

**Behavioural and morphological aspects of
feed intake in spiny lobsters *Panulirus
ornatus* and *Sagmariasus verreauxi***

by

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Statements and declarations

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General abstract

The unique characteristics of spiny lobster behaviour and morphology impact feed intake and are crucial factors influencing growth, survival, and feeding efficiency in culture. This thesis explores key aspects of juvenile spiny lobster nutritional behaviour and morphology by investigating culture type, mouthparts morphology, feed characteristics and feeding frequency in context of growth, survival and feeding efficiency in culture. This improved understanding of lobster nutritional physiology will aid in the development of economical and effective formulated feeds and feeding strategies for lobster aquaculture. Furthermore, the holistic understanding of feed intake and behaviour will aid in the development of improved commercial production systems, which remain a significant bottleneck for lobster aquaculture. The two spiny lobster species, *Panulirus ornatus* and *Sagmariasus verreauxi*, examined in this thesis, are promising candidates for spiny lobster aquaculture.

This thesis includes an investigation of culture type effect on growth, survival and behaviour in early juvenile *P. ornatus*. The three culture types examined segregated or allowed particular conspecific interactions and included isolated, separated and communal culture. In the isolated culture (individual vessels), lobsters were deprived of all conspecific interactions; separated culture (adjacent cages) eliminated physical interactions, and communal culture allowed all sensory and physical interactions. The influence of chemical cues intensity on growth and survival was also investigated, with two water exchange rates. The feeding behaviour and feed preferences for either mussel gonad, commercial prawn feed or moist feed were investigated using time-series photography. The study showed that physical interactions between conspecifics are essential for optimal culture performance. The communally cultured lobsters exhibited improved growth and moulting frequency. These results may be related to the complex social structures of this gregarious species. Behavioural observations revealed circadian rhythm of interactions with feeds, feed preferences and intake. Observations also revealed differences in lobster behaviour in the differing culture types. Lobsters reared in separation displayed a higher level of interactions with feeds; however, this was not associated with higher feed intake. The study also demonstrated an increase of daylight activity (interactions with feeds and feed intake) with ontogeny from juvenile instar 2 to 4.

The focal point in advancing the aquaculture of spiny lobster species is the development of formulated feeds. This thesis includes an investigation of the functional morphology of two spiny lobsters, *P. ornatus* and *S. verreauxi*, by analysing the mouthpart morphology and mouth aperture. The analysis is focussed on determining likely feed preference based on morphology to provide knowledge to use in the development of formulated feed. Mouth aperture correlates with lobster

carapace length (CL) and is equal to approximately 4 and 7.5% for *S. verreauxi* and *P. ornatus* lobsters, respectively. This finding provides a species-specific tool that could be used in experimental studies to define optimal dimensions of pellets, and later inform routine adjustments of formulated feed pellet size optimal to the size of the lobster. Differences in mouthpart morphology through ontogeny included an increase of calcification and robustness, which likely reflects a different requirement in the texture (softness/hardness) of the pellet as the animals develop. Spines and setation are a prominent feature of the mouthpart morphology and differ between species and during ontogeny. Spines on maxillipeds III and II and crista dentata on maxillipeds III are likely to play an important role in holding and manipulation of feed before ingestion. Hence, pellet texture will probably need to accommodate spinal puncturing and grasping in a species and age specific manner. Ontogenetic changes in setation were found, with much richer setation proportionally to the size of mouthpart present in first instar juvenile lobsters as well as species-specific differences. This indicates shifts in functions of particular mouthparts at different life stages which likely implies differences in filter feeding with age and between species. This is unlikely to be of high importance in pellet feeding development but could represent an avenue of investigation for the difficult first feeding juvenile stage. Collectively, the differences during ontogeny and between species suggest that formulated feeds may need to tailor to species and life stage, particularly regarding pellet dimensions and texture. Morphological investigations allowed for the establishment of biologically coupled feed pellet diameters in two experiments which examined the amount of feed waste produced by *S. verreauxi* juveniles in relation to pellet diameter, length and texture. The study examined the relationship between feed waste composition and feeding efficiency by juvenile spiny lobster, *S. verreauxi*. Lobsters were fed seven different

pellet diameters in each of two tested textures (hard and dry, HDP; soft and moist, SMP) or seven pellet lengths (HDP only), in two separate experiments. After feeding lobsters at 0.5% BW for 6 h, feed waste was collected and categorised as either feeding related waste (FRW), which was resulting from manipulation and maceration or non-feeding related waste (NFRW), which was uneaten/unmanipulated feed. In all tested feeds, particularly HDP, increasing pellet diameter or length corresponded in an increase of FRW and decrease of NFRW resulting in no difference in total feed waste in any treatment investigated. Thus, even if there was a clear improvement in feeding efficiency with small feed dimensions (i.e. low FRW), feed intake was not improved. Feed leaching rate decreased with pellet size indicating more rapid decline in feed attractiveness for smaller pellets. The findings indicate a counteractive interaction exists between pellet size and feed attractiveness and suggests that improved sustained feed attractiveness would further enhance feeding on small pellets. Future research should aim at optimising feed dimensions simultaneously to support efficient feeding whilst enhancing prolonged attraction/gustatory stimulation. The sensory stimuli including olfactory, visual, acoustic and tactile, are important aspects of feed exposure and the optimisation of feeding schedules. Feed attractiveness and availability are key drivers for maximising feed intake by juvenile lobsters. These aspects were investigated in a study focussed on the effect of feed frequency on survival, growth and behaviour of early-stage (instars 2-6) juvenile *P. ornatus*. The lobsters were exposed to one of six feeding frequencies with either one, two, four, eight, sixteen and thirty-two feed supplies per day with overall daily ration the same between treatments. The study showed that increasing feed frequency from one to around a maximum of sixteen feeds per day, improved lobster growth by increasing feed intake. Growth and feed intake maximisation at 16 feeds per day is

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Collectively, this thesis exposed the behavioural and morphological complexity of juvenile spiny lobsters in context of commercial production systems and formulated feed intake. Findings include the importance of gregariousness and physical interactions for growth, and a convoluted interaction between feed intake, growth rate and cannibalism. This thesis highlights the importance of further work to better understand and resolve the apparent negative consequence of growth (cannibalism) for the development of spiny lobster aquaculture. The morphological investigation relating to pellet dimensions showed that the feed shape should be adapted to the size of lobster. However, it has to be considered in context of the wide behavioural repertoire, affecting feed intake in spiny lobsters. The importance of feed attractiveness and availability highlights importance of even subtle improvements to chemosensory and visual exposure of feeds.

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Chapter 1 General introduction

1.1 Introduction

Spiny lobsters (Family; Palinuridae) are amongst the world's most valuable seafood commodities with growing market demand. The fishery is the main source of lobsters, with global capture value above US \$3.8 billion in 2018 (FAO, 2020). The aquaculture of juvenile lobsters has been undertaken in South-east Asia since the 1990s, using wild caught seed (puerulus and early juveniles) (Radhakrishnan, 2015; Smith, 2017). These grow-out systems are fed with "trash fish" or "low valued fish", including snails, mussels, clams, crabs, lizardfish and red big-eye (Hung et al., 2010); and feeding practices have remained unchanged for the last two decades (Perera and Simon, 2015). This unpredictable diet is nutritionally imbalanced and deficient, and in consequence may cause nutritional problems, leading to poor feed conversion ratio (FCR), which is currently estimated at 17-30 (Lai Van and Le Anh, 2009; Priyambodo, 2009). In the attempt to satisfy lobster nutritional needs, an overabundance of feed is commonly supplied to sea-cages leading to large amounts of uneaten feed, which along with lobster faecal and urinary wastes, affects the environmental quality of surrounding waters and marine habitat (Priyambodo, 2009). The formation of sediment in the areas of lobster farming also creates a favourable environment for the growth of pathogenic bacteria. The consequences are severe negative ecological changes, including reduction of the oxygen concentrations in bottom waters and anoxic sediments which results in unfavourable conditions for the aquaculture of the lobsters and increases the risks of diseases (Lai Van and Le Anh, 2009; Vinh and Huong, 2009). These current lobster aquaculture practices are not sustainable since they exploit natural resources for seedstock and feed, have negative impacts on the environment and are unreliable for aquaculture, in context of lobster health and

production (Bell, 2004; Ngoc et al., 2009; Williams, 2004). Consequently, there is considerable interest in developing sustainable spiny lobster aquaculture. A key objective of sustainable aquaculture is the development of closed cycle aquaculture systems, consistent with global goals to decrease reliance on wild capture as a source for aquaculture seed as outlined in the 2030 Agenda for Sustainable Development and the Sustainable Development Goals (SDGs), (FAO, 2016). This agenda aims to preserve wildlife and limit impact on wild ecosystems caused by providing food for human consumption. The attempts to develop closed cycle aquaculture for spiny lobsters have been hampered by difficulties in rearing the larvae and early juveniles. Recent breakthroughs in spiny lobster larval culture at Institute for Marine and Antarctic Studies (IMAS), has made the mass production of juvenile seed stock possible (Carter et al., 2014; Fitzgibbon, 2010; Fitzgibbon et al., 2012; Fitzgibbon et al., 2014; Jensen et al., 2013a; Jensen et al., 2013b; c; d; Simon et al., 2012). These advances have made sustainable lobster aquaculture a step closer. However, a next critical step in the development of a sustainable industry is refining technologies for mass aquaculture of the early juvenile stages including the development of effective formulated feeds and efficient feeding strategies.

While there are developed formulated feeds and strategies in other commercial aquaculture crustaceans, including shrimp and crabs, poor performance remains an obstacle for aquaculture development in spiny lobsters (Perera and Simon, 2015). This indicates that current lobster feeds and strategies remain inadequate to their behaviour and morphology. The formulated feeds dimensions, texture and attractiveness remain yet to be established and need to mitigate persistent problems, including unsuitable feed attractiveness resulting in short or lack of motivation to feed and the production of high amounts of feed waste (Irvin and Williams, 2009b; Jones

and Shanks, 2009; Williams, 2009). The holistic understanding of behavioural and morphological aspects of feed intake may be a step towards successful spiny lobster aquaculture.

1.2 Distribution and lifecycle

Panulirus ornatus and *Sagmariasus verreauxi* are excellent candidates for aquaculture. *Panulirus ornatus* is characterised by rapid growth, a relatively short larval development phase and is one of the fastest growing *Panulirus* species, which can reach up to 1 kg (10.5 cm CL) in 20 months of culture (Holthuis, 1991; Kenway et al., 2009; Williams, 2007; Williams, 2009). *Sagmariasus verreauxi* is relatively robust species, which is highly gregarious in nature and can withstand wide range of temperatures (Crear et al., 2000; Fitzgibbon et al., 2017; Jensen, 2012; Radhakrishnan et al., 2019b). *Panulirus ornatus* is distributed through most of tropical and subtropical Indo-Pacific, however the density of occurrence over this range is variable (Chakraborty and Radhakrishnan, 2015). Areas with highest density in Australia are the tropical Queensland east coast and Torres Strait (Kenway et al., 2009). *Panulirus ornatus* prefers shallow water habitats, up to 8 m deep and can be found on various substrates such as sand, muds, rocks and coral reefs (Holthuis, 1991). *Sagmariasus verreauxi* is distributed in the temperate Indo-West Pacific in the north of New Zealand and Australia (from southern Queensland to Tasmania) (Holthuis, 1991). This species can be found in depths from 0 to 155 m, on sand, gravel and rocks (Holthuis, 1991). Long-distance migrations can be observed in both species, however when not in mass migration, lobsters reside in shelters and migrate short distances in small groups, in search for food and shelter suitable for their increasing body size (George, 2005; Skeer et al., 2020). Both species have a complex life cycle,

which includes 11 and 17 larval (phyllosoma) stages in *P. ornatus* and *S. verreauxi* respectively (Jensen, 2012; Johnston et al., 2008; Ventura et al., 2015). The larval stages of *S. verreauxi* is punctuated by single moults (Kittaka, 1997), however *P. ornatus* can experience variable numbers of moults within each larval stage (Smith et al., 2009b). Each larval stage can be defined as achieving specific morphological development whereas instar can be described as a growth moult. Final stage phyllosoma metamorphosis to the pueruli, which is a non-feeding larval stage that more resembles juvenile lobsters. Puerulus migrate from beyond the shelf break, to coastal settlement areas, where the juvenile lobsters can find suitable habitats and start feeding (Fitzgibbon et al., 2014).

1.3 Behaviour

Spiny lobsters have very wide behavioural repertoire, which is subject to shifts during their complex life cycle (Childress and Herrnkind, 1994). Major behaviours including feeding behaviour and preferences, choice of habitat, cannibalism, gregariousness, mass migrations, queuing, learning abilities, shelter location and defence against predators can all change through ontogeny (Carter et al., 2014; Childress and Herrnkind, 1996; Radhakrishnan et al., 2019b; Thomas et al., 2003; Tuzan et al., 2019). After hatching, the phyllosoma are planktonic and move with ocean currents, the non-feeding puerulus is nektonic and migrates towards shore for settlement and finally the juvenile lobsters progress through ontogenetic shifts from early juvenile to the adult stage. Early juveniles are vulnerable to the predation due to their small size and typically live solitarily and feed in areas providing uninterrupted shelter, including macroalgae (Childress and Herrnkind, 1994). With increasing size and robustness, lobsters become more nocturnal, hide in available shelters during daylight and are

active at night (Childress and Herrnkind, 1994). The sub-adult and adult lifestyle of spiny lobsters can include an equilibrium of gregariousness and aggression, with different levels of each between species. Aggressive encounters between conspecifics can include sweeping with antennae, grappling with pereopods or lunging, and retreat of a defeated competitor by moving backward by clapping the telson (Berrill, 1975). The consequences of a defeat may include losing shelter and the defeated lobster may walk away and start a queue of conspecifics. Lobsters are attracted to the queues, even when only one lobster is walking and this can include the winner of the fight (Berrill, 1975). Aggressive encounters are typically not observed in queuing, which involves cooperation between conspecifics and is beneficial by reducing drag and increasing chances of survival by group defence and dilution of risk (Dolan III and Butler IV, 2006). When in shelter, lobster response to predators varies depending on the attacker. When attacked by octopus, lobsters often abandon shelter but remain in shelter when attacked by triggerfish (Weiss et al., 2008). The abandonment of shelter during octopus attack may be motivated by either the ability of octopus to extract lobsters from shelter or the fact that octopus and lobsters prefer similar shelters types (Gristina et al., 2011). Hence, abandoning shelter by a lobster may motivate octopus to stay and discontinue the pursue. The learning abilities of spiny lobster is likely a critical factor influencing life success. For *Palinurus elephas*, the exposure to a predator (*Octopus vulgaris*) threat can alter subsequent defensive strategies, including avoiding areas and shelters with predator cues (Gristina et al., 2011). Clawed lobsters, *Homarus americanus*, use urine signals to recognise their previous opponents and react as dominants or subordinates depending if they won or lost a previous conflict, respectively (Karavanich and Atema, 1998). Similarly, dominant *P. argus* release frequent urine signals to advertise and maintain their social

status (Shabani et al., 2009). Lobsters can also learn to adjust their behaviour to gain more feeding opportunities, *P. argus* was shown to increase their daylight activity when exposed to daytime feeding and would learn to feed directly from the hand of the diver supplying feed (Lozano-Alvarez, 1996). Understanding behavioural shifts and complexity through ontogeny will be crucial for the development of effective spiny lobster aquaculture systems including stage and species-specific feeds and feeding regimes.

