Marron	$_1 27.2 \pm 2.1^a$	$_1 32.9 \pm 2.3^b$	$_1 28.8 \pm 2.7^a$	$_1 27.8 \pm 2.9^a$	$_1 21.2 \pm 1.8^c$
		Yabbies	$_1 26+2 \pm 1.3^a$	$_1 33.1 \pm 1.6^b$	$_1 29.2 \pm 2.1^a$	$_1 27.1 \pm 2.0^a$	$_1 20.1 \pm 1.3^c$

	61-75	Marron	$_1$27.5 ± 1.7^a	$_1$34.3 ± 2.1^b	$_1$29.9 ± 2.6^a	$_1$28.8 ± 2.6^a	$_1$22.7 ± 2.3^c
		Yabbies	$_1$27.9 ± 1.6^a	$_1$35.4 ± 3.2^b	$_1$28.8 ± 1.8^a	$_1$28.7 ± 3.1^a	$_1$21.1 ± 2.1^c
Lipid (%)	2-15	Marron	$_1$2.66 ± 0.20^a	$_1$2.52 ± 0.11^a	$_1$2.45 ± 0.12^a	$_1$2.55 ± 0.14^a	$_1$2.67 ± 0.13^a
		Yabbies	$_1$2.59 ± 0.11^a	$_1$2.49 ± 0.16^a	$_1$2.55 ± 0.13^a	$_1$2.61 ± 0.10^a	$_1$2.63 ± 0.14^a
	61-75	Marron	$_1$2.68 ± 0.17^a	$_1$2.67 ± 0.16^a	$_1$2.62 ± 0.20^a	$_1$2.65 ± 0.11^a	$_1$2.45 ± 0.13^a
		Yabbies	$_1$2.57 ± 0.13^a	$_1$2.51 ± 0.21^a	$_1$2.58 ± 0.19^a	$_1$2.56 ± 0.18^a	$_1$2.52 ± 0.17^a
Ash (%)	2-15	Marron	$_1$36.6 ± 2.6^a	$_1$44.7 ± 1.6^b	$_1$39.9 ± 2.4^b	$_1$34.5 ± 2.5^a	$_1$35.8 ± 2.5^a
		Yabbies	$_1$35.5 ± 2.7^a	$_1$43.5 ± 2.6^b	$_1$40.8 ± 2.1^b	$_1$36.7 ± 2.0^a	$_1$34.8 ± 2.8^a
	61-75	Marron	$_2$30.1 ± 1.7^a	$_2$36.5 ± 2.8^b	$_2$35.8 ± 3.2^b	$_2$30.5 ± 2.6^a	$_2$28.9 ± 2.1^a
		Yabbies	$_2$31.0 ± 1.2^a	$_2$37.7 ± 2.7^b	$_2$34.7 ± 2.8^b	$_2$29.4 ± 1.4^a	$_2$29.1 ± 1.2^a
Gross energy (kJ g^{-1})	2-15	Marron	$_1$8.1 ± 2.6^a	$_1$15.8 ± 1.8^b	$_1$14.5 ± 2.4^b	$_1$8.8 ± 1.5^a	$_1$10.5 ± 2.3^c
		Yabbies	$_1$8.3 ± 2.3^a	$_1$14.6 ± 1.6^b	$_1$14.2 ± 2.1^b	$_1$8.9 ± 2.0^a	$_1$9.8 ± 2.6^c
	61-75	Marron	$_1$12.7 ± 3.0^a	$_1$17.4 ± 3.6^b	$_1$16.9 ± 1.4^b	$_1$10.6 ± 2.6^a	$_1$10.0 ± 2.1^c
		Yabbies	$_1$12.2 ± 2.3^a	$_1$18.8 ± 2.6^b	$_1$15.0 ± 1.9^b	$_1$9.9 ± 2.6^a	$_1$10.7 ± 1.0^c
Carbo-hydrate (%)	2-15	Marron	$_1$12.9 ± 1.2^a	$_1$3.5 ± 1.6^b	$_1$13.5 ± 3.1^a	$_1$20.5 ± 2.7^c	$_1$19.8 ± 2.6^c
		Yabbies	$_1$13.9 ± 2.0^a	$_1$3.9 ± 2.3^b	$_1$10.6 ± 2.5^a	$_1$17.3 ± 2.3^c	$_1$22.8 ± 2.8^c
	61-75	Marron	$_2$20.6 ± 2.6^a	$_2$10.6 ± 2.1^b	$_2$16.1 ± 2.3^a	$_2$23.3 ± 1.8^a	$_2$34.7 ± 3.9^c
		Yabbies	$_2$18.5 ± 2.8^a	$_2$8.0 ± 1.5^b	$_2$18.1 ± 1.7^a	$_2$24.7 ± 3.5^a	$_2$35.7 ± 3.9^c

Same alphabetical superscripts (a, b, c) in the same row (comparisons among moult stages) and numerical subscripts (1, 2) in the same column of each factor (comparisons between weight classes) are not significantly different at the $\alpha = 0.05$ level. n = 6.

There was a significant difference in protein content between moult stages (Table 7.2), but no difference was observed between species and weight class at any moult stage. Protein from the exoskeleton was the highest during the postmoult stage (AB). A significant reduction of protein from the intermoult stage (C) to the premoult stages (D_0, D_1, D_2) was observed.

Chitin content was highest during the intermoult (C), and minimal during the late premoult (D_2) (Table 7.2). Body weight of crayfish had no effect on chitin

concentration in their exoskeletons in any species. In both marron and yabbies, the total lipid in the exoskeleton remained constant over the moult cycle, irrespective of body weight class.

Ash content was lower during the postmoult (AB) and late premoult stages (D_1, D_2) (Table 7.2), while it was significantly higher during intermoult (C), and the early premoult stage (D_0). Species had no effect on the ash level at any moult stage. However, ash was weight class-dependent in every moult stage; larger crayfish had lower ash contents in their exoskeleton.

Gross energy was maximal during intermoult stage (C) and early premoult (D_0) (Table 7.2). In both species, gross energy of exoskeleton was weight class independent during the moult cycle.

Table 7.3. The percentage of the dry exoskeleton to body weight of intermoult staged marron and yabbies for different moult stages and weight classes

Weight	Species	AB	C	D_0	D_1	D_2	
2-15	Marron	$_1$2.83 0.22^a	± $_1$7.28 0.25^b	± $_1$7.17 0.25^b	± $_1$5.15 0.22^c	± $_1$4.66 0.28^c	±
	Yabbies	$_1$2.79 0.13^a	± $_1$7.42 0.14^b	± $_1$6.98 0.15^b	± $_1$4.81	± 0.28^c $_1$4.55 0.23^c	±
61-75	Marron	$_2$2.02 0.19^a	± $_2$5.37 0.23^b	± $_2$5.04 0.34^b	± $_2$3.62 0.17^c	± $_2$3.52 0.29^c	±
	Yabbies	$_2$2.21 0.13^a	± $_2$5.42 0.13^b	± $_2$5.45 0.12^b	± $_2$4.13 0.12^c	± $_2$3.62 0.14^c	±

Same alphabetical superscripts (a, b, c) in the same row (comparisons among moult stages) and numerical subscripts (1, 2) in the same column (comparisons between species) are not significantly different at the $\alpha = 0.05$ level. Number of samples are same as Table 7.1.

The percentage of dry exoskeleton to intermoult body weight was significantly different between moult stages and weight classes (Table 7.3) and was independent of species during the entire moult cycle.

Table 7.4: The P_E during postmoult (AB) - intermoult (C) as weight gain and during intermoult (C) - late premoult (D_2) as weight loss of the exoskeleton for different weight classes of marron and yabbies

Weight	Species	Gain	Loss
2-15	Marron	$_1 4.15 \pm 0.24^a$	$_1 2.62 \pm 0.23^b$
	Yabbies	$_1 4.33 \pm 0.26^a$	$_1 2.87 \pm 0.19^b$
61-75	Marron	$_2 3.16 \pm 0.13^a$	$_2 1.86 \pm 0.13^b$
	Yabbies	$_2 3.01 \pm 0.21^a$	$_2 1.80 \pm 0.20^b$

Same alphabetical superscripts (a, b) in the same row (comparisons between gain and loss) and numerical subscripts (1, 2) in the same column (comparisons between weight classes) are not significantly different at the $\alpha = 0.05$ level. n= 30.

A significant difference in P_E was found between gain and loss (Table 7.4). The P_E was weight class-dependent and species-independent.

7.4 DISCUSSION

The anecdysial exoskeleton of crustaceans consists of 4 layers, from the most external to the most internal: the epicuticle, the exocuticle, the endocuticle and the membranous layer (Travis 1963). Each layer contains three major components, chitin, protein and calcium carbonate, together with various minor components such as proteoglycans, lipids and inorganic materials (Travis 1955, 1965; Green and Neff 1972; Welinder 1975; Hegdahl et al. 1977a; Hegdahl et al. 1977b; Roer and Dillaman 1984; Lowenstam and Weiner 1989; Simkiss and Wilbur 1989). The relative proportion of these components varies depending on the species and location on the body (Ekpenyong et al. 2013), sex (Abdul-Sahib and Ajeel 2005), moult stage (Pratoomchat et al. 2002b) and even in a single individual (Cribb et al. 2009). The cycling of materials during the moult cycle can, in most cases, be explained with reference to the known physiology of the moult cycle (Nicol et al. 1992; Roer et al. 2015).

The higher protein level during postmoult stage (AB) of marron and yabbies agrees with the result of Pratoomchat et al. (2002b) on the exoskeleton of mud crab. The

unhardened exoskeleton of brown crab, *Cancer pagurus,* European green crab, *Carcinus maenas* and spiny spider crab, *Maia squinado* after ecdysis or postmoult (AB) also have higher protein content (Drach and Lafon 1942). The minimum level of protein at late premoult (D_2) is the result of the resorption of protein from old exoskeleton for pre-exuvial deposition during the premoult stages (D_0, D_1, D_2) (Drach 1939; Travis 1965; Roer 1980; O'Brien et al. 1991; Roer et al. 2015).

The highest protein content in the exoskeleton during the postmoult (AB) reported in this study is considerably lower than the levels reported by other authors in other crustaceans (Table 7.5). The difference could be due to the delayed sampling time after ecdysis. Late sampling (on day 2 after ecdysis) of the postmoult exoskeleton would result in a lower proportion of the protein in exoskeleton as calcification and the deposition of minerals after ecdysis in crayfish had already commenced immediately after ecdysis (Willig and Keller 1973; Wheatly and Ayers 1995).

In the current study, ash content was significantly lower during the late premoult (D_2) and postmoult (AB) stages as calcium is resorbed from the exoskeleton and is lost to the environment or stored within the body (Greenaway 1985). There is a conversion of calcium carbonate to gastroliths in the stomach during the premoult stages (D_0, D_1, D_2) that is typical for any crayfish species (Greenaway 1985; Nagasawa 2012).

During premoult stages (D_0, D_1, D_2), calcium carbonate in crustaceans is reduced in the exoskeleton before moulting to increase their flexibility as a preparation for moult (Nagasawa 2012), while the deposition of calcium salt increases from the postmoult (AB) until the intermoult stage (C), resulting in higher ash content during the intermoult (C) and early premoult (D_0). In general, higher calcium carbonate content in the harder exoskeleton during intermoult (C) and early premoult (D_0) results in higher ash content (Nagasawa 2012). Although the two outer layers (epi- and exocuticle) of the new exoskeleton are created during premoult, these new layers do not calcify as they contain unbound proteins that inhibit calcification until after the moult (Paul and Sharpe 1916; Travis 1963; Travis and Friberg 1963).

The lower ash content in larger marron and yabbies is due to their higher demineralisation rate during premoult stages (D_0, D_1, D_2) and lower rate of

mineralisation from postmoult (AB) to intermoult (C), while the opposite trend is adapted by smaller crayfish (Wheatly and Ayers 1995), as larger crayfish have a longer time, relative to smaller crayfish, available during their intermoult stage (C). For example, in larger red swamp crayfish, there is a higher efflux of calcium (demineralisation) during premoult stages (D_0, D_1, D_2) and lower influx (mineralisation) during postmoult (AB) and intermoult (C) (Wheatly and Ayers 1995).