1.3.1 Communication

The behavioural complexity in spiny lobsters is orchestrated with multiple communication paths, including chemical, hydrodynamic, acoustic, visual, tactile and their combinations (Childress et al., 2007). Chemical communication is a prevalent path for multiple behaviours, including messaging about position in hierarchy, recognition of conspecifics, mate detection, aggregation and avoidance, shelter location, migrations and feeding (Aggio and Derby, 2010; Hernández-Prior et al., 2020). Crustacean chemoreceptors are widely distributed on the body and in general can be divided into olfactory and distributed chemoreception (Derby and Weissburg, 2014). The chemical signals or cues are messages used in bilateral communication (signals) or be informative for the receiver (cues) and are expressed with responsive behaviour. These respectively may include pheromones in mate search and chemicals emitted by injured or attacked animal (Derby and Weissburg, 2014). After identification of the chemosensory stimuli, the behavioural response varies depending on the chemicals (Corotto et al., 2007; Hernández-Prior et al., 2020). Other paths, including visual, acoustic and mechanical have limitations, including constant physical values and can be easily disrupted by surrounding environment (Thiel and Breithaupt, 2010).

With chemical communication, an organism can create wide range of specific messages directed to conspecifics (Derby and Weissburg, 2014). Recent findings however indicate the importance of acoustic communication for lobsters, which can be detected over long distances (Jézéquel et al., 2020). The spiny lobsters analysed in this thesis belong to two evolutionary lineages distinguished by acoustic capability: Stridentes (*P. ornatus*) and Silentes (*S. verreauxi*) (Daning Tuzan, 2018; Jeffs et al., 2013; Staaterman et al., 2010). The Stridentes (*P. ornatus*) produce loud sounds using the plectrum and file located at the base of the antenna (Patek, 2001). The loud rasps are emitted in situations such as predator attack and can be produced even by soft shelled post-moult lobsters (Buscaino et al., 2011; Patek, 2001). The ultrasound signals (screech) are commonly used in non stressful situations, indicating possibility of communication with conspecifics (Buscaino et al., 2011). Tactile sensory anatomy is widespread on the lobster body but apart from the unimodal chemosensilla located on antennular lateral flagella, all other known chemosensilla have mechanosensory functions (Cate and Derby, 2001; Derby et al., 2001). Bimodal sensilla are distributed on antennae, mouthparts, pereopods, cephalothorax, abdomen and telson (Derby, 2021; Derby et al., 2001; Tidau and Briffa, 2016). The tactile contact is important in defence techniques, by detecting water currents created by large predatory fish (Wilkins et al., 1996). For juvenile lobsters in culture, direct tactile contact is thought to be important component of growth and feeding stimulation (Marchese et al., 2019; Vijayakumaran et al., 2010). The mass migrations of spiny lobsters are a good example of their behavioural complexity and importance of the mechanical communication path. Migrations include queuing where lobsters are in physical contact with conspecifics, which reduces hydrodynamic drag and aid with migration (Kanciruk, 1978). Tactile and visual communication is a component of mate selection,

in estimation of potential mate size (Raethke et al., 2004). Furthermore, the visual aspects of conspecific interactions include threatening displays by elevating position and presenting appendages (Hernández-Prior et al., 2020). Spiny lobsters have ability to discriminate colours and show preference and avoidance to particular colours (Lesmana et al., 2021). It is also an important component of defence strategies when attacked by predators.

1.3.2 Gregariousness

Gregariousness is behaviour which can be described as non-random aggregation and is observed with variable levels in different spiny lobsters species (Behringer and Butler IV, 2006). It includes attraction of individual to a group, caused by the group properties (Cobb, 1981). Different levels of gregariousness may lead to variability in densities of spiny lobsters in nature. Lobsters are attracted to conspecifics and when a strong odour is emitted by large group, social lobsters can aggregate in higher numbers, irrespective of shelter capacity (Behringer and Butler IV, 2006). The correlation between shelter availability, gregariousness and aggressive encounters is complex (Berrill, 1975). Aggressive behaviours are important in social groups, as tool for defence, resources protection and hierarchy formation (Briones-Fourzán et al., 2015; Huber and Kravitz, 2010). Lobsters often display short aggressive contacts with conspecifics, specifically when seeking to occupy or defending their place in shelter (Berrill, 1975). The balance between gregarious and aggressive encounters may be manipulated to certain level with feed availability (Thomas et al., 2003). In *Jasus edwardsii*, the increase of daily feed supplies and ration size, decrease aggressive behaviours and competition for feed (Thomas et al., 2003). Social interactions are also an important component in spiny lobster growth optimisation. In the study of Tuzan et

al. (2019), *S. verreauxi* juveniles with either high or low metabolic rate showed that the best growth was in communal culture, suggesting prevailing role of social interactions in growth improvement. Similarly, juvenile *P. ornatus* reared communally grow faster than in individual culture (Irvin and Williams, 2009a; Marchese et al., 2019; Ratunil Jr, 2017). The growth increase was driven by conspecific interaction and feeding competition in communal group (Irvin and Williams, 2009a; Marchese et al., 2019; Ratunil Jr, 2017). Cannibalism in communal culture and nutrition from consumed conspecifics, could be another explanation of growth improvement (Irvin and Williams, 2009a). In sub-adult *P. homarus*, close tactile contact between conspecifics was suggested to increase growth and moulting frequency in communal group (Vijayakumaran et al., 2010).

Gregarious cooperation is also exhibited in lobster mass migrations, which require aggregation and orderly queuing. When a queue is disturbed by a predator, some spiny lobster species can form a rosette, with lobsters coordinating a protective ring of antennae (Briones-Fourzán et al., 2006; Kanciruk, 1978). Spiny lobsters rich chemical sensory also allows for detection of diseased conspecifics. In these circumstances lobsters do not show gregariousness and avoid diseased conspecifics (Behringer et al., 2006). Gregariousness and conspecific interaction is thus an important aspect of spiny lobster life which has been shown to both be affected by culture conditions and in turn influences growth performance. Understanding the roles and shifts of gregarious behaviours through ontogeny is an important consideration in the development effective formulated feeds and feeding regimes for lobster aquaculture.

1.3.3 Cannibalism

Cannibalism is a significant impediment for spiny lobster aquaculture, particularly

during the early juvenile stages where it can severely impact survival rates and productivity (Romano and Zeng, 2017). Cannibalism in crustaceans is known to be a complex behaviour that may be caused by multiple factors which can vary between species and life stages (Romano and Zeng, 2017). In natural environments, cannibalism is considered an adaptation for times of feed deficiency (Romano and Zeng, 2017). Cannibalism in culture has previously been investigated in a few studies, with conflicting results. Irvin and Williams (2009a) suggest that cannibalism in *P. ornatus* was opportunistic and particularly occurred with vulnerable individuals that took longer than 30 min to moult. In this study, long or failed moulting was suggested to be an artifact of an unknown nutritional deficiency (Irvin and Williams, 2009a). Chau and Ngoc (2009) found that availability of shelter was important for increasing survival and reducing cannibalism and suggested a link between physical exposure of vulnerable individuals to the dominant individuals. However, Ratunil Jr (2017), showed no difference in *P. ornatus* survival between lobsters reared individually or communally and suggested that unspecified nutritional deficiency and water quality may cause mortality in both culture types. Likewise, Vijayakumaran et al. (2010) found similar survival in *Panulirus homarus* reared in either individual or communal cultures. These studies reported the presence of cannibalism but do not show a direct link of this behaviour to survival. In the study by Jones et al. (2001), *P. ornatus* exposed to different densities showed similar survival. These authors suggested that cannibalism of post-moult specimens was a main reason of mortality and that an inappropriate feeding schedule could have contributed. In this study, feed availability was limited before and at dawn, which was suggested to contribute to motivation for cannibalism (Jones et al., 2001). These studies highlight the complexity of cannibalism and its potential impact on spiny lobster aquaculture. Gaining an improved understanding of

the mechanisms stimulating this behaviour will provide valuable insight into potential methods to mitigate this behaviour in culture.

1.3.4 Feeding behaviour

The feeding behaviour in spiny lobsters includes detection and recognition of odours, decision whether to search for the source, location and approach to the feed (Derby et al., 2001). The senses used in feeding behaviour utilise chemoreceptors located on antennules, legs and mouthparts (Derby et al., 2001). Antennular chemoreceptors function in the search and orientation toward source of stimulus. Chemoreceptors located on legs and mouthparts are more responsible for deciding if it will be consumed (Derby et al., 2001). Lobster feeding behaviour also includes mechanosensory in detection of hydrodynamic cues from potential prey (Derby et al., 2001). This complexity allows lobsters to find, catch and ingest feed in the aquatic environment. In aquaculture, formulated feeds and feeding schedules for spiny lobsters need to capitalize on all sensory pathways to promote maximum feed consumption. The attractiveness of the feed is a first critical step to utilising chemosensory pathway to promote feeding. Feeding schedule is another important aspect, which when optimal, may concurrently improve exposure to feed when most attractive. Moreover, it may increase feeding opportunities to both dominant and submissive lobsters.

In culture, the feeding behaviour of spiny lobsters is more complex than most other aquaculture species because feeding is often delayed and crustaceans macerate feeds instead of swallowing pellets whole (Simon, 2009). Lobsters “messy feeding” is due to external handling and maceration of pellet diets which often leads to the total breakdown of dry formulated pellets and up to 50% food wastage (Sheppard et al., 2002). Experiments examining juvenile *J. edwardsii* confirmed that feed pellets

dimensions, consistency and fragmentation characteristics are significant factors affecting efficient feeding (Sheppard et al., 2002). Cox et al. (2008) suggested that significant feed loss can be caused by inappropriate feeding relative to shifts in feeding behaviours and mouthparts development through ontogeny, indicating that feed formats should be tailored for species and life stage. Furthermore, these behavioural and mechanical aspects of lobster feeding can complicate typical assessments of formulated feed quality including measurements of nutrient ingestion (such as apparent feed intake) and the evaluation of the feeding efficiencies (such as feed conversion ratios). These complications highlight the importance of formulated feeds format, in context of lobster species, feeding behaviour and ontogeny. The establishment of assessment method to adjust formulated feed to the lobster species, behaviour and ontogeny may improve feeding in culture.

The natural behaviour of spiny lobsters is to seek shelter, when disturbed lobsters retreat and stay hidden (Davidson et al., 2002). This cryptic nature makes it difficult to perform casual observations, and thus it is difficult to gain an understanding of the feeding response and behaviour. Previous video observation studies, demonstrate the value of observational work for assessing the appropriate feed form in the development of artificial diets for spiny lobsters at differing life stages (Sheppard et al., 2002; Smith et al., 2009a).

The analysis of feeding schedules and ration size in context of feeding behaviour, is an important component in further development of aquaculture. In *J. edwardsii*, the increase of feeding ration to excess and frequent supplies (4 day^{-1}) resulted in a decrease of aggressive encounters with conspecifics and competition for feed (Thomas et al., 2003). The feeding behaviour in juvenile *P. ornatus*, was influenced by feed type with levels of lobster interactions differing between tested feeds which

was related to growth performance (Marchese et al., 2019). These studies highlight lobster behavioural complexity, which can be manipulated to advantage using different feed types and schedules. Finding the optimal feed types, ration sizes and schedules adequate to the behaviour, is likely to be the important step towards spiny lobster culture.

1.4 Feeding morphology

Anatomical changes of lobster feeding morphology with growth suggests variability in the feed preferences and behaviour. In nature, lobsters are known to prey on feeds of differing texture and size with development which likely reflects changes to their feeding morphology. The early juvenile stages mostly feed on soft and pulpy feeds (Cox et al., 2008). With development, their increasing size and robustness of appendages allows for extension of their diet to include larger and tougher prey and even armoured prey (Juanes and Hartwick, 1990).

Observational studies can provide important insight into dietary preferences particularly for life stages. Mouthpart morphology of juvenile lobsters has been shown to change through early development, which corresponds with ontogenetic changes in formulated feed preference and performance. Differences in feeding behaviour and morphology between lobster species suggest that formulated feeds need to be tailored for the specific species as well as life stage. Cox et al. (2008), described mouthparts in juvenile *P. argus* from pueruli to adult and demonstrated progressive development of density, robustness, pigmentation and calcification during ontogeny and that these morphological and behavioural changes are reflected in choice of prey. Behavioural observations showed mouthparts moving in sequence and functions when feeding and grooming activities were observed when the feed was not accessible. Smith et al.

(2009a) compared *P. ornatus* feeding behaviour and morphology between three different growth stages. Their findings confirmed that soft diets are more efficient in early juvenile animals and analysis of pellet size showed that diameter is the most important dimension and should suit the lobsters mouth aperture. These studies indicate importance of formulated feeds format, including texture and dimensions in context of lobster species, feeding behaviour and growth stage. The lobster ontogeny is an important aspect to consider in formulated feeds development (Cox et al., 2008).

1.5 Formulated feeds for spiny lobsters

Essential component of success in animal rearing is nutritionally suitable and economical feed (Vijayakumaran, 2004). Despite considerable research effort, poor performance on formulated feeds remains a major obstacle to commercial spiny lobster aquaculture (Perera and Simon, 2015). Much of the previous nutrition research on lobsters has focussed on assessing performance of formulated feeds and dietary requirements of lobsters based on the feed nutrient composition. However, this assessment assumes that the feed nutrients are bio-available to the lobster and there is growing evidence that many nutrients presented in formulated diets, may not be effectively ingested or digested by spiny lobsters (Perera and Simon, 2015).

Francis et al. (2014) suggested that the moisture content is an important parameter to consider in phyllosoma feed composition, because it mimics natural prey. Early juvenile lobster have been shown to choose soft and pulpy feeds, however; feed preferences change throughout development (Cox et al., 2008). Early juvenile lobsters prefer small and soft feeds, while late juveniles prefer larger and armoured prey. The different juvenile stages show variable capabilities for feed handling which for a formulated feed to be effective should be reflected in format characteristics, such as

size, shape and texture. Key in the assessment of formulated diets is the consideration of the unique feeding behaviours and anatomy of spiny lobsters in aquaculture.

Chemical attraction is a vital part of feed intake in lobsters, attractive substances are detected with antennules and all body areas and stimulate perception, identification, searching and ingestion (Derby and Atema, 1982b; Derby et al., 2001; Kozma et al., 2018). Free amino acids and organic acids were indicated as substances stimulating feed intake in lobsters (Derby and Atema, 1982b; Derby and Atema, 1988; Williams, 2007; Zimmer-Faust et al., 1984).

Gaining a holistic understanding of feeding will provide valuable knowledge required for the development of the effective formulated feeds and feeding strategies.

1.6 Feeding schedules

Feeding behaviours can greatly impact the degree of feed or nutrient losses arising from poor feed stability and wastage. Leaching is dependent on a range of factors including the dry matter water stability of the feed, levels of water-soluble nutrients, and the time spent in the culture water before consumption (Marchetti et al., 1999; Palma et al., 2008). The timing of the feeding response is thus an important consideration in the development of formulated feeds (Marchese et al., 2019; Tolomei et al., 2003). Timing of the feed response can be influenced by numerous factors including attractiveness, form, feeding regularity and appetite revival of the lobster at particular life stages (Tolomei et al., 2003). At present we have little understanding about the timing of feeding responses and the duration that feeds remain attractive and nutritious to juvenile lobsters. By understanding the feeding response of juvenile lobster on differing formulated feeds and under differing feeding schedules, it may be possible to optimise various aspects of the feed formulation and feed management

strategies to limit nutrient loss resulting from leaching and wastage.