Chitin is known for its fibrous architecture, as a scaffold for the deposition of calcium carbonate during mineralisation (Roer and Dillaman 1984; Simkiss and Wilbur 1989; Lowenstam and Weiner 1989), and is synthesised by chitin synthase at the apical side of epithelial cell membranes. The protein is also synthesised by epithelial cells, and is subsequently secreted and deposited to the exoskeleton (Nagasawa 2012). From the complex structure made of chitin and protein, some different proteins have the ability to bind to chitin (Cribb et al. 2009). The accompanied deposition of protein and chitin during postmoult (AB) and early intermoult (C) (Welinder 1974) explains the reasons for the same pattern of variation in chitin and protein during the moult stages. The synthesis of chitin during the hardening period of Antarctic krill has been shown by Buchholz (1989). The higher gross energy value of the crayfish exoskeleton during intermoult (C) and early premoult (D_0) may be due to the presence of higher content of chitin (Morganti et al. 2011).

The gradual decline in chitin throughout the premoult stages (D_0, D_1, D_2) in the current study is due to the partial decomposition to separate the old exoskeleton from the new exoskeleton (Nagasawa 2012) and maximal breakdown of chitin controlled by the chitinase and betaN-acetyl glucosaminase enzymes during late premoult (D_2) (Buchholz and Buchholz 1989). Up to 50% of chitin, during the late premoult stage of crayfish, is resorbed to be stored as acetylglucosamine to form a chitin complex in the new exoskeleton (Gwinn and Stevenson 1973).

A relatively greater change in chitin content was reported in other crustacean species. For example, the chitin content in dry weight was 40-45% of the exoskeleton in the premoult, while 71-72% in the post-exuviate exoskeleton of brown crab and spiny spider crab (Drach and Lafon 1942). Similarly, small sized noble crayfish had a wide

range of chitin concentration from 36.5% to 71.0% in their exoskeleton during the postmoult stage (AB) (Welinder 1974). In the current study, a lower content and narrower range of chitin over a moult cycle were reported, 20.1-35.4% for marron and yabbies.

The lipid is only found in the outermost epicuticle layer where it could act as a water-shield (Promwikorn et al. 2005; Nagasawa 2012). Lipid content in the marron and yabbies exoskeleton remained constant over a moult cycle in all weight classes. There has been limited literature on the cyclic change in exoskeleton lipid in crustacean species; most of the lipid estimation was analysed for only exoskeleton wastes (Table 7.5). Chandumpai et al. (1991) reported that total exoskeletal lipid of the giant tiger prawn was significantly higher at day 0 after ecdysis (postmoult-AB). The sampling time can be used as an explanation for the difference between these two results as our postmoult exoskeleton samples were collected 2 days after the ecdysis instead of on day 0, as by Chandumpai et al. (1991).

The variation of carbohydrate was similar to that of protein due to its role in the formation of the exoskeleton over the moult cycle. Carbohydrate is utilised during structuring and building up exoskeleton while contributing to the polymerisation of chitin (Gwinn and Stevenson 1973). The current results suggest that a decrease of carbohydrates during the late premoult (D_2) to intermoult stage (C) is correlated with the proportional increase of chitin and protein. Thus, carbohydrate content is reduced by excretion while protein is gradually transferred to the formation of a new exoskeleton from D_2 stage. The finding agrees with the suggestions of Pratoomchat et al. (2002b), Stevenson (1985) and Skinner (1985) on the formation of the new exoskeleton.

The proximate composition of the exoskeleton in various species of crustacean has been reported by several authors and a summary of the relevant published results are presented in Table 7.5. Our results fall within the ranges of earlier reports and closer to Johnson and Peniston (1982), who summarised that protein and chitin in crustacean exoskeleton were 30-40% and 13-42%, respectively.

Table 7.5: Comparison of proximate exoskeleton compositions (% dry weight) from different crustacean species

Species	Protein	Lipid	Chitin	Ash	Carbohydrate	Reference(s)
Macrobrachium macrobranchion	14.0	4.0	-	7.1	-	Ekpenyong et al. (2013)
Chionoecetes opilio	34.2	17.1	-	-	-	Asunción et al. (2011)
Penaeus indicus	32.5	9.8	-	26.6	-	Ravichandran et al. (2009)
Metapenaeus affinis	11.3	2.8	-	8.5	-	Abdul-Sahib and Ajeel (2005)
Collinectes sapidus	25.1	2.1	13.5	58.6	-	Elaborated according to Muzzarelli et al. (1997), Naczk et al. (1981), Shahidi and Synowiecki (1991), Synowiecki and Al-Khateeb (2000)
Chinoecetes opilio	29.2	1.3	26.6	40.6	-	
Pandalus borealis	41.9	5.2	17.0	34.2	-	
Cragon crangon	40.6	9.9	17.8	27.5	-	
Peneaus monodon	47.4	1.3	40.4	23.0	-	
Procamborus clarkii	29.8	5.6	13.2	46.6	-	
Euphausia superba	41.0	11.6	24.0	23.0	-	
Cancer pagurus	-	-	-	40-45	-	Drach and Lafon (1942)
Maia squinado	-	-	-	71-72	-	
*Cherax cainii**	20.6	2.4	38.3	54.5	34.7	Current study
*Cherax albidus**	21.7	2.7	39.4	55.7	35.7	Current study

** Maximum contents were selected among moult stages from the current study*

The percentage of dry exoskeleton to wet body weight of the intermoult staged crayfish was significantly higher in intermoult (C) and early premoult (D_0), which can be supported by calcification, mineralisation and organic deposits during the postmoult (AB) to intermoult (C), while degradation and resorption of these components take place during the premoult (D_0, D_1, D_2) (Nicol et al. 1992; Wheatly and Ayers 1995; Roer et al. 2015). Crayfish lost 1.5% of organic and inorganic matters in the form of protein, chitin and ash from the exuviae at ecdysis, due to the

higher P_E gain than loss. The higher P_E in smaller crayfish is explained by the fact that smaller size crayfish mineralise their exoskeleton quicker and with a higher calcium deposition rate during the postmoult calcification process (Wheatly and Ayers 1995).