Feeding schedule can have significant influence on growth of lobsters. Research on *J. edwardsii* showed optimal growth when juvenile lobsters were fed once daily, after dusk in excess (Thomas et al., 2003). Also it was demonstrated that feeding once daily, at dusk, provides improvement in growth in juvenile *P. argus* (Cox and Davis, 2006). In contrast, the conclusion from study on juvenile *Panulirus versicolor* indicated that feeding lobsters more than once per day may improve growth but further research is required (Syafrizal et al., 2018). The investigation of feeding schedules should include lobsters circadian rhythm, which is influenced by photoperiod, feed availability, presence of conspecifics and predators (Zenone et al., 2020). Hence an improved understanding of the impacts of feeding schedule in lobster culture will likely provide valuable information for effective feeding practices to maximize feeding and minimise feed wastage. The inclusion of feed leaching analysis will provide information about the optimal time within which the feed keeps its nutritional value. This parameter will be an important aspect of feeding schedule analysis as it allows the assessment of an effective feeding schedule from the animal behaviour and feed quality point of view. Gaining an understanding of lobster feeding response and behaviours can therefore provide important insights for assessing appropriate feed formulations and the influence of feeding schedules on dietary nutrient intake.

The holistic understanding of behavioural and morphological aspects of feed intake and their relationship in spiny lobsters, will add knowledge to the development of aquaculture. The adjustment of formulated feeds characteristics, including dimensions, texture and attractants content, suitable to the spiny lobster needs may improve culture performance and decrease levels of feed waste produced. The wide approach of this thesis will add knowledge of spiny lobster behavioural complexity in general.

1.7 Aims of the thesis

This thesis focusses on two species, *P. ornatus* (tropical rock lobster) and *S. verreauxi* (eastern rock lobster), which have been identified as viable Australian lobster species for closed cycle aquaculture due to favourable biological characteristics and high market value and demand (Fitzgibbon et al., 2017; Smith et al., 2009b).

This thesis aims to investigate the feeding behaviour in combination with morphological factors influencing nutrient intake in spiny lobsters, with a focus on the early juvenile stages. The thesis also explores general behaviour, with an emphasis on activity and cannibalism. A key objective of the thesis is to provide information that will support the development of economical and effective formulated feeds and feeding strategies for juvenile lobsters. The holistic understanding of behaviour and feed intake will also aid in the development of commercial production systems for the juvenile culture of lobsters, which remain a significant bottleneck for aquaculture.

The aims of this thesis are to:

- Examine the role of conspecific interaction either through physical contact or visual and chemical cues, on growth, survival and feeding behaviour of early juvenile *P. ornatus*. Through the examination of three culture types (isolated culture, eliminating all interactions; separated culture, eliminating physical interactions; and communal culture, allowing all interactions) the influence of particular conspecific interactions will be investigated in context of optimal growth and survival, important to aquaculture (Chapter 2).
- Investigate the ontogeny of mouthpart morphology of *P. ornatus* and *S. verreauxi*, through the analysis of early juvenile, juvenile, sub-adult and adult

stages. To describe the mouthparts, measure mouth aperture and compare the two species in the context of providing insight into suitable formulated feed formats for aquaculture (Chapter 3).

- Examine the influence of formulated feed pellet dimensions (diameter and length) and texture (hard and soft) on feed wastage in juvenile *S. verreauxi* and to determine optimal dimensions and texture to minimise wastage (Chapter 4).
- Determine the influence of feed frequency on growth, survival, feeding behaviour, feed waste and overall nutrient intake in juvenile *P. ornatus*, to provide insight into the optimal feeding schedules for spiny lobster aquaculture (Chapter 5).

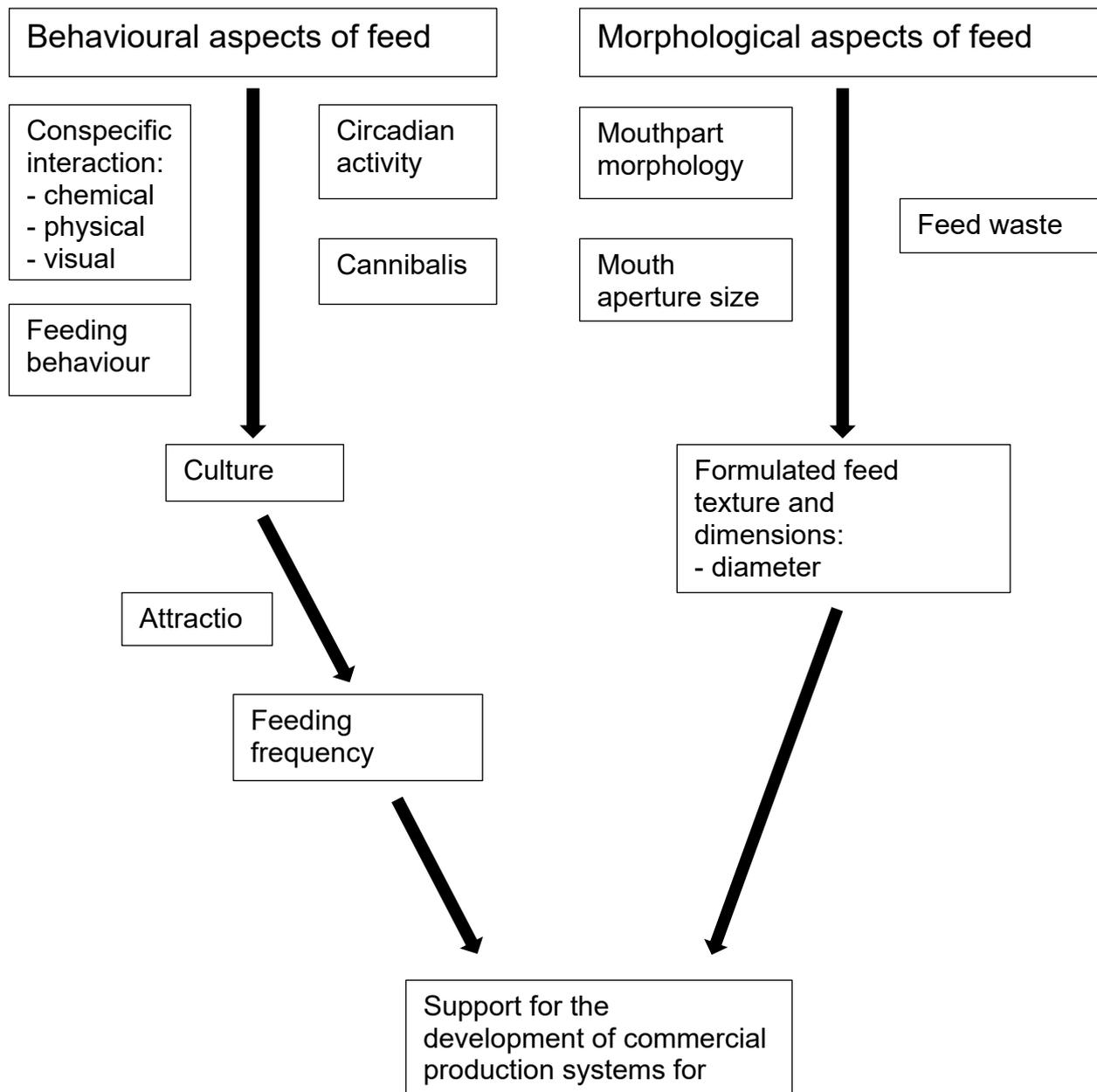


Figure 1.1. Logic flowchart of the Thesis experimental design.

Chapter 2 The effect of conspecific interaction on survival, growth and feeding behaviour of early juvenile tropical spiny lobster *Panulirus ornatus*

Abstract

Behaviour underpins many facets of the performance of animals in aquaculture. By manipulating culture systems to segregate or allow particular aspects of conspecific interaction, we found physical interactions between *P. ornatus* individuals to be essential for better culture performance. Three culture types were used to control conspecific interactions: isolated culture (individual vessels) excluded all conspecific interactions, separated culture (lobsters cultured in adjacent cages) excluded physical interactions, and communal culture allowed for all interactions. Two water exchange rates were introduced to investigate the influence of chemical cue intensity on growth and survival. Time-series photography was used to determine the feeding behaviour and preference for different feeds including mussel gonad, commercial prawn feed and moist feed. The experiment showed improved growth and moulting frequency in communally cultured lobsters. These results suggest that direct physical contact between conspecifics is required to optimise growth of lobsters, which may be related to the complex social structures of this gregarious species. Behavioural observations of two juvenile instars (2 and 4), revealed circadian rhythm of interactions with feeds, feed preferences and intake. Observations revealed differing behaviours between the different culture types, where lobsters reared in separation displayed higher level of interactions with feeds; however this was not associated with higher feed intake. Observations of two juvenile instars (2 and 4) exhibited increase of daylight activity (interactions with feeds and feed intake) in older lobsters (instar 4).

2.1 Introduction

Spiny lobsters (family Palinuridae) display a wide range of social behaviours in various contexts. These include complex social interactions such as avoiding diseased conspecifics, den sharing, non-random aggregation in open areas, mass migrations, and establishment of social hierarchies (Candia-Zulbarán et al., 2015; Childress et al., 2007; Shabani et al., 2009; Zimmer-Faust and Spanier, 1987). Non-random aggregation, known as gregariousness is observed in most spiny lobsters and is expressed with variable intensity in different species, but overall spiny lobsters are considered a social taxa (Atema and Cobb, 1980; Behringer et al., 2006). The benefits of being gregarious are varied and include protection from predators, energy conservation by reducing drag in queuing during mass migrations, quicker shelter location by attraction to conspecifics in occupied dens (“guide effect”), increased reproduction opportunities, and shelter optimisation (Bill and Herrnkind, 1976; Childress et al., 2007; Childress and Herrnkind, 1998; 2001; Herrnkind, 1969). Such social behaviours are underpinned by several communications pathways, including acoustic, visual, tactile and hydrodynamic (current production), chemical and multimodal combinations of the aforementioned (Childress et al., 2007).

Understanding the role of lobster communication in social behaviours will be fundamental in the development of new aquaculture technologies, which are needed to expand the developing lobster farming industry. Spiny lobsters are fished in over 90 countries with a global capture value over US \$2.7 billion in 2015 (FAO, 2017; Phillips, 2006; Plagányi et al., 2018). Whilst fisheries are the primary source of lobsters, the practice of catching and culturing wild caught seed to market size has been undertaken since the 1990s, mainly in Vietnam, Philippines and Indonesia (Radhakrishnan, 2015; Smith, 2017). Current wild seed collection practices strongly

impact lobster ecosystems by exploiting natural resources (Bell, 2004; Ngoc et al., 2009). However to date, the commercial production of hatchery-reared spiny lobster seed has not been possible, due to difficulties in rearing lobsters through their extended larval phase (Dennis et al., 2004; Jeffs, 2010; Kenway et al., 2009; Kittaka and Booth, 2000; Lai Van and Le Anh, 2009; Smith, 2017; Thuy and Ngoc, 2004). Significant research effort has focused on the development of hatchery technologies for spiny lobsters (Fitzgibbon and Battaglione, 2012; Fitzgibbon et al., 2012; Fitzgibbon et al., 2017; Francis et al., 2014; Jensen et al., 2013a; Kittaka, 1988; 1994; Kittaka and Ikegami, 1988; Kittaka et al., 1997). At the Institute for Marine and Antarctic Studies (IMAS) in Australia, spiny lobster hatchery research has recently been focused on the tropical spiny lobster *P. ornatus*, which is one of the largest species in the genus and can grow to 1 kg in 20 months of post-larval culture (Holthuis, 1991; Kenway et al., 2009). Significant progress has been made in the hatchery production of *P. ornatus* seedstock enabling large numbers of juveniles to be reared from egg, suggesting that commercial closed-cycle spiny lobster aquaculture may soon be possible. Juveniles cultured from wild seed collection often suffer high mortality and similar losses using hatchery seed would likely represent a major impediment to commercial productivity. Mortality during culture of early juvenile stages has been attributed to cannibalism, and also a lack of post-larval fitness (Chau and Ngoc, 2009; Irvin and Williams, 2009a). The development of systems to limit the impacts of cannibalism and optimise early juvenile survival and growth will be crucial to the successful development of closed cycle *P. ornatus* aquaculture (Smith, 2017).

As in most crustacean species, cannibalism in spiny lobsters typically occurs during and shortly after moulting (Jones et al., 2001; Romano and Zeng, 2017; Thomas et al., 2000). Previous studies have attempted to decipher the impact of cannibalism on

growth and survival by culturing animals communally and individually. Three studies used communal and individual cultures in testing growth and survival of juvenile *P. ornatus*. Irvin and Williams (2009a) and Ratunil Jr (2017), both used communal and individual culture to exclude tactile communication by keeping lobsters separately in adjacent cages. These experiments showed significantly improved growth in communal culture and no significant difference in survival between the culture types. The third study on *P. ornatus*, performed by Marchese et al. (2019), investigated two culture types, individual, where lobsters were held in unconnected vessels eliminating all communication paths (isolation), and communal. The growth was improved in animals held communally. Marchese et al. (2019) surmise that growth increase is an outcome of social interactions with related stimulation of feeding response. Similarly, Vijayakumaran et al. (2010) analysed influence of individual (separated in adjacent cages) and communal culture on growth and moulting of sub-adult *P. homarus*. The growth and moulting frequency was significantly higher in communal culture. Vijayakumaran et al. (2010), concluded that physical contact was important in inducing moulting and growth. However, no previous study has directly compared growth performance of spiny lobsters between communal, separated and isolated culture and thus the growth benefit of the differing conspecific communication channels remains unclear.

Like other complex behaviours observed in spiny lobsters, cannibalism is driven by multiple of communication paths, i.e. chemical, tactile, visual, acoustic and multimodal (Barki et al., 2010; Heldt, 2013; Romano and Zeng, 2017). Analysis of communication based on observations of conspecific interactions is likely to provide valuable insight towards understanding cannibalism. Further research is now required to better interpret the significance of cannibalism in the mortality observed in early juveniles,

and to examine the role of communication in the apparent differences in feeding and growth when animals are reared communally or separately.

Behaviour changes through lobster ontogeny, and is influenced by environmental factors including food accessibility, habitat types, shelter availability, presence and density of conspecifics and predators (Anger, 2001; Childress, 1995; Childress and Herrkind, 1996; Fox, 1975; Fürtbauer and Fry, 2018; Lipcius and Herrkind, 1982; Tidau and Briffa, 2016). During ontogeny, two shifts are important to aquaculture including the shift in levels of gregariousness from solitary to communal, and the concomitant shift in prey preference. Optimising the culture environment by understanding behavioural needs through ontogeny, is likely to improve growth, survival and productivity. Understanding shifts in gregariousness and changes in feeding preferences during early juvenile development, will be a valuable component of developing formulated feeds and appropriate feeding regimes, an important component of aquaculture economics.

This study aims to investigate the role of conspecific interaction either through physical contact or visual and chemical cues, in growth, survival and feeding behaviour of early juvenile *P. ornatus*. Three different rearing cultures allow or isolate particular conspecific interactions. Communal culture allows all conspecific interactions, separated culture (lobsters cultured in adjacent cages) excludes physical interactions, and isolated culture (individual vessels) excludes all interactions. The experimental cultures allowed for control and analysis of influence of cannibalism on survival. Two water exchange rates were assessed in an attempt to further investigate the influence of chemical cues intensity on growth, survival and behaviour of early juvenile *P. ornatus*. Time-series photography analysis incorporated behavioural observations

without disrupting the lobsters natural activity, in an attempt to present circadian rhythm of interactions with feeds and intake.