In conclusion, the chitin, ash and gross energy show similar variations over a moult cycle in marron and yabbies. Their level reaches a peak during the intermoult (C) and early premoult (D_0) stages. Protein and carbohydrate content are highest during the postmoult (AB) and late premoult (D_2), respectively. Lipid level stays at a constant level during the moult cycle. Ash and carbohydrate are higher in larger crayfish. The proportion of dry exoskeleton to the wet body weight of intermoult staged crayfish is both moult stage- and weight class-dependent.

CHAPTER 8: GENERAL DISCUSSION

8.1 BACKGROUND INFORMATION

Within the broader aim of the current research theme, the research has highlighted four different aspects of weight-dependent physiological parameters of the haemolymph in farmed freshwater crayfish in WA. In addition, one of the related objectives was to study the natural population structure of yabbies in a man-made ecosystem or earthen ponds constructed for fishing/farming of yabbies. However, the second objective targeted the effects of crayfish moult stages on the physiological status of crayfish, while the third objective focused on the effects of feeding and starvation on the haemolymph osmolality of these crayfish. Figure 8.1 conceptualises the overall research themes of the current study.

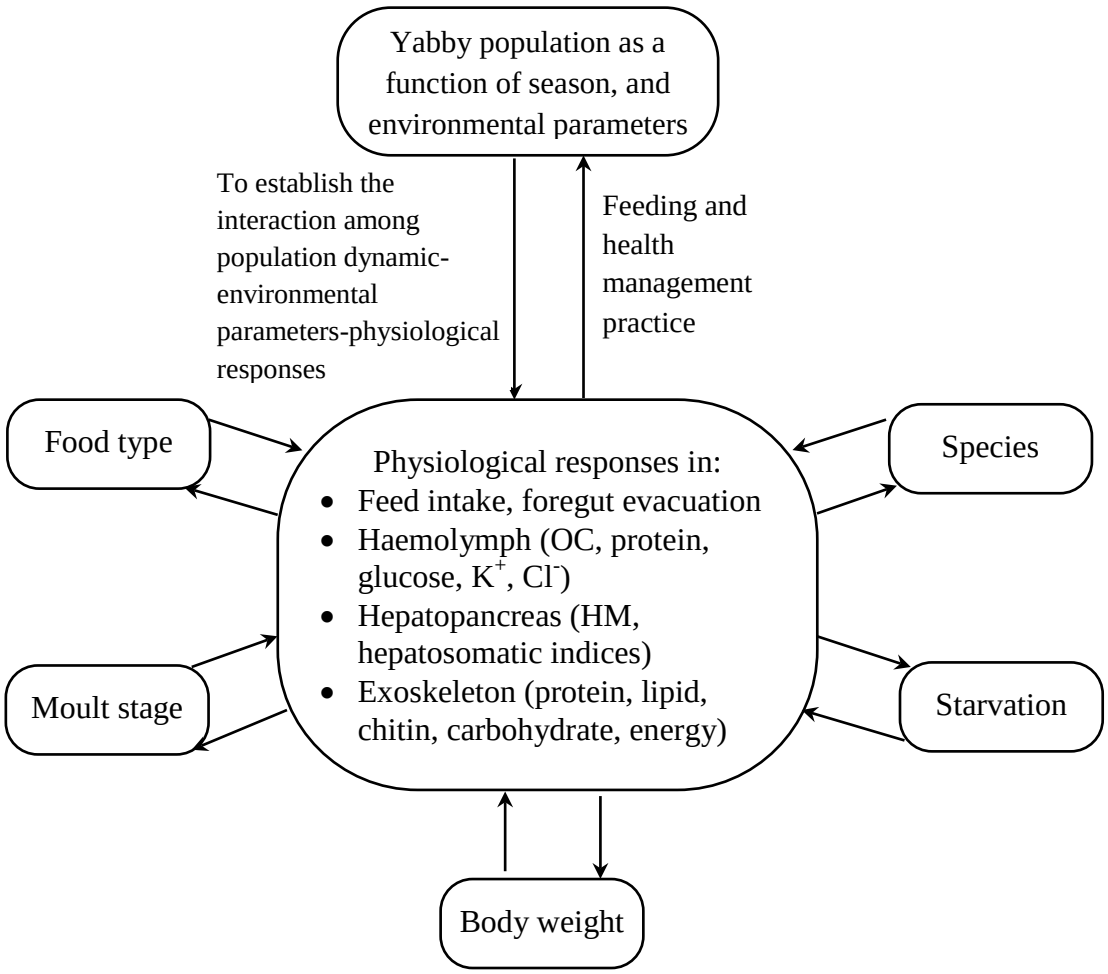


Figure 8.1: Schematic diagram of the current research showing the five variables (moult stage, body weight, food type, starvation and species) and the overarching objective (population structure).

Figure 8.1 also attempts to bring the perceived discrete objectives under one broad theme and the subsequent discussion explains this attempt.

Understanding the impacts of internal and external factors on crayfish physiology is key to improving crustacean productivity under farming conditions (Lignot et al. 2000). Only OC and haemolymph constituents among the independent variables were evaluated and were subjects of food type and starvation, while all other physiological statuses of the haemolymph in both crayfish species were compared, and contrasted by all moult stages, weight classes and species. Along with the result from the population structure of yabbies, the impact of the five variables (moult stage, weight class, food types, starvation and species) will be used to define physiology status of the crayfish population.

The correlation between environmental parameters and yabby population size may be considered an objective not directly related to the measured physiological parameters of this study; however, the growth of yabbies has a strong bearing on the studied physiology of the haemolymph of crayfish (Chapter 4). In addition, there is an indirect relation between the changes in the yabby population structure and growth performance, which in turn are dependent on the soil- and water-related biotic and abiotic parameters (Chapter 3).

Lawrence et al. (1998) and De Graaf et al. (2010) studied yabbies and marron for their population dynamics and the impact on the latter by physicochemical parameters. Biological factors such as phytoplankton and zooplankton were not included in their research, although they are deemed important food sources for crayfish (Jones 1997; Meakin et al. 2008; Meakin et al. 2009). There was an attempt to fill this gap in existing knowledge by conducting research on the population dynamics of yabbies as influenced by biological factors including phytoplankton and zooplankton abundance. However, no similar research on the population structure of marron was conducted as marron have a different production system and management system in WA. The commercial production of marron originates from

semi-intensive management systems where marron are farmed in drainable earthen ponds that house single-cohort populations of marron, whereas multiple cohort populations exist in yabby farm dams as a result of continuous recruitment and harvesting of yabbies. Therefore, the mark-recapture method of population estimation is a more applicable technique for quantifying the size of crayfish populations (Skudal et al. 1992; Nowicki et al. 2008; Bolat et al. 2011) where natural populations exist including an extensive management style in yabby farm dams. In spite of this, previous and current studies conclude that the population structure of marron and yabbies is significantly influenced by the physicochemical properties of water, bottom soil and biological parameters.

Table 8.1: Summary of the relationship among independent and dependent variables (moult stage, weight class, feeding status, food types, species), and the moult stage-specific highest and lowest values of independent factors

Studied factors	Moult stages	Weight classes	Feeding status	Food types	Species	Moult stage(s) showing highest value	Moult stage(s) showing lowest value
Osmolality	yes	yes	yes	no	yes	C	AB
Interval of individual moult stage	yes	yes	na	na	yes	C	AB
Weight increment of individual moult stage	yes	yes	na	na	yes	AB	D_2
HM%	yes	yes	na	na	yes	AB	C, D_0, D_1, D_2
HI_{wet}	yes	yes	na	na	yes	C	AB, D_0, D_1, D_2
HI_{dry}	yes	yes	na	na	yes	C	AB, D_0, D_1, D_2
Haemolymph protein	yes	yes	yes	na	no	C, D_0	AB,D_1, D_2
Haemolymph glucose	yes	no	yes	na	no	C, D_0	AB, D_1, D_2
Haemolymph K^+	yes	no	no	na	no	C	AB, D_0,

							D_1, D_2
Haemolymph Cl^-	yes	no	no	na	no	C	AB, D_0, D_1, D_2
FC	na	yes	na	yes	no	na	na
FGE	na	yes	na	yes	no	na	na
Exoskeleton protein	yes	no	na	na	no	AB	C, D_0, D_1, D_2
Exoskeleton chitin	yes	no	na	na	no	C, D_0	AB, D_1, D_2
Exoskeleton lipid	no	no	na	na	no	na	na
Exoskeleton ash	yes	yes	na	na	no	C	AB, D_0, D_1, D_2
Carbohydrate	yes	yes	na	na	no	D_2	C
Exoskeleton gross energy	yes	no	na	na	no	C, D_0	AB, D_2
P_E	yes	yes	na	na	no	C, D_0	AB, D_2

P_E: the percentage of dry exoskeleton in relation to total wet body weight; FC: food consumption rate; FGE: foregut evacuation rate; yes: a significant difference found; no: no significant difference (T-test, $P < 0.05$); na: no data available, AB: postmoult, C: intermoult, D_0, D_1, D_2: premoult stages are five moult stages defined from a moult cycle of marron and yabbies.

The various moult stages, among the selected variables, are important variables influencing most of the studied parameters (Table 8.1) (Heath and Barnes 1970; Richard 1980; Vargas-Albores and Ochoa 1982; Skinner 1985; Lignot et al. 1999; Pratoomchat et al. 2002b; Pascual et al. 2006; Marcy et al. 2009; Durliat and Vranckx 1982). Intermoult stage C is the least affected by the studied parameters, making this stage the preferred stage to be used for many experiments. Therefore, in the current study, feeding and starvation experiments were restricted to intermoult stage C. On the other hand, premoult and postmoult stages encompass greater fluctuations in their physiological status, making them unsuitable stages for selection for feeding and starvation experiments.

The variations in all the studied physiological parameters from various body parts/tissues/organs over a moult cycle were dependent on moult stages, similarly to

other crustaceans (Passano 1960; Bliss 1985; Garcia 1988; Aiken and Waddy 1992; Chen and Chia 1997; Cheng et al. 2002; Franco et al. 2006; Galindo et al. 2009; Ha and Fotedar 2016). Most of these physiological parameters peaked at the intermoult (C) and bottomed out at postmoult (AB) and/or late premoult (D_2). Similarly, haemolymph osmolality of marron and yabbies reached the highest level during the intermoult stage (C) and was lowest in the postmoult stage (AB). The same pattern was reported in the blue shrimp, *Litopenaeus stylirostris* by Mugnier and Justou (2004) and Lignot et al. (1999), the giant tiger prawn, *Penaeus monodon* by Ferraris et al. (1987) and white leg shrimp, *P. vannamei* by Charmantier and Soyez (1994), though these species live in different water salinities. Therefore, irrespective of marine and freshwater habitats, crustaceans experience a similar mechanism of haemolymph homeostasis.

The integument permeability decreases, creating an impermeable barrier between the internal and external environment during the intermoult stage (C), while an increase in ATPase activity mobilises ionic secretion into the haemolymph (Charmantier and Soyez 1994). Moreover, water absorption as a result of an increase in the permeability of the integument during late premoult and early postmoult stages (Ferraris et al. 1987; Hunter and Uglow 1993) dilutes the haemolymph and causes a reduction in the osmotic capacity during postmoult (AB) (Galindo et al. 2009).

The higher HI_{dry} with low HM% of the hepatopancreas during intermoult stage C in the current study supports the claim by McClain (1995a) that a large hepatopancreas with a low moisture content is usually an indicator of well-conditioned animals. Similar results are reported by Lindqvist and Louekari (1975) in noble crayfish, *Astacus astacus* and by Magalhães et al. (2012) in bristled river shrimp, *Macrobrachium olfersii*. There is a greater accumulation of reserves in the hepatopancreas during the intermoult stage (C) to be used later during ecdysis (Passano 1960). Lower HI_{wet} and HI_{dry} during premoult (D_0, D_1, D_2) denotes the spending of these reserves for new exoskeleton formation and an abrupt spurt in the growth (Magalhães et al. 2012).