2.2 Materials and methods

2.2.1 Experimental animals

First instar juvenile *P. ornatus* were reared from egg at IMAS using procedures similar to those described by Fitzgibbon and Battaglione (2012); Fitzgibbon et al. (2012); Ikeda et al. (2011). Post metamorphosis to first instar, juvenile lobsters were kept communally in 18-L blue plastic vessels. One mini-Mills collector (a spherical mesh shelter of approximately 20 cm diameter (Mills and Crear, 2004)) was located in the centre of the vessel, and opaque tubes (diameter 2.5 cm) with mesh inside were placed on the bottom, as additional shelters. The seawater was obtained from River Derwent (Taroona, Tasmania), filtered, ozonated and used in flow through system. Water quality was maintained at $27.47\text{ }^{\circ}\text{C} \pm 0.07\text{ S.E.}$, $\text{pH } 8.16 \pm 0.03\text{ S.E.}$, salinity $34.62\text{ ppt} \pm 0.04\text{ S.E.}$, and dissolved oxygen $126.17\% \text{ sat.} \pm 1.25\text{ S.E.}$. Prior to stocking, animals were fed twice daily, with moist feed (IMAS commercial-in-confidence manufactured moist feed). Vessels were siphoned clean and uneaten feeds were removed before feeding.

2.2.2 Experimental design

A factorial design examined the effects of three types of animal rearing (isolated, separated and communal) and two water exchange rates (low flow; 6 exchanges h^{-1} , and high flow; 15 exchanges h^{-1}) on growth, survival and behaviour of emergent juvenile lobsters from first juvenile instar. The three animal rearing cultures are described below. Each treatment contained five replicates (Table 2.1.).

Table 2.1 Experimental rearing cultures design.

Rearing culture	Total number of animals	Treatment description	Water exchange rate	Number of animals	Number of replicates
Isolated	60	6 animals in individual isolated blue 3-L vessels	Low - 6 exchanges h ⁻¹	30	5
			High - 15 exchanges h ⁻¹	30	5
Separated	60	6 animals in individual 3-L perforated vessels within 35L vessel	Low - 6 exchanges h ⁻¹	30	5
			High - 15 exchanges h ⁻¹	30	5
Communal	60	6 animals in communal 18-L vessel	Low - 6 exchanges h ⁻¹	30	5
			High - 15 exchanges h ⁻¹	30	5

Lobsters were randomly stocked into experimental culture on the third day after they had moulted from a puerulus into the first juvenile instar (1), the day of the moult to instar 1 was noted as day zero. Following stocking, lobsters were acclimated for three days and subsequently the experiment was conducted for 49 days. Stocking animals into rearing vessels and completion of the experiment, was performed within three consecutive days.

2.2.3 Experimental cultures

The three rearing cultures examined were as follows:

- 1) Isolated culture: each of five replicate units comprised of six isolated 3-L blue plastic vessels (13.5 cm length x 13.5 cm width x 18 cm height), equipped with four mesh circles (9 cm diameter) on each wall to provide climbing substrate. Each vessel had an individual flow through water exchange system, to keep animals totally isolated from each other. A single lobster was maintained in each 3-L vessel.
- 2) Separated culture: each of five replicate units comprised of a 35-L blue plastic vessel, which contained six transparent plastic 3-L vessels similar to the isolated treatment (13.5 cm length x 13.5 cm width x 18 cm height). Each 3-L vessel was equipped with round mesh openings (9 cm diameter per one mesh opening) on each

wall, which allowed water to circulate between vessels and match the climbing substrate in the isolated treatment. Water flow through exchange system included an inlet in each transparent vessel, and two outlets draining from the bottom of the 35-L holding vessel. A single animal was maintained in each 3-L vessel. The culture allowed for visual and olfactory interactions between animals within each replicate.

3) Communal culture: each of five replicate units consisted of 18-L blue plastic vessel (38 cm length x 24 cm width x 24.8 cm height). The container walls were equipped with mesh rectangles on each wall (two rectangles on large walls, dimensions 37 cm x 15 cm, one on small wall, dimensions 23 cm x 15 cm and two on the opposite small wall, dimensions 8.5 cm x 15 cm), to match the climbing substrate per animal, as in the isolated and separated cultures. Each 18-L vessel had an individual flow through water exchange system consisting of one inlet and one outlet. Six animals were held communally in each 18-L vessel.

Rearing conditions were standardised regardless of culture treatment. The climbing substrate (mesh on vessel walls) combined with the walking area of the vessel bottom was uniform for all cultures and equalled 437 cm² per animal. Each animal rearing culture system was equipped with one round mesh shelter per lobster, (mini Mills collector, 10 cm in diameter) (Mills and Crear, 2004; Phillips et al., 2001). The available vessel volume was 3-L per animal in all cultures. Vessels in all cultures were equipped with transparent lids, placed above the water surface.

Each rearing culture (isolated, separated and communal), was examined at two water exchange rates 6 and 15 exchanges h⁻¹ (n = 5, Table 2.1). Light was provided in two spectra: 1) actinic fluorescent light of wavelength 420 nm (on for 10 h day⁻¹); and 2) red fluorescent light of wavelength 640 nm (on for 24 h day⁻¹). Red light was provided to satisfy the quality of the time-series photography for analysis. Red light does not

affect nocturnal activity of lobsters and thus the experimental photoperiod was 10:14 L:D (Endler, 1978; Fitzgibbon and Battaglione, 2012; Mills, 2005). The photoperiod at 10:14 L:D was in the range observed in natural environment of lobsters and was set to accommodate feeding and maintenance work in the experiment (Simon and James, 2007). The used photoperiod does not have negative impact on juvenile lobsters feed intake, growth and survival (Chittleborough, 1975; Crear et al., 2003). Light-phase intensity was $6.78 \mu\text{moles s}^{-1}\text{m}^{-2} \pm 0.11 \text{ S.E.}$ and intensity of red light during the dark-phase was $2.17 \mu\text{moles s}^{-1}\text{m}^{-2} \pm 0.05 \text{ S.E.}$, all light measurements were taken at the water surface. Seawater used in the experiment was filtered to a nominal $40 \mu\text{m}$ and ozonated to 750 mV prior to use. Water quality in the culture vessels was maintained at $27.67 \text{ }^\circ\text{C} \pm 0.03 \text{ S.E.}$, pH $8.15 \pm 0.00 \text{ S.E.}$, salinity $34.34 \text{ ppt} \pm 0.01 \text{ S.E.}$, and dissolved oxygen $9.10 \text{ mg L}^{-1} \pm 0.02 \text{ S.E.}$ ($115.82\% \text{ sat.} \pm 0.27 \text{ S.E.}$), slightly supersaturated due to ozonation. Water quality was recorded daily with a WTW Multiline 3430 meter.

2.2.4 Experimental feeds and feeding

All experimental animals were simultaneously fed in excess with three feed types: 1) gonad, finely chopped ($4\text{-}10 \text{ mm}^2$) blue mussel (*Mytilus galloprovincialis*) gonad; 2) pellet, Kuruma prawn pellets (Higashimaru, Tokyo); and 3) moist feed (IMAS feed noted above). Feed amounts per animal were calculated on a dry weight (DW) basis as specified in Table 2.2.

Table 2.2 DW (dry weight) in experimental feeds.

Feed	Weight of 1 piece (mg)	DW (%)	Pieces (n) feeding ⁻¹ individual ⁻¹	DW (mg) feeding ⁻¹ individual ⁻¹	Pieces (n) feeding ⁻¹ replicate ⁻¹	DW (mg) feeding ⁻¹ replicate ⁻¹
Pellet	15.16	0.8	1	12.13	6	72.8
Moist feed	20.5	0.15	4	12.3	24	73.8

Animals were fed twice daily, 30 min following the change in light cycle. Experimental vessels were siphoned clean and uneaten feed removed before each feeding. At the same time, survival and any moulting activity were recorded. Cleaning and feeding order was random among three culture types and ten replicates within each culture type. The individual randomisation sequence was prepared for each day of the experiment.

2.2.5 Growth and survival

Initial lobster carapace length (CL_0) and initial wet weight (WW_0) were measured on a separate subsample of fifteen juvenile instar 1 lobsters from the same cohort at the start of the experiment. Subsampled lobsters were rinsed in de-ionised water, blotted with paper towel to remove excess moisture and frozen in $-20\text{ }^{\circ}\text{C}$, for later analysis of initial dry weight (DW_0).

Upon completion of the experiment, all animals were blotted with paper towel, and weighed for whole body wet weight. Carapace length was measured using Vernier caliper, lobsters were then rinsed with de-ionised water and frozen at $-20\text{ }^{\circ}\text{C}$ for later analysis. Lobster samples were subsequently freeze dried and weighed.

Growth and survival variables analysed were as follows; juvenile survival (S), biomass gain (BG), productivity (P), final carapace length (CL_t), carapace length gain (CLG), final wet weight (WW_t), wet weight gain (WWG), final dry weight (DW_t), dry weight gain (DWG), specific growth rate (SGR) (Fitzgibbon et al., 2017; Simon and Jeffs, 2011; Zhang et al., 2016) and average moults per day (AMD), were calculated using the following formulae:

$$1) S (\%) = (n_t/n_0) \times 100$$

- 2) $CLG \text{ (mm)} = CL_t - CL_0$
- 3) $WWG = WW_t - WW_0$
- 4) $DWG = DW_t - DW_0$
- 5) $SGR \text{ (% W d}^{-1}\text{)} = (\ln DW_t - \ln DW_0)/t \times 100$
- 6) $BG \text{ (%)} = (B_t - B_0)/B_0 \times 100$
- 7) $P \text{ (g m}^{-3} \text{ day}^{-1}\text{)} = (B_t - B_0) \times n_t/(V \times t)$
- 8) AMD

$$AMD = \frac{X_{R1} + X_{R2} + X_{R3} + X_{R4} + X_{R5}}{5}$$

$$X_R = \frac{\left(\frac{N_R}{S_R}\right)_{Day 1} + \left(\frac{N_R}{S_R}\right)_{Day 2} + \dots + \left(\frac{N_R}{S_R}\right)_{Day 49}}{t}$$

where n is the number of surviving juveniles; t is the duration of experiment (49 days); 0 is initial stocking; B is the sum of dry weight; V is the water volume per 1 animal (3-L=0.003 m³). For AMD (average number of moults *lobster⁻¹*day⁻¹), X_R is calculated first (average daily number of moults *animal⁻¹ *replicate⁻¹), R is replicate, N_R is number of moults in a replicate, S_R is the number of surviving animals in a replicate; 5 is the number of replicates.

2.2.6 Behavioural observations

Behavioural observations were recorded with time-series photography using a similar method as described by Marchese et al. (2019). Images were captured with GoPro Hero 5 Black cameras. The cameras were installed in transparent plastic cylinders embedded in the centre of the transparent vessels lids and placed above the water surface. Behavioural observations were recorded at two juvenile instars (2 and 4), starting from the third day after moult. For each juvenile instar, an image was recorded

every 30 s over a 72 h period. Images were captured over white feeding trays (4.8 cm x 5 cm in isolated and separated cultures and 12 cm x 12 cm in communal culture), which provided a contrasting background for analysis. Observations were made in the low water exchange rate treatments on five randomly chosen animals from each rearing culture. Images were not recorded in the high water exchange rate treatment because the high water flow acted to dislocate the feed particles from the feed trays. In the communal culture the randomly chosen animal was painted with a yellow spot for identification. To account for any influence of this handling procedure, all selected individuals across all cultures were tagged using identical procedures and in addition, a random animal was also painted in the same way from each replicate of the high flow treatment even though no behavioural observations were made.

Time-series photographs were analysed for 1) feed interactions and 2) feed intake. Feed interactions were defined as: when an animal was manipulating or interacting with the various feed types, with a nominal score of one given to each interaction within each 30 sec recorded image. Feed intake was visually estimated based on observed consumption or feed pieces removed from field of view. Feeding activity was assigned to a fractional figure representing the amount of feed consumed or removed from the field of view, with 1 representing one piece of feed consumed or removed. Intake was recorded at the end of the process of feed consumption or removal from the field of view. Feed intake was subsequently recalculated to DW (mg) for each of the three feeds and taking into account the differing feed piece mass and dry matter content detailed in Table 2.2. The time that animals spent interacting with feeds was calculated in min, with each min of observation comprised of two photographs. The data for graphic presentation of behavioural observations was summed into 30 min intervals, where each interval was comprised of observations from preceding 30 min.

Time-series image analysis was processed manually in Adobe Photoshop CC2017. The 72 h material was analysed and averaged into 24 h periods. Subsequently the average 24 h observations of five individual lobsters from one rearing culture and instar were averaged into rearing culture and instar average for 24 h.

2.2.7 Statistical analysis

The data were analysed with two-way ANOVA (growth and survival) and one-way ANOVA (behavioural observations). The factors considered in data analysis were fixed and included culture type and water exchange rate in two-way ANOVA. One-way ANOVA considered culture type only. Levene's test was used for evaluation of homoscedasticity, normality was tested by the Shapiro-Wilk W-test. In cases where normality was not met, the data were inverse or natural logarithm transformed to provide a normal distribution prior to analysis. When Levene's test showed that variances were not homogeneous, the data were square root transformed before performing ANOVA. When a significant difference was observed, Tukey's HSD test was used for post hoc analysis of means. When significant interaction was observed, pairwise multiple comparison was performed. Significance was accepted at level $\alpha < 0.05$. Analyses were calculated using SPSS statistics software v. 24.

2.3 Results

2.3.1 Growth and survival

Lobsters in communal culture obtained highest WW_t and WWG , but the difference was not significant (Table 2.3). Culture type had a significant effect on CL_t ($F=5.23$; d.f. 2,24; $p=0.013$), CLG ($F=5.23$; d.f. 2,24; $p=0.013$), DW_t ($F=4.154$; d.f. 2,24; $p=0.028$), DWG ($F=4.154$; d.f. 2,24; $p=0.028$) and AMD ($F=4.163$; d.f. 2,24; $p=0.028$),

with faster growth in animals cultured communally than animals in isolated and separated cultures (Figure 2.1).