The length of the moult interval in marron and yabbies in the current study was dependent on the moult stages, body weights and species. Like many other crustacean species, intermoult (C) and early premoult (D_0) stages of marron and

yabbies took a longer time to complete, irrespective of the body weight class and species. Intermoult stage (C) accounted for up to 55% of the entire moult cycle, and crayfish with a larger body weight required more time to complete a moult stage than small ones. Similar results were previously reported by Ackefors et al. (1995) and Westman et al. (1993) for noble crayfish and the signal crayfish, *Pacifastacus leniusculus*.

Growth in crustaceans, described in terms of percentage intramoult weight increment, is a function of a number of internal and external factors (Botsford 1985). In the current research, the weight increment of crayfish varied significantly among moult stages, body weights and species. The percentage intramoult weight increment was highest over postmoult (AB) due to the absorption of water (Bliss et al. 1966; Mantel and Farmer 1983; Cameron 1989; Neufeld and Cameron 1992; Chen and Chia 1997; Cheng et al. 2001; Cheng et al. 2002; Charmantier-Daures and Vernet 2004) and hardening of exoskeleton using the calcium stored in gastroliths (Aiken and Waddy 1987; Aiken and Waddy 1992; Nicol et al. 1992; Wheatly and Ayers 1995; Jussila 1996; Nagasawa 2012; Roer et al. 2015).

Feeding is another important variable affecting the osmotic regulation of the crayfish. Under controlled laboratory conditions, the results of feeding marron and yabbies in both Chapter 5 and Chapter 6 showed an increase in haemolymph osmolality (66.0 and 47.0 mOsm kg^{-1} for marron and yabbies, respectively), protein (18.3 and 18.2 mg mL^{-1}) and glucose (18.0 and 13.0 mg mL^{-1}), though Na^{+}, K^{+} and Cl^{-} ions, known to make up more than 90% of the osmotic pressure of the haemolymph in most crustaceans (Prossner 1973; Castille and Lawrence 1981b, a; Mantel and Farmer 1983), were stable after feeding and showed no difference between fed and starved crayfish (Lignot et al. 1999; Ha and Fotedar 2016). The results from the current study validated the fact that the haemolymph protein level (Stewart et al. 1967; Adiyodi 1969; Uglow 1969; Djangmah 1970) and glucose (Lignot et al. 1999) decrease during starvation. Therefore, the increase in haemolymph osmolality after feeding should originate from the increased protein and glucose level due to a rapid and massive transport of digested nutrients such as protein and glucose from the gut to the haemolymph (Lignot et al. 1999; Rosas et al. 2000). Similar results have been observed by Ahearn and Maginniss (1977) in the

giant freshwater prawn, by Rosas et al. (1995) in Atlantic white shrimp and by Lignot et al. (1999) in blue shrimp.

The highest level of osmolality of marron and yabbies was reported at 7 hours after feeding, irrespective of food types (Chapter 5 and Chapter 6). The higher FGE (50%) within 3 hours from T4 to T7 would likely result in the highest osmolality at T7 due to a higher content of glucose and protein making up the osmolality (Ahearn and Maginniss 1977; Rosas et al. 1995; Lignot et al. 1999; Ha and Fotedar 2016). The results showed that the osmolality level of crayfish is food evacuation dependent. This also supports the higher level of haemolymph osmolality in fed crayfish (Chapter 5 and Chapter 6) than in starved crayfish. However, no effect from different food types was observed on the osmolality of marron and yabbies at various starvation times (1, 4, 7, 10 hours).

Table 8.2: Comparison of haemolymph osmolality (mOsm kg^{-1}) at sampling times of T7 and T10 post-feeding from different chapters for crayfish of similar body weights under fed and starved conditions

Species	Feeding status	Mean weight	Osmolality (mOsm kg^{-1})			
			T7* Chapter 5	T7** Chapter 6	T10* Chapter 5	T10** Chapter 6
Marron	Starved	67.5 ± 2.4	$_1460.2 \pm 5.2^a$	na	$_1450.5 \pm 4.6^a$	na
	Fed	61.5 ± 1.3	$_2543.7 \pm 4.2^a$	$_1548.7 \pm 4.2^a$	$_1454.4 \pm 5.3^b$	450.7 ± 4.0^b
Yabbies	Starved	63.5 ± 1.9	$_3429.4 \pm 3.9^a$	na	$_2417.5 \pm 5.4^b$	na
	Fed	60.7 ± 2.6	$_4478.7 \pm 2.9^a$	$_2485.7 \pm 2.9^a$	$_2424.7 \pm 4.1^b$	421.7 ± 4.3^b

*Same alphabetical superscripts in the same row and numerical subscripts in the same column are not significantly different (T-test). na: no data available; *: n=11; **: n=27.*

Table 8.3: Comparison of haemolymph osmolality at sampling time T4 (4 hours post-feeding) from different chapters for crayfish of similar body weight under fed and starved conditions

Species	Feeding	Mean	Osmolality (mOsm kg^{-1})

	status	weight	T4 Exp.2* Chapter 5	T4 Exp.3** Chapter 5	T4** Chapter 6
Marron	Starved	67.5 ± 2.4	$_1$476.3 ± 5.6^a	$_1$470.5 ± 4.2^a	na
	Fed	61.5 ± 1.3	$_2$535.4 ± 6.3^a	$_2$530.6 ± 4.2^a	$_2$529.7 ± 4.2^a
Yabbies	Starved	63.5 ± 1.9	$_3$418.7 ± 4.9^a	$_3$410.4 ± 3.3^a	na
	Fed	60.7 ± 2.6	$_4$475.6 ± 5.1^a	$_4$480.4 ± 4.9^a	$_4$473.7 ± 2.9^a

*Same alphabetical superscripts in the same row and numerical subscripts in the same column are not significantly different (T-test). na: no data available; *: n=20; **: n=27.*

A similar osmolality from different experiments at each sampling time (hour) after feeding (Table 8.2, Table 8.3) confirms the reliability of osmolality variations after feeding. The conclusion that osmolality is dependent on moult stage, weight class, feeding status and species, but not the type of food used, is summarised in Table 8.1.