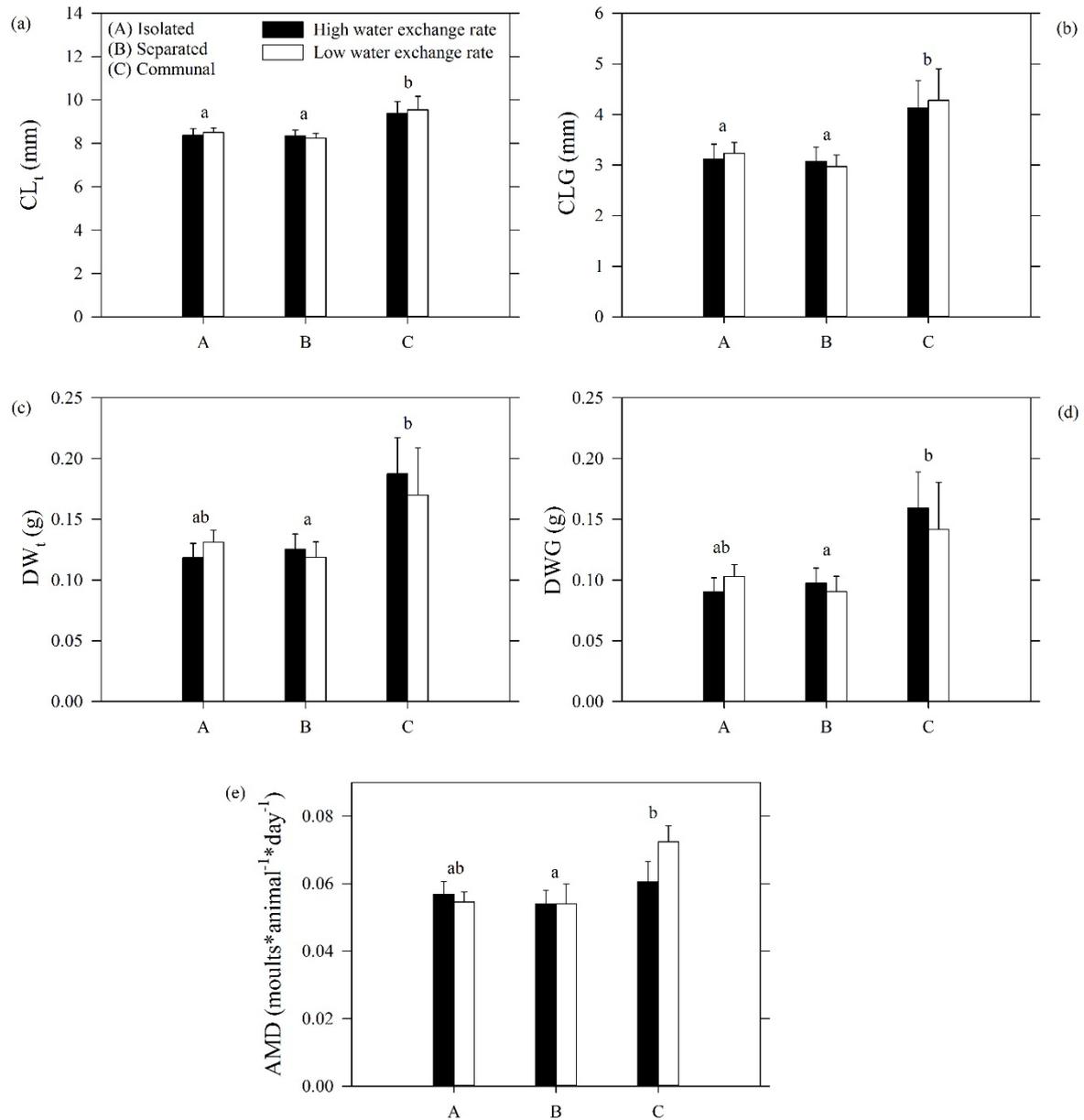


Figure 2.1. (a) final carapace length; CL_t (mm), (b) carapace length gain; CLG (mm), (c) final dry weight; DW_t (g), (d) dry weight gain; DWG (g), (e) average moults per day; AMD ($\text{moults} \cdot \text{animal}^{-1} \cdot \text{day}^{-1}$). The juvenile *P. ornatus* lobsters were reared in three culture types; isolated (A), separated (B) and communal (C), and two water exchange rates; high (15

exchanges h^{-1}) and low (6 exchanges h^{-1}). Values are mean \pm SE, significant differences in response to the culture type are indicated with superscripts. Initial carapace length (mmCL₀) = 5.26 ± 0.12 ; initial dry weight (gDW₀) = 0.02 ± 0.00 .

In terms of DW_t, DWG and AMD, significant difference was observed between separated and communal cultures only. There was significant effect of culture type on SGR, but significance was not observed in the means comparison (Table 2.3). Analysis of BG and P showed no significant differences. A significant interaction effect of culture type and water exchange rate was found for S, but significance was not observed in the means comparison. Water exchange rate had no significant effect on growth and survival.

2.3.2 Time-series photography

2.3.2.1 Daily rhythm of interactions with feeds

Subsequent to the onset of dark phase and afternoon feeding, lobsters in both instars (2 and 4) subject to time-series photography, in all culture types (isolated, separated and communal) were frequently interacting with feeds (Figures 2.2 and 2.3). Animals commenced feed interactions within 30 min after addition of the afternoon ration. Feed interactions increased quickly and peaked within one h in isolated culture and 90 min in communal culture after the onset of feeding, for both juvenile instars. Animals in separated culture were actively interacting with feeds for an extended time, in contrast with remaining culture types. High levels of feed interactions in separated culture was reached within one h after the onset of feeding, and steadily increased thereafter. The peak of feed interactions in separated culture was extended between fourth and fifth, and second and third h after feeding in instars 2 and 4 respectively. Following the

peak, feed interactions in all cultures and instars gradually decreased in slightly fluctuating pattern until the end of the dark phase.

After the beginning of the daylight phase and morning feeding, lobsters in all cultures exhibited low levels of feed interactions. Animals in all cultures commenced feed interactions within 30 min after the addition of the morning feed ration in both instars. The pattern of feed interactions was slightly fluctuating, the peaks were reached within two and three h in isolated culture for juvenile instars 2 and 4 respectively. Animals in separated culture reached the peak of feed interactions within 90 and 60 min, and communal within 150 and 120 min for instars 2 and 4 respectively. Occasional feed interactions, separated by zero activity periods were observed in communal instar 2, during the daylight phase. Juvenile instar 4 lobsters in all cultures displayed higher feed interaction levels during the daylight phase compared to instar 2 lobsters.

2.3.2.2 Feed preferences and intake

The sum of feed interaction time in instar 2 (Table 2.4), was significantly higher for gonad in isolated culture and for moist feed in separated culture ($F=6.697$; d.f. 2,12; $p=0.011$ and $F=16.196$; d.f. 2,12; $p=0$ respectively). There was no significant difference found for sum of interaction time with pellet. The total interaction time with all feeds for instar 2 was significantly higher in separated culture ($F=14.191$; d.f. 2,12; $p=0.001$). Instar 2 lobsters in communal culture exhibited highest DW intake from gonad, pellet and in total, but the difference was not significant (Table 2.4). The highest consumption of DW from moist feed in instar 2 lobsters was displayed in separated culture, but again the difference was not significant.

Instar 4 lobsters exhibited the highest sum of feed interactions with gonad, moist feed and in total, in separated culture (Table 2.4). Frequency of interactions with pellet was

higher in communal culture. The DW intake from gonad, pellet and total was higher in communal culture, and from moist feed in isolated culture (Table 2.4). No significant differences were found for sum of feed interaction and DW intake between juvenile instar 4 cultures. Overall, juvenile instar 2 and 4 lobsters exhibited a similar pattern of feed intake, where the total sum of interaction with feeds was highest in separated and total DW intake in communal culture. The lobsters in communal culture displayed considerably higher feed interaction frequency and DW intake from pellet in instar 4 compared to instar 2.

Table 2.3 Effects of rearing cultures (isolated, separated, and communal) and water exchange rates (low, 6 exchanges h⁻¹; high, 15 exchanges h⁻¹) on growth and survival of juvenile *P. ornatus* (data are presented as mean ± s.e.). Initial wet weight (gWW₀) = 0.11 ± 0.004; initial carapace length (mmCL₀) = 5.26 ± 0.12; initial dry weight (gDW₀) = 0.02 ± 0.001.

Animal rearing culture	Isolated		Separated		Communal		Two way ANOVA			
Water exchange rate (L ^{-h})	High	Low	High	Low	High	Low	F	d.f.	P	
WW _t (g)	0.47 ± 0.05	0.49 ± 0.03	0.46 ± 0.04	0.46 ± 0.05	0.72 ± 0.13	0.68 ± 0.16	Interaction	0.158	2,24	0.855
							Culture type	3.341	2,24	0.052
							Water exchange rate	0.005	1,24	0.945
WWG (g)	0.35 ± 0.05	0.37 ± 0.03	0.35 ± 0.04	0.34 ± 0.05	0.60 ± 0.13	0.57 ± 0.16	Interaction	0.204	2,24	0.817
							Culture type	2.919	2,24	0.073
							Water exchange rate	0.001	1,24	0.978
SGR (%BW/day)	2.88 ± 0.21	3.11 ± 0.16	3.00 ± 0.20	2.88 ± 0.21	3.75 ± 0.34	3.47 ± 0.41	Interaction	0.432	2,24	0.654
							Culture type	3.675	2,24	0.041*
							Water exchange rate	0.058	1,24	0.811
BG (%)	104.81 ± 48.14	105.14 ± 47.57	216.23 ± 59.78	39.45 ± 61.13	194.43 ± 44.24	288.23 ± 119.28	Interaction	2.018	2,24	0.155
							Culture type	2.279	2,24	0.124
							Water exchange rate	0.243	1,24	0.626
P (g m ⁻³ day ⁻¹)	4.61 ± 1.93	4.23 ± 2.02	11.85 ± 4.33	2.44 ± 2.61	6.42 ± 1.79	15.26 ± 6.32	Interaction	2.867	2,24	0.076
							Culture type	1.449	2,24	0.255
							Water exchange rate	0.521	1,24	0.477
S (%)	46 ± 9.71	43 ± 8.49	70 ± 9.71	30 ± 9.71	46 ± 6.23	60 ± 13.54	Interaction	3.865	2,24	0.035*
							Culture type	0.365	2,24	0.698
							Water exchange rate	1.557	1,24	0.224

Two way ANOVA effects; WW_t-final wet weight, WWG-wet weight gain, SGR-specific growth rate, BG-biomass gain, P-productivity, S-survival, ANOVA significance level P<0.05;

*significant differences

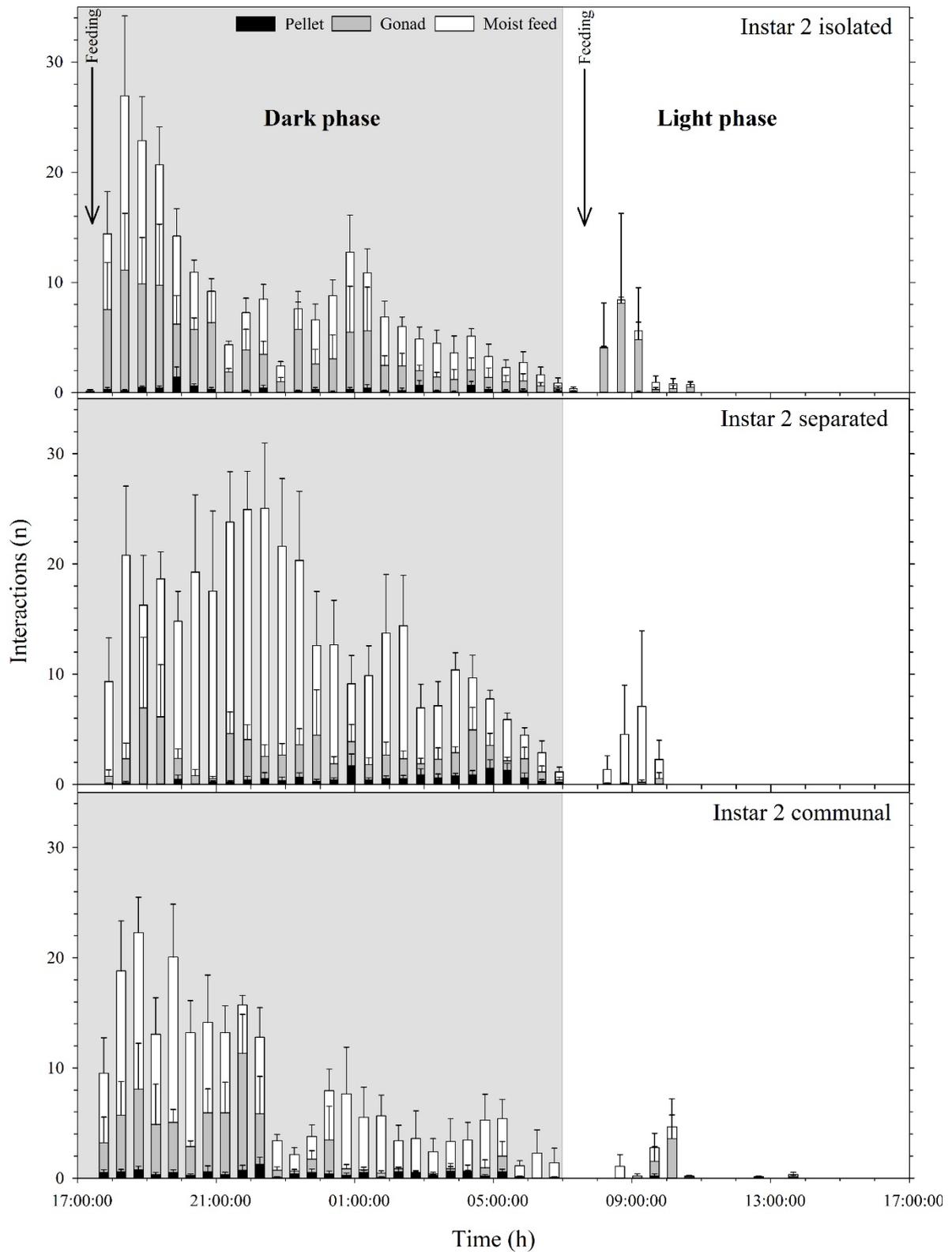


Figure 2.2. Feed interactions (n), shown for each culture; isolated, separated and communal in instar 2, average 24 h, error bars denote s.e..

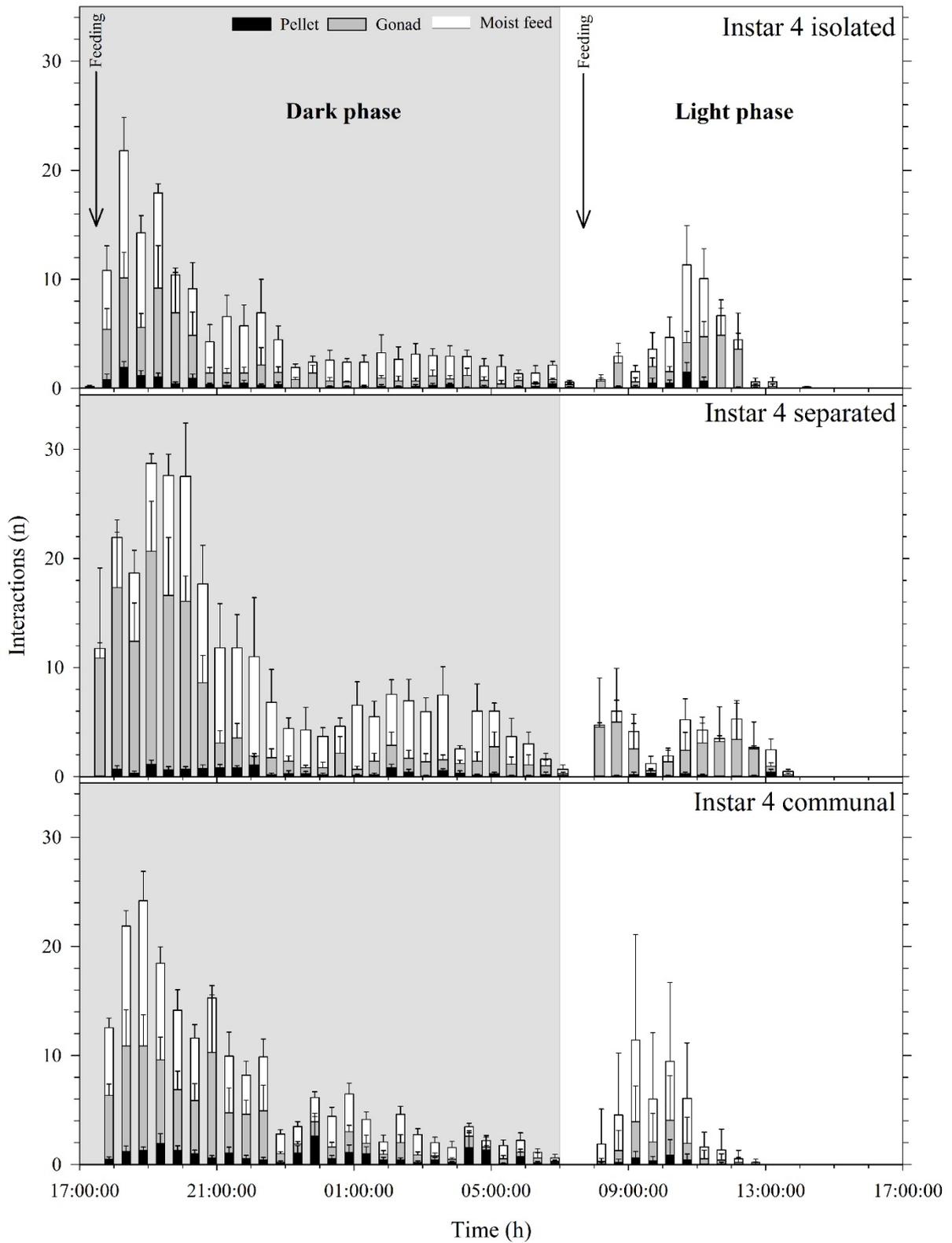


Figure 2.3. Feed interactions (n), shown for each culture; isolated, separated and communal in instar 4, average 24h, error bars denote s.e..

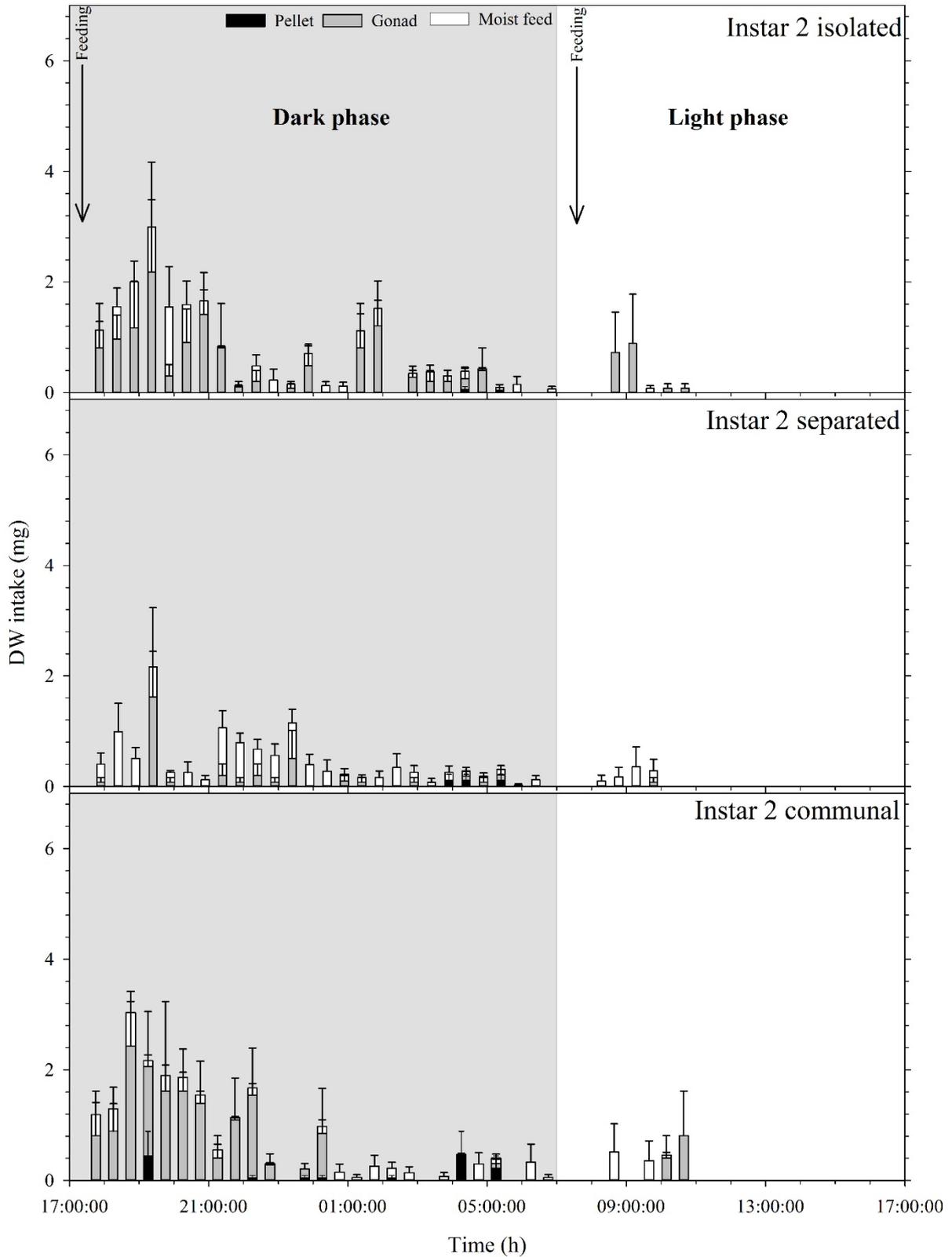


Figure 2.4. DW (dry weight) intake (mg), shown for each culture; isolated, separated and communal in instar 2, average 24h, error bars denote s.e..

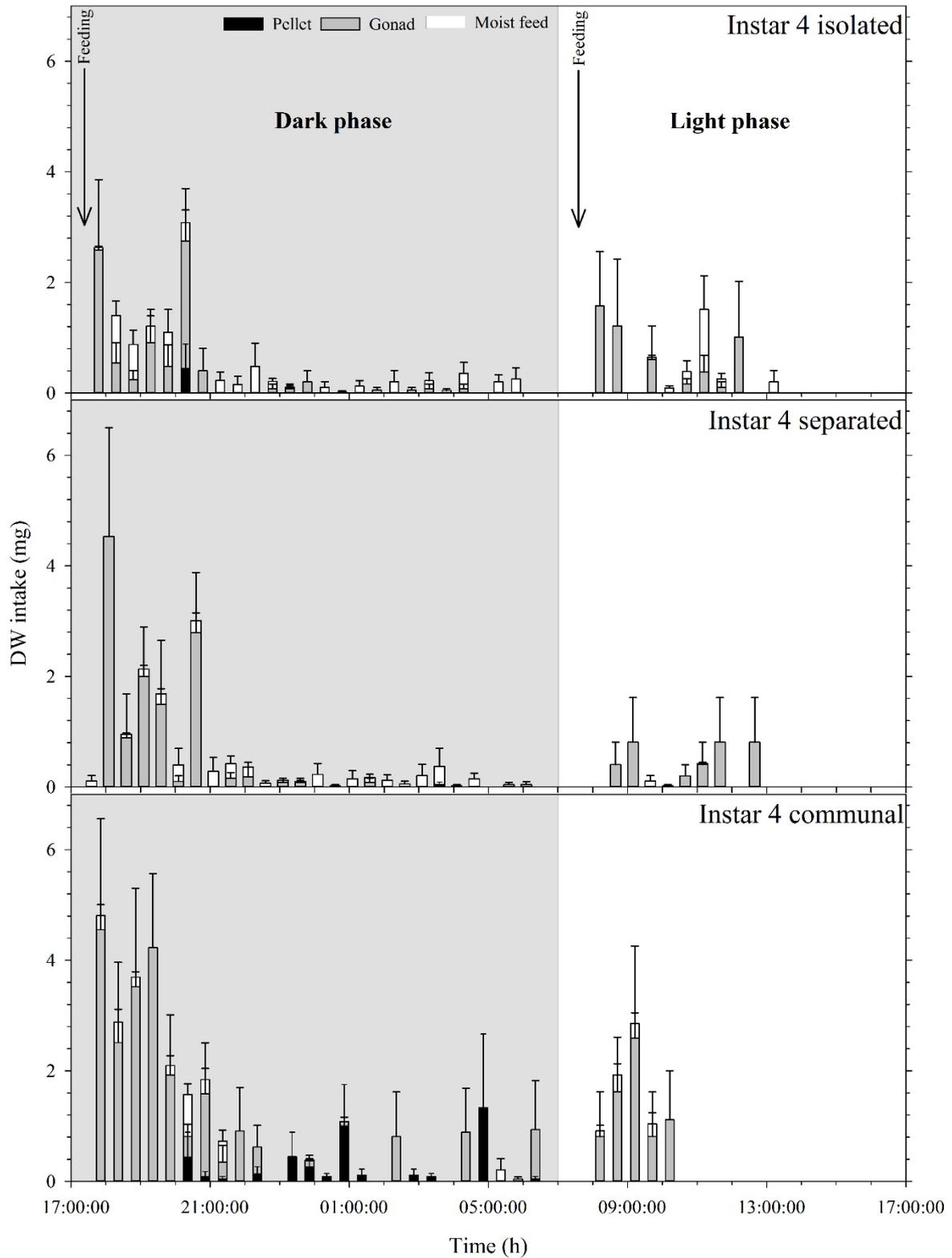


Figure 2.5. DW (dry weight) intake (mg) shown for each culture: isolated, separated and communal in instar 4, average 24h, error bars denote s.e..

Table 2.4 Effects of three culture types (isolated, separated, and communal), on behaviour and DW (dry weight) intake of juvenile *P. ornatus*, in instar 2 and 4.

Animal rearing culture			Isolated	Separated	Communal	One way ANOVA		
						F	d.f.	P
Instar 2	Sum of feed interaction time (min)	Gonad	67.2 ± 8.45 ^a	31.83 ± 8.80 ^b	33.86 ± 5.27 ^b	6.697	2,12	0.011
		Moist feed	64.16 ± 11.91 ^b	153.20 ± 15.43 ^a	75.13 ± 7.54 ^b	16.196	2,12	0
		Pellet	4.03 ± 1.31	6.83 ± 1.88	6.00 ± 1.77	0.736	2,12	0.5
		Total	135.40 ± 12.09 ^b	191.87 ± 11.52 ^a	115.00 ± 7.48 ^b	14.191	2,12	0.001
	DW intake (mg)	Gonad	14.54 ± 3.72	3.46 ± 2.61	15.91 ± 4.08	3.754	2,12	0.054
		Moist feed	7.27 ± 2.21	9.14 ± 1.66	5.18 ± 0.83	1.41	2,12	0.282
		Pellet	0.10 ± 0.10	0.33 ± 0.33	1.29 ± 1.29	Not enough data		
		Total	21.91 ± 5.00	12.93 ± 2.71	22.38 ± 5.17	1.436	2,12	0.276
Instar 4	Sum of feed interaction time (min)	Gonad	44.13 ± 14.78	76.56 ± 15.06	45.23 ± 9.46	1.902	2,12	0.192
		Moist feed	66.16 ± 9.01	75.73 ± 20.10	62.96 ± 9.06	0.233	2,12	0.795
		Pellet	7.96 ± 0.99	6.33 ± 1.32	12.16 ± 4.69	0.609	2,12	0.56
		Total	118.27 ± 21.70	158.63 ± 19.50	120.37 ± 18.90	1.287	2,12	0.312
	DW intake (mg)	Gonad	13.06 ± 4.24	15.83 ± 4.03	29.99 ± 5.87	3.6	2,12	0.06
		Moist feed	7.12 ± 1.65	3.43 ± 2.09	3.54 ± 0.58	2.433	2,12	0.13
		Pellet	0.44 ± 0.44	0.04 ± 0.04	4.20 ± 3.09	Not enough data		
		Total	20.63 ± 3.23	19.31 ± 2.56	37.73 ± 8.64	2.294	2,12	0.143

One way ANOVA effects; DW-dry weight, ANOVA significance level P<0.05; significant differences between means are marked with superscripts.

2.4 Discussion

Our findings present the importance of all conspecific interactions, in optimisation of growth and survival in early juvenile *P. ornatus* lobsters. The multiple communication channels utilized by spiny lobsters, include visual, chemical, mechanical and acoustic (Bushman and Atema, 2000; Herberholz, 2007; Kozma et al., 2018; Schmitz, 2002; Thiel and Breithaupt, 2010). This complexity of chemosensory physiology is responsible for a broad range of behaviours (Derby and Sorensen, 2008). Limitations to communication introduced in the present study resulted in reduced growth and affected behaviour of this gregarious and simultaneously cannibalistic species. Behavioural observations demonstrated the lobsters circadian rhythm of feeding interactions, feed preferences and intake, together with differences resulting from culture type. Experimental cultures allowed for control of cannibalism, however this was not the primary cause of mortality.

2.4.1 Growth and survival

In the present study, culture type had a significant effect on juvenile *P. ornatus* growth, which was greater in communal culture. Our growth results concur with Irvin and Williams (2009a), Ratunil Jr (2017) and Marchese et al. (2019) studies, where growth of *P. ornatus* juveniles was significantly higher in communal culture, compared with lobsters reared in individual adjacent cages (Irvin and Williams, 2009a; Ratunil Jr, 2017) and in unconnected individual vessels (Marchese et al., 2019). Similarly, Vijayakumaran et al. (2010), showed that subadult *P. homarus* grew faster and moulted more often when kept communally. In the same study, *P. homarus* subadults showed improved growth when shifted from individual to communal culture, and

conversely decreased growth when moved to individual culture. These results confirm the importance of conspecific interactions, noted in our study, specifically tactile close contact, on the growth and moulting frequency of lobsters. Eliminating physical contact while maintaining other communication paths, including chemical cues, does not appear sufficient to stimulate maximum growth. The mechanism of improved growth of lobsters in communal culture may include feeding stimulation that results in greater feed consumption, perhaps driven by competitive pressures. Another potential mechanism stimulating growth may be cannibalism and moult consumption. In the present experiment, shedded exuvia and deceased lobsters were often found partly eaten, and the time-series photography analysis revealed that animals were readily consuming exuvia and deceased individuals. This behaviour was possible only in communal culture, with conspecific lobsters consuming exuvia of recently moulted individuals, as spiny lobsters do not feed approximately two days before and after moulting (Lipcus and Herrnkind, 1982; Musgrove, 2000). In the present experiment, all shedded exuvia and mortalities were removed from the rearing cultures twice daily, which would have prevented newly moulted lobsters from consumption of their own moults in isolated and separated cultures.

There was no significant differences in survival between culture types, which suggests no influence of cannibalism on lobster survival. Spiny lobsters, including *P. ornatus*, are known to have cannibalistic behaviours, specifically in early juvenile instars (Carter et al., 2014; Irvin and Williams, 2009a; Jones et al., 2001; Jones, 2009; Romano and Zeng, 2017; Skewes et al., 1994). According to Claessen et al. (2004), there are four aspects of cannibalism: 1) victim mortality (victim is killed by cannibal); 2) energy extraction (victim is consumed by cannibal); 3) size dependence (victims are usually smaller than cannibals); and 4) competition (victims and cannibals compete for

common resources). These aspects may occur in various configurations and may influence one another. Cannibalism may be the cause of mortality when it involves killing the victim or be the consequence of mortality when a conspecific is already dead. Cannibalism has been commonly implicated in reduced survival in cultured early juvenile *P. ornatus*, but this hypothesis has not been properly tested (Irvin and Williams, 2009a). Survival results in the present study tend to disagree with this hypothesis and suggest that cannibalism is a response to mortality and not the cause of mortality. This result corresponds with the outcome of experiments performed by Ratunil Jr (2017) and Irvin and Williams (2009a), where survival of juvenile *P. ornatus* lobsters was not significantly different between communal and individual culture. Similarly, Vijayakumaran et al. (2010), demonstrated that *P. homarus* had similar survival in communal and individual culture, again suggesting that cannibalism is not a primary cause of mortality in a palinurid species. Mortality may relate to a nutritional deficiency, resulting in moult failure, which can partly be overcome via nutritional supplementation by cannibalism of moults and dead or dying individuals (Irvin and Williams, 2009a; Thomas et al., 2003). Likewise, improved growth in communal culture may be the result of the added source of nutrients, by cannibalism and eating exuviae. The effect of dispersion of olfactory cues in water, on lobsters growth and survival was tested with two water exchange rates. It was hypothesised that higher water flow may act to better communicate olfactory cues, and lead to improved culture performance of lobsters in separated culture. Previously it was suggested that insufficient dispersal of olfactory cues may have been responsible for poor growth and lower moulting frequency of *P. homarus* in individual culture (Vijayakumaran et al., 2010). Our results showed no significant differences in growth between animals cultured under either low or high water flow. This suggests that the dispersal of olfactory cues is not a primary

factor that limits growth performance. However, it is acknowledged that higher water flow and exchange rates may have also acted to dilute water borne chemical cues, and further research is required to better understand the influence of olfactory cues on lobster growth.