Similarly to responses of haemolymph physiology and hepatopancreatic indices to the studied variables, change in exoskeleton compositions was also a function of moult stage, body weight class and species. The underlying mechanism of the variations in exoskeleton composition is explained by mineralisation/deposition during postmoult and decomposition during premoult of exoskeleton over a moult cycle (Nicol et al. 1992; Wheatly and Ayers 1995; Roer et al. 2015). The content of exoskeleton lipid was the only component that was independent of all the variables (Promwikorn et al. 2005; Nagasawa 2012), which is different from the result of Chandumpai et al. (1991), who stated that the highest lipid content was after ecdysis.

8.2 FEEDING PHYSIOLOGY IN CRAYFISH

Feed and feeding management based on the rate of food ingestion and foregut evacuation are critical for optimal crayfish growth in a culture environment (Jory 1995; Jory et al. 2001), and are functions of a number of variables such as food type, development stages and environmental conditions (Hill and Wassenberg 1992; Maguire and Allan 1992; Nunes and Parsons 2000; Gonzalez-Pena et al. 2002; Wasielesky et al. 2003; Soares et al. 2005; Simon and Jeffs 2008). Due to a reduction

in the metabolic rate at a larger body weight (Musgrove 1993), the total quantity of feed consumption is higher in larger marron and yabbies, but inversely in percentage terms (Loya-Javellana et al. 1995; Nunes and Parsons 2000; Simon and Jeffs 2008).

The results from the current and previous studies have indicated that the selection of feed and feeding strategy for a higher growth of marron and yabbies is dependent on multiple factors. The first criteria to consider are the protein level and the protein to energy (P:E) ratio (Lee and Wickins 1992). Thirty per cent protein is considered the suitable level for crayfish (Hubbart et al. 1986; Tsvetnenko et al. 1995; Jones et al. 1997) to achieve optimum growth rates, while the recommended P:E ratio should be close to 120 (30% protein and 2,500 kcal kg^{-1}) (D'Abramo and Robinson 1989). Among three tested food types, only algae granules have a protein level (30%) and P:E ratio (70) close to the above recommendations.

Marron and yabbies are able to ingest a wide variety of diets with wider physical properties (Jones 1997) due to their relatively larger hepatopancreas (Lorman and Magnuson 1978; Momot et al. 1978; Sokol 1988; D'Abramo and Robinson 1989; Tacon 1993), a feature of omnivores. This explains why formulated pellet, algal granules and prawn flesh, which have distinct differences in terms of nutrient profiles and physical structure, were selected for the current study. The chosen food types represent a wide food spectrum/niche available in a water body. Phytoplankton is a major component of any natural primary productivity in an earthen pond environment as a consequence of the application of inorganic fertilisers (Boyd and Tucker 1998) and plays a key role in the food chains of an aquatic animal (Dede and Deshmukh 2015). Algae also constitute a natural diet as well as a supplementary source of nutrients for crayfish (O'Brien 1995; Tidwell et al. 1996). The importance of phytoplankton to the growth of crayfish in extensive culture ponds is confirmed in Chapter 3. The cannibalistic characteristics of crayfish suggest prawn flesh as an extreme carnivore diet to be tested, while formulated pellet is frequently used in semi-intensive crayfish culture systems in WA.

Zooplankton, either in the form of live food (Jones 1997) or detritus (Mills et al. 1994), is an important nutrient source for crayfish. Irrespective of body weight class, both marron and yabbies prefer to prey upon live zooplankton, including *Daphnia* spp, rather than commercial pellet if both food types are presented (Meakin et al.

2008; Meakin et al. 2009). Jones (1997) concluded that yabbies significantly increase their growth rate with a supplementation of zooplankton in their diet. Similarly, a significant correlation between zooplankton density and population size (Chapter 3) added to the importance of zooplankton to crayfish production, though the relative contribution of zooplankton to the nutritional budget of the studied ponds was not investigated.

8.3 SUMMARY

Given that the performance of a living organism is a function of multiple internal and external parameters, in the current study, the physiology and feeding ecology (multiple internal parameters) of marron and yabbies were functions of moult stage, weight class and starvation. However, the physiology of osmoregulatory capacity was not food type dependent. The haemolymph physiology of marron and yabbies was sensitive during premoult and postmoult or before and after ecdysis, while health indices had their highest values during the intermoult stage. Marron and yabbies of juvenile size experienced the same patterns of changes in their haemolytic physiological measurements over a moult cycle. Osmolality is dependent on moult stage, weight class, feeding status and species, but not the type of food used. Without a dependence on food type, there was a critical change in OC in both marron and yabbies at 7 hours after feeding as a result of the evacuation time of the ingested food.

While the effect of external environment parameters, including light regime, water quality parameters and food availability, was not studied, the haemolymph physiology of marron and yabbies in the study is explained in the light of moult stages during their life cycles. The underlying mechanisms associated with moult stages and haemolymph physiology are related to inflow and outflow of water, organic materials and minerals controlled by moult-related hormones (Chang 1985). Under controlled laboratory conditions, the results of the periodic change in OC, hepatosomatic indices, haemolymph constituents and exoskeleton compositions are indicators and references for defining the health condition and development stage of crayfish (Chang and O'Connor 1983; Skinner 1985).

Provided that studying the population dynamic of crayfish is more applicable under the extensive farm dam situation of yabbies, the dependence of yabby populations on water quality parameters will lead to changes in the physiological status arising from the impacts from abiotic and biotic environmental parameters. Dissolved inorganic nitrogen, soluble reactive phosphorus and zooplankton density play an important role in defining the population size as they are significantly correlated and have a significantly positive effect on yabby populations, while pH and hardness have a negative influence on yabby populations.