2.4.2 Behaviour

Behavioural observations made on two juvenile instars (2 and 4) in three culture types, provided valuable insight into the cryptic patterns of feed interactions, preferences and intake. Time-series photography showed high levels of nocturnal activity and feed interactions within one to five h after beginning of dark phase and the addition of the afternoon ration. Likewise, Cox et al. (1997) showed that adult *P. argus* have high nocturnal activity in the early-evening. Wild adult clawed lobsters, both *H. gammarus* and *H. americanus* display high levels of nocturnal activity with a peak one to three h after sunset (Karnofsky et al., 1989; Moland et al., 2011). Research on *P. cygnus* (Jernakoff, 1987) and on *H. americanus* (Golet et al., 2006), showed significantly higher activity at night and dawn, than at dusk and during the day. The natural activity rhythm in arthropoda is modulated by hormones, and can be modified by developmental, environmental and internal changes (Skiebe, 2002). Our observations illustrate the activity pattern of early juvenile lobsters held in laboratory conditions. The artificial laboratory environment and juvenile age of observed lobsters did not interrupt expression of circadian activity. In the present study *P. ornatus* feed interactions gradually decreased through the night following the high intensity peak that occurred after beginning of dark phase and offering the feed ration. Further to the nocturnal activity patterns of lobster, the attractiveness of feeds may be an additional factor affecting the peak activity of lobsters in the early evening. Williams et al. (2005)

showed that the attractiveness of three different feeds (mussel and two pelleted feeds) for juvenile *P. ornatus* decreased with immersion time. The loss of attractiveness post immersion is due to leakage of water soluble compounds (Williams et al., 2005). Likewise, the study of Marchese et al. (2019) showed increased frequency of feed interactions, followed by decrease or termination within two to three h after feeding with pellet. It is thus likely that freshly supplied feeds may stimulate greater foraging behaviour immediately after the feed is added and result in decreased feed activity associated with increased feed soak times. The daylight interactions with feeds, after the morning addition of feed ration could be motivated by presence of fresh, attractive feeds as was noted when adult *H. americanus*, were supplied fresh feeds during daylight (Wang et al., 2016).

The observed pattern of interactions with feeds was similar between communal and isolated culture; in contrast lobsters in separated culture displayed a higher frequency of feed interactions, compared to isolated and communal cultures. While the reason of increased activity of lobsters in separated culture is unclear, it may have been motivated by sensing presence of conspecifics and searching for further interaction; whereby lobsters may have been interacting with feeds by actively moving. This behaviour may be motivated by gregariousness, expressed as readiness to aggregate with conspecifics. Sensing the presence of conspecifics may have influenced the lobsters behaviour, as was noted in the crab, *Carcinus maenas* (Fürtbauer and Fry (2018). That experiment analysed activity response, measured as distance travelled by crabs held in solitary or in paired housing. The crabs held in solitary revealed considerably differing levels of activity between individuals, and more uniform when paired. After pairing with conspecifics, crabs aligned their activity, mostly by decreasing activity by the more active individual. Fürtbauer and Fry (2018) suggest a

social conformity effect in crab activity, which occurs in social animals and is an adaptation of individual to another one or to a group, by behaviour modification.

The present study demonstrated that during daylight the frequency of interactions with feeds was considerably higher in instar 4, when compared with instar 2 across all cultures. The older animals may become less cautious because they are larger and less vulnerable to predators (Kanciruk, 1980). Spiny lobsters are cryptic and avoid predators in the wild by hiding during the day and being active at night. Moreover their activity can be modulated by predator experience. Briceño et al. (2018) found differences in metabolic rate of *J. edwardsii*, exposed to olfactory cues of *Octopus maorum*. Lobsters decreased their metabolism and stayed inactive, as an anti-predatory behaviour. Weiss et al. (2008) observed different behavioural response of *P. argus* to two different predators. Lobsters remained in shelter when attacked by triggerfish but left the shelter and escaped when attacked by octopus. In culture it is possible that feeding behaviour may be modified with the ongoing absence of predators, leading to modification of the circadian rhythm and increasing the daylight activity, expressed as leaving shelter and feeding. This conclusion is consistent with study of Marchese et al. (2019), who used juvenile *P. ornatus* lobsters, reared in the predator free laboratory environment. The initial wet weight of lobsters in their experiment (1.75 to 2.10 g), corresponds approximately to instar 6 to 7 and post-juvenile age of approximately 2 months (based on own observations). The behavioural observations revealed continuous 24 h interacting with preferred feeds (mussel). This may reflect a progression of our observation of increasing feeding interactions from instar 2 to 4. Similarly the behaviour modification in absence of predators was observed by Lozano-Alvarez (1996) on *P. argus* juveniles. Lobsters were captured from wild, held in cages with access to shelters and fed in abundance.

Initially, the displayed behaviour included typical gregariousness and staying in shelters during daytime. After two weeks of experiment, lobsters increased their daytime activity, by feeding, moving and climbing on cage sides and top.

Although not significantly different from other culture types, lobsters in communal culture displayed highest feed intake for both juvenile instars. Time-series photography in the present study exhibited a discrepancy between feed intake and feed interactions results, suggesting that feed interacting is not directly related to feed intake. Animals in separated culture displayed high levels of feed interactions, but the total feed intake was lowest amongst all culture types for both instars. In contrast, lobsters in communal culture exhibited relatively low feed interaction levels, and high feed intake. This observation corresponds well with a study on *Astacus astacus*, which showed no direct evidence of influence of activity time on feed intake (Franke and Hörstgen-Schwark, 2015). Our observation of high feed interaction levels and low feed intake exhibited by lobsters in separated culture, may be linked to awaiting for conspecific interaction, instead of foraging. Alternatively, the differences in feed intake between two instars in three experimental cultures, may be the result of complex learning abilities and social structures in crustaceans (Daniel and Derby, 1988; Derby, 2000; Derby et al., 2001). The animals in communal culture showed increasing feed intake from instar 2 to 4 and feed intake was lower in isolated and separated cultures. We presume that lobsters across all culture types adapted their feeding behaviour to the surrounding conditions. Lobsters in communal culture were competing for feed, which may have encouraged rapid and more efficient feeding, whereas this behaviour was unnecessary in isolated and separated cultures. Furthermore, separated culture may have encouraged excessive feed interaction with the aim of encouraging conspecific interaction. This adaptive behaviour may be linked to crustacean learning

abilities. In the study of Lozano-Alvarez (1996), after spending two weeks in communal cages and being fed every second day, *P. argus* juveniles began to approach and grab food directly from hand of a diver supplying feed ration. This behaviour may be another expression of learning abilities, by adapting to the surrounding conditions, including feeding method and presumably intraspecific competition for food. Similarly, learning abilities were indicated in crab *C. maenas* and *Callinectes sapidus* captured from wildlife, which were increasing speed of finding hidden feed on consecutive days and in consequence increasing their chances of survival (Roudez et al., 2008).

The lobsters feeding preferences observed for both instars (2 and 4), showed highest interaction and consumption levels for mussel gonad and moist feed while interactions with and consumption of pellet were rare. Likewise, Williams et al. (2005) and Marchese et al. (2019) observed *P. ornatus* preference for mussel in comparison with formulated feeds. The mussel is a vital part of spiny lobsters natural diet, also preferred over other prey such as sea urchin and abalone (Mayfield et al., 2001). This is an important observation for further work, on the palatability and texture of diet for lobsters in aquaculture.

2.5. Conclusion

The present study provides observations of three culture types, which showed a profound effect on *P. ornatus* growth and behaviour. The improved growth in communal culture suggests the importance of conspecific interactions and competition in the optimisation of juvenile *P. ornatus* growth. The survival results showed no significant differences between culture types, indicating that cannibalism was not the primary cause of mortality. The growth and survival results in communal culture, suggest that under these conditions this is the optimal method of rearing early juvenile

P. ornatus lobsters. Further research is required to investigate mortality and cannibalism, in the context of nutritional deficiency and behaviour of older lobsters. Time-series photography analysis revealed that lobsters have a defined circadian rhythm, primarily with nocturnal activity, but extending into daylight activity during instar 4, which may be an adaptation to the non-predator environment. This observation is a valuable input to the optimisation of feeding schedules, with consideration of lobsters changing behaviour through ontogeny and adaptation capabilities. The commencement of feed interactions and intake within 30 min to one h after the addition of feed rations, reaching a peak within one to three h revealed lobsters quick response to the presence of feed. The following decrease of feed interactions and feeding highlight a need for research aimed to improve and lengthen diet palatability, to encourage increased and extended feeding.

Chapter 3 Functional morphology and ontogeny of mouthparts and mouth aperture of *Panulirus ornatus* and *Sagmariasus verreauxi*: implications for formulated feeds development

Abstract

Development of formulated feed is now a focal point in advancing the aquaculture of spiny lobster species. This study investigated the functional morphology of two spiny lobsters, *Panulirus ornatus* and *Sagmariasus verreauxi*, by analysing the mouthpart morphology and mouth aperture. The analysis is focussed on determining likely feed preference based on morphology with the aim to provide knowledge to use in the development of formulated feeds. Mouth aperture correlates with lobster carapace

length and is equal to approximately 4 and 7.5% for *S. verreauxi* and *P. ornatus* lobsters, respectively. This finding provides a species-specific tool that could be used in experimental studies to define optimal dimensions of pellets in relation to the size of the lobster. Differences in mouthpart morphology through ontogeny included increase of calcification and robustness, and this is likely to reflect a different requirement in the texture (softness/hardness) of the pellet as the animals develop. Spines and setation are a prominent feature of the mouthpart morphology and differ between species and during ontogeny. Spines on maxillipeds III and II and crista dentata on maxillipeds III are likely to play an important role in holding and manipulation of feed prior to ingestion. Pellet texture may need to accommodate these differences in species and age specific mouthpart ontogeny. Ontogenetic changes in setation were found, with much richer setation proportionally to the size of mouthpart in first instar juvenile lobsters as well as species-specific differences indicating shifts in functions of mouthparts with life stages. This may indicate differences in filter feeding capacity with age and between species, as reported for other decapods. This is unlikely to be of high importance in pellet feeding but may represent an avenue of investigation for first feeds in juveniles. Collectively, the differences during ontogeny and between species suggest that formulated feeds may need to be tailored to species and life stage, particularly regarding pellet dimensions and texture.

3.1 Introduction

Spiny lobsters are a highly valued seafood, with global capture value over US\$3.8 billion (FAO, 2020). Lobsters are mainly sourced from fisheries, but limited culture of wild juvenile seed has been undertaken since the 1990s (Radhakrishnan, 2015; Smith, 2017). Currently spiny lobster aquaculture is based on feeding “trash fish”, leading to

low feed conversion ratios, disease and environmental damage (Vinh and Huong, 2009). Commercial feeds are needed to resolve these problems, but existing formulated feeds are poorly ingested leading to between 19 to 50% wastage of the feeds provided (Sheppard et al., 2002; Smith et al., 2009a). Spiny lobster feeding is often described as “messy” due to external fragmentation of feed (Sheppard et al., 2002; Zoutendyk, 1988). Unlike Atlantic salmon that rapidly respond to a feeding event by consuming whole pellets in the water column (Stradmeyer, 1989; Stradmeyer et al., 1988), spiny lobsters display a protracted feeding response, locating pellets on a substrate and ingesting them over a prolonged period that includes external manipulation with their complex mouthparts. Consequently, ingestion and mouthpart morphology of individual species and their growth stages are important factors to consider in development of formulated feeds (Aaqillah-Amr et al., 2021).

Spiny lobsters undergo considerable morphological changes through ontogeny. After a complex oceanic larval phase, juveniles emerge from the non-feeding puerulus that swims from offshore into coastal reefs to settle. Juveniles become increasingly large and robust with subsequent instars, with concomitant gains in setation, pigmentation, behaviour, habitat and feeding preferences (Cox et al., 2008; Factor, 1989). Cox et al. (2008) found that mouthparts in early juvenile, juvenile and late juvenile-adult *Panulirus argus*, increase in size, robustness, calcification, setation and pigmentation in tandem with increasing body size. The mandibles show the most extensive alterations, including appearance of tubercles, defined apexes and a shift from two to three segments in the mandibular palp. In the first instar juveniles, the small size and fragility of mouthparts indicate that soft and pulpy feeds are the optimum choice, which corresponds with analysis of stomach contents, containing predominantly (96%) gastropods (Cox et al., 2008; Marx and Herrnkind, 1985b). Increasing robustness and

size of mouthparts, including development of teeth on mandibles in older juveniles (45-80 mm carapace length (CL)), allow for ingestion of firmer feeds.

Spiny lobster mouthparts are involved in ingestion process, which includes introduction and delivery of feed to the pre-oral cavity, setting between the mandibles followed by crushing and swallowing (Johnston, 1995; Parra-Flores et al., 2019). The three pairs of maxillipeds secure, manipulate, transport feed towards mouth and position the feed for manipulation (Cox et al., 2008; Francis et al., 2014). Maxillae II in *P. argus*, function as part of exhalant system, assist in transporting feed to the mandibles and may also function as filter feeding mouthpart due to rich setation (Cox et al., 2008). Maxillae I tear and mandibles cut and crush feed in a single movement (Francis et al., 2014; Watling, 2013). The mouth aperture in spiny lobsters is restricted with mandibles on the lateral sides, with the labrum and metastoma on anterior and posterior sides, respectively. The oesophagus is located dorsally from the mouth and leads to foregut where feed is pulled using suction created by expanding foregut (Paterson, 1968; Watling, 2013).

Recently settled *P. argus* juveniles (10 – 15 mm (CL)), preferentially feed on small (1 – 2 mm diameter), soft, fleshy feeds, but their diet also includes small gastropods, bivalves and crustaceans (Cox et al., 2008; Marx and Herrnkind, 1985a). They are able to efficiently shred larger soft, fleshy feed pieces and pass them through a relatively small mouth aperture (Cox et al., 2008). With juvenile development, their increasing mouthpart size and strength allows extension of their diet to include more robust prey, such as large bivalves, urchins, gastropods and crustaceans (Barkai et al., 1996; Dumas et al., 2013; Mayfield et al., 2001).