8.4 LIMITATIONS OF THE STUDY

The main limitations of the current study were:

- The research does not explain the direct effects of water- and soil-related quality parameters on the haemolymph physiology of crayfish.
- The research only targeted limited haematological variables. More haemolymph-based physiological variables should be included in future studies.
- The impact of ionic profile on haemolymph osmolality was beyond the scope of the current research as osmolality can have a strong influence on the ionic profile that contributes up to 90% of osmolality in crustaceans
- The study does not find relationships among food type and the haemolymph constituents, hepatosomatic indices and exoskeleton compositions.
- The research does not examine the relative contribution of zooplankton to the nutritional budget of the yabbies in the studied ponds.

CHAPTER 9: CONCLUSIONS AND RECOMMENDATIONS

9.1 CONCLUSIONS

Based on the results, the following summary reflects the achievement of the objectives of the research:

1. The density of yabbies in purpose-built ponds in Western Australia was lower than previously reported, ranging from 0.05 to 0.14 individuals m^{-2} depending on pond location, seasons and water quality parameters (Objective 1).

2. In the pond system, irrespective of sampling time and pond location, the number of male yabbies was 2.5-3.0 times higher than that of females (Objective 1).

3. Dissolved inorganic nitrogen, soluble reactive phosphorus and phytoplankton in the pond water had a stronger positive correlation with yabby population size/density, while pH and hardness were negatively related to yabby population density (Objective 2).

4. Total nitrogen and organic matter between the bottom soil and water column were significantly correlated during spring and summer. A significant correlation of these parameters with yabby population was observed (Objective 2).

5. Osmoregulatory capacity was highest during intermoult (C) in both marron and yabbies. It increased with an increase in body weight in every moult stage (Objective 3) and was not affected by the different food types within the first 10 hours post-feeding (Objective 6).

6. More weight was gained during postmoult stage (AB) irrespective of the body weight classes (Objective 3).

7. The time required to complete a moult cycle was longer in larger crayfish than smaller crayfish. Intermoult stage (C) was the longest, at up to 50% of the moult cycle, while the shortest at 10% was recorded for postmoult stage (AB) (Objective 3).

8. Percentage dry matter of whole body carcass was highest in intermoult stage (C) and lowest during postmoult (AB) with a significant difference between the two species (Objective 3).

9. Hepatosomatic indices of marron and yabbies were significantly influenced by their respective moult stages, while body weight class did not show an effect on the indices (Objective 3).

10. OC, moult intervals and growth rates of marron larger than 15 g were higher than those of yabbies, while hepatosomatic indices and moult frequency were higher in yabbies (Objective 3).

11. Haemolymph osmolality, protein, potassium, chloride and glucose concentrations showed similar fluctuations during a moult cycle in both marron and yabby. They were higher in fed crayfish, while no differences in potassium and chloride were found between fed and starved ones (Objective 4).

12. The food consumption and foregut evacuation rate of larger crayfish fed shrimp flesh were lowest while they were higher in smaller crayfish fed algal granules and commercial formulated pellet (Objective 5).

13. In the condition of constant water temperature of 23^{o}C, a significant reduction in the foregut evacuation rate in marron and yabbies occurred 7 hours post-feeding (Objective 6) and resulted in the highest haemolymph osmolality being recorded at the same time (Objective 7), irrespective of food types and body weight class.

14. A period of more than 10 hours was required to complete the foregut evacuation and for haemolymph osmolality to return to the minimum stable level during starvation of crayfish (Objective 6).

15. The content of protein, chitin, ash, gross energy and carobohydrate in both marron and yabby's exoskeleton was significantly different among moult stages, while lipid was constant over a moult cycle (Objective 8).

16. The level of ash, chitin and gross energy from exoskeleton reached a peak during intermoult (C) and early premoult (D_0). Protein and carbohydrate were highest during postmoult (AB) and late premoult (D_2), respectively (Objective 8).

17. The ash and carbohydrate were higher in larger crayfish's exoskeleton. The protein, chitin, ash and gross energy were weight class-independent (Objective 8).

18. The proportion of dry exoskeleton to the wet body weight of intermoult staged crayfish is both moult stage- and weight class-dependent (Objective 8) as the result of different rate of deposition and resorption during a moult cycle and and development stage of crayfish.

9.2 RECOMMENDATIONS

The recommendations are based on the limitations and conclusions of the research (Objective 9):

1. The effects of ions such as Ca^{2+}, Mg^{2+} and Na^{+} as well as the Na^{+}/K^{+} ratio from ambient water, which can be important in understanding ATPase activity-dependent OC of crayfish should be studied.

2. The effect of different environmental parameters during a culture such as water and soil quality factors on physiological responses of marron and yabby needs to be investigated.

3. Research needs to be carried out on the correlation between physiology indications and the growth rate of crayfish.

4. The influence of commercially available food types on the physiological responses of the haemolymph of marron and yabby needs to be studied.

5. The relative contribution of zooplankton to the nutritional budget of crayfish culture systems needs further investigation.

6. When applying the results of FC and FGE in a real production of pond system, it is necessary to study the effect of environment conditions that

would be different from controlled optimal laboratory settings as in the current study on feeding-related activities of crayfish.

7. A feeding frequency of 10 hours approximately depending on water temperature is recommended for both marron and yabby crayfish to maintain a healthy condition based on the result of time for completion of foregut evacuation and the stability of osmoregulatory capacity.

8. Successful feeding is a function of both internal and external factors (Nunes and Parsons 2000). The former have been studied, while the latter, such as water quality, light intensity, and natural and artificial food availability, need careful investigation when applied in the reality of production.

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Followings are papers which has been published

1. Mai, V.H., Fotedar, R. 2018. Haemolymph constituents and osmolality as functions of moult stage, body weight, and feeding status in marron, *Cherax cainii* (Austin and Ryan, 2002) and yabbies, *Cherax destructor* (Clark, 1936). Saudi Journal of Biological Sciences, 25(4), 689-696.

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2. Mai, V.H., Fotedar, R. 2017. Osmoregulatory capacity, health status and growth as functions of moult stages from various weight classes in marron, *Cherax cainii* (Austin and Ryan, 2002) and yabbies, *Cherax destructor*. (Clark, 1936). Marine and Freshwater Behaviour and Physiology, 50:1, 1-16.

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