The ingestion of different types of prey includes wide range of complex techniques. Feeding on mussels involves using maxillipeds III to detach prey from the substrate,

mandibles to crush the shell, while maxillipeds and pereopods are involved with positioning. Finally, the maxillipeds and pereopods open the mussel and remove the flesh (Lau, 1987). Feeding on more robust gastropods includes grinding a hole in the shell before extracting the flesh using suction, while feeding on urchins involves removing the chewing organ (Aristotle lantern) and extracting the flesh with the maxillipeds (Carnevali et al., 1993; Mayfield et al., 2001; Radhakrishnan et al., 2019a). Setae and spines play multiple roles in the feeding, including chemo and mechanoreception, detection, capture, feed manipulation and filtering (Garm, 2004b; Lavalli and Factor, 1992; Rocha et al., 2018). The filtering is one of feeding strategies used by decapods, including Hermit Crab *Pagurus bernhardus* and clawed lobster *Homarus americanus* (Garm, 2004a; Gerlach et al., 1976). This technique utilises flagella present on exopods of maxillipeds III and II, and setae on maxillipeds III and both maxillae. The function of flagella is to create currents transporting small particles to the mouth proximity and setae to catch and deliver small feed particles to mouth aperture (Garm, 2004a; Gerlach et al., 1976). The setae and spines also assist to direct food into the mouth aperture, trap small food particles and hold larger pieces during ingestion (Watling, 2013).

Mouthpart morphology and mouth aperture are also important considerations in the context of formulated feeds characteristics and efficiency of feeding. The importance of pellet texture was demonstrated by Kawamura et al. (2018) in a study of two decapods *Litopenaeus vannamei* and *Macrobrachium rosenbergii* whereby contrasting feeding behaviour and efficiency were found when fed either pellet, squid or fish. *Macrobrachium rosenbergii* can easily crush pellets with mandibles, which are too weak in *L. vannamei* for this function, demonstrating the suitability of soft feed for *L. vannamei* and hard feed for *M. rosenbergii* (Kawamura et al., 2018). Likewise, the

mud crab *Scylla serrata* when fed with hard, dry feeds demonstrated reduced waste and faster growth, compared to when fed with soft, moist feeds (Ali et al., 2011). Efficiency of feed of *Panulirus ornatus* is dependent on development stage with juvenile lobsters (~ 2 g) producing less waste on soft pellets while adults produce less waste when feed on hard pellets (Smith et al., 2009a). This study also showed that both juvenile and adult lobsters more readily accept long pellets, which are easier to secure and handle than short pellets and that pellet diameter is crucial factor which should fit mouth aperture to minimise waste (Smith et al., 2009a).

As shown previously for several decapods including spiny lobsters, characterisation of mouthpart morphology and mouth aperture can provide valuable information for the development of formulated feeds as a predictor of pellet dimensions, texture and consistency. After establishing optimal culture type for better performance in spiny lobsters (Chapter 2), the next step was to investigate morphological aspects influencing feed intake. This study characterises the mouth aperture and mouthpart morphology through ontogeny from the first juvenile instar to adult in *P. ornatus* and *S. verreauxi* with the aim to identify key features needed in formulated feeds. These two species, have been identified as viable Australian lobster species for closed cycle aquaculture due to favourable biological characteristics, including fast growth (*P. ornatus*) and ability to withstand large temperature variations (*S. verreauxi*) (Fitzgibbon et al., 2017; Smith et al., 2009b).

3.2 Materials and methods

3.2.1 Experimental animals

Palinurus ornatus and *S. verreauxi* were reared from hatching at IMAS. Lobsters utilised for mouth aperture measurements were collected from large juveniles, sub-

adults and adults (Table 3.1). Lobsters were sampled for morphological analysis from first instar juvenile, small juvenile, large juvenile and sub-adult and adult groups (Table 3.1). All specimens were wet weighed (WW) and measured for carapace length (CL) before euthanising in an ice slurry prior to terminal sampling.

3.2.2 Mouth aperture measurements

Whole fresh specimens were measured for maximum opening range of mandibles and oesophagus diameter. The measurements were taken with Castroviejo angled caliper 0 – 40 mm. The mandibles were opened with Kelly forceps and the opening range was measured at the narrowest point (between tubercles). Likewise, the caliper was inserted into the oesophagus and the measurement was taken in an elongated position. The oesophagus measurement was treated as circumference² (mm) and recalculated to diameter (mm) using the formula: diameter (mm) = circumference (mm)/ π . Both mandibles opening range (mm) and oesophagus diameter (mm) were converted to percent of experimental lobsters CL.

3.2.3 Statistical analysis

The mouth aperture measurements (mandibles opening range to CL (%) and oesophagus diameter to CL (%)) were analysed with one-way ANOVA. Shapiro Wilk W-test and Levene's test were used for normality and homoscedasticity testing, respectively. When a significant difference was found, post hoc analysis of means was performed (Tukey's HSD test). Significance was accepted at $p < 0.05$. SPSS statistics software v. 24 was used for calculations.

3.2.4 Specimen preservation

Lobster samples from first instar juvenile, small juvenile and large juvenile groups were preserved whole, in the large juveniles, thorax (plastron) was incised in the middle to facilitate penetration of fixative and preservative. In the sub-adult and adult group, the abdomen was removed from samples, and only cephalothorax was fixed and preserved. Prepared samples were immersed in 7% formalin (7 ml of 37 – 40% formaldehyde and 93 ml of filtered seawater) and fixed for 24 h, then rinsed twice with filtered seawater. The samples were then stored in 70% ethanol (70 ml of 100% ethanol, 25 ml of deionised water and 5 ml of glycerol) until analysis. The preservative was replaced after seven days to ensure a standard 70% concentration of ethanol was maintained.

3.1.1 Morphology analysis

Mouthparts from collected specimens were dissected as detailed in Figure 3.1 and photographed. Samples in the smaller size range (first instar juveniles, small and large juveniles) were dissected under a stereomicroscope Leica M80, on base Leica TL 3000 Ergo, using electrolytically sharpened needles and microscalpels (Brady, 1965; Conrad et al., 1993) and photographed with Leica DFC 450 C camera. Mouthparts from large specimens (sub-adults and adults) were dissected with scalpels (blade sizes 11 and 22), forceps (Kelly forceps), and dissecting scissors and photographed with stand-mounted Canon EOS 400D with EFS 17-85 mm M 0.35 m/1.2 ft lens. The mouthparts from each age group were examined using a Leica MZ16 stereo dissecting microscope and Leica DM2500C Nomarski Interference compound microscope with N-PLAN objectives. Representative examples were illustrated (line drawings on

tracing paper) with the aid of a camera lucida. The terminology used in descriptions follows Paterson (1968) and Cox et al. (2008).

Table 3.1. Carapace length (CL) and wet weight (WW) of *Panulirus ornatus* and *Sagmariasus verreauxi* used for mouth aperture and mouthparts morphology analysis; N, number of animals (unbalanced due to availability).

Analysis	<i>Panulirus ornatus</i>				<i>Sagmariasus verreauxi</i>		
	N	CL (mm)	WW (g)	N	CL (mm)	WW (g)	
First instar juvenile	Mouthparts	3	5.75 ± 0.17	0.11 ± 0.01	3	10.22 ± 0.67	0.41 ± 0.07
Small juvenile	Mouthparts	3	9.30 ± 1.84	0.70 ± 0.46			
Large juvenile	Mouthparts	3	20.06 ± 1.11	7.16 ± 0.85	3	37.56 ± 0.19	28.78 ± 1.18
	Mouth aperture	6	30.79 ± 0.79	29.27 ± 2.12	7	40.57 ± 1.80	35.81 ± 4.94
Sub-adult and adult	Mouthparts	10	93.77 ± 6.92	798.25 ± 135.45	12	120.10 ± 13.85	1,004.34 ± 197.39
	Mouth aperture	5	83.24 ± 6.72	551.71 ± 138.33	9	109.02 ± 17.12	779.01 ± 218.69

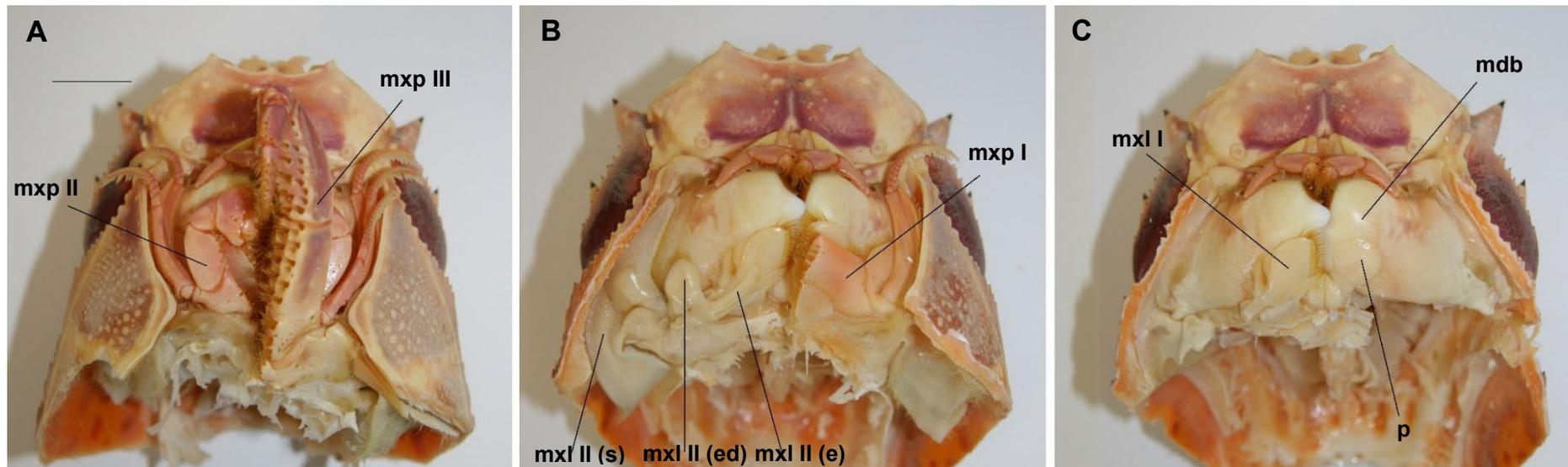


Figure 3.1. Schematic showing steps of mouthpart removal on the *Sagmariasus verreauxi*; (A) left maxilliped III (mxp III); right maxilliped II (mxp II), exposed by removing right mxp III; (B) left maxilliped I (mxp I), exposed by removing left mxp III and II; right maxillae II scaphognathite (mxl II (s)) located in exhalant passage, endopodite (mxl II (ed)) and endites (mxl II (e)), exposed by removing right mxp III, II and I; (C) right maxillae I (mxl I), exposed by removing right mxp III, II, I and mxl II; left mandible (mdb) and paragnath (p), exposed by removing left mxp III, II, I and mxl II, I. Scale bar: 20 mm.

3.1 Results

3.1.1 Mouth aperture

Mouth aperture was significantly greater for *P. ornatus* compared to *S. verreauxi* in both measured variables (mandibles opening range to CL (%), $F = 36.457$, d.f. 3,22, $p = 0.000$; oesophagus diameter to CL (%), $F = 15.367$, d.f. 3,22, $p = 0.000$) (Figure 3.2). The effect of size group on mouth aperture dimensions was not found (Figure 3.2).

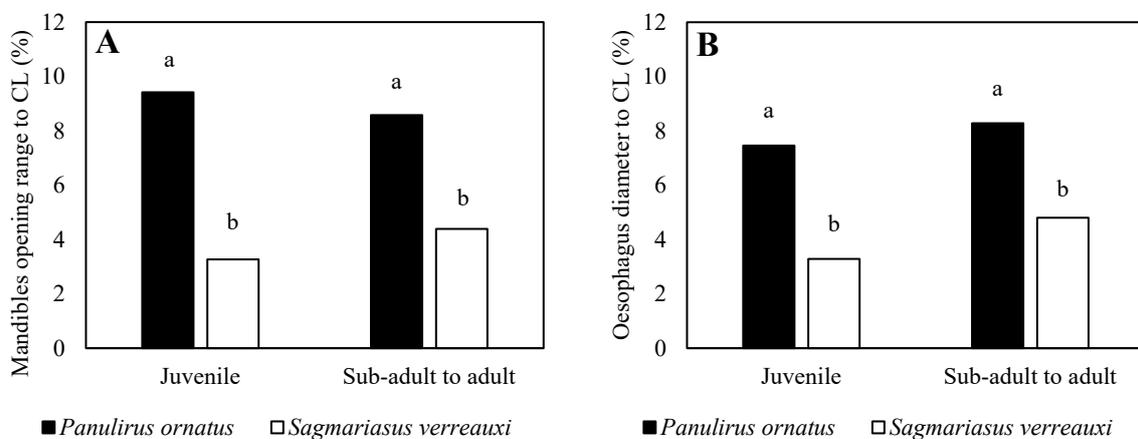


Figure 3.2. *Panulirus ornatus* large juvenile ($n = 6$) and sub-adult to adult ($n = 5$); and *Sagmariasus verreauxi* large juvenile ($n = 7$) and sub-adult to adult ($n = 9$); mouth aperture dimensions (mandibles opening range (A); oesophagus diameter (B)), presented as percent (%) of their carapace length (CL). Values are mean \pm S.E., significant differences are marked with superscripts.

3.1.2 Mouthparts

Mandibles

Mandibles for both species examined are wide and asymmetrical in the gnathal lobe, with a deep indentation between incisor and molar process in the left mandible, and a slight protrusion on the right mandible, where incisor and molar process are fused (Figure. 3.3A and 3.3B for *P. ornatus* and *S. verreauxi*, respectively). The indentation

and protrusion on the mandibles are more pronounced for *P. ornatus*. The molar process of mandibles carry tubercles, which resemble teeth in both species (Figure 3.3E and 3.3F). The incisor process in the right mandible slightly overlaps the left incisor in the proximal part in both species. Three segmented mandibular palps curve inwards and carry setation, including spine-like setae. Setation is predominantly at the distal segment of mandibular palps in both species but is more abundant in *S. verreauxi*. The mandibular palps in *P. ornatus* are thinner with a larger distal segment. The paragnaths, labrum and metastoma are similar across both species (Figure 3.3C and 3.3D).

The mandibles and mandibular palps remain similar through ontogeny, with varying degrees of calcification. In the first instar juveniles, mandible calcification increases from the anterior apodeme, which is transparent, to a heavily calcified proximal end near incisor process/masticator endite. The mandibular palps are transparent with light pigmentation (Figure 3.3G and 3.3H).

Maxilla I

Maxilla I in both species is flattened and consists of two endites and endopodite. The shape of endites and endopodites is notably different between species. In *P. ornatus*, endites are bent and elongated towards mouth aperture and in *S. verreauxi* the endites are shorter and wider (Figure 3.4A and 3.4B). The endopodite of *P. ornatus* is large, oval shaped and is directed laterally. In *S. verreauxi*, the endopodite is smaller, pointed and directed anteriorly, along the anterior endite. Setation includes spines located on the proximal side of endites and setae on the endopodite. The setation is richer and longer in *P. ornatus*, specifically on the anterior sides of the endites and endopodite. Through ontogeny, few differences in maxilla morphology were observed (Figure 3.4D and 3.4E). In first instar *S. verreauxi*, maxilla I is less pronounced than that present in

later juveniles, likewise, endites are less angled and spines point anteriorly (Figure 3.4C). This was not observed in *P. ornatus* maxilla I which are the same throughout the ontogeny documented in this study.

Maxilla II

Maxilla II in both species is flat and consists of a large scaphognathite located in the exhalant passage (Figure 3.1B), endites and endopodite located posteriorly from the maxilla I (Figure 3.5A and 3.5B). Three narrow endites are present in the *S. verreauxi* with two wide endites in *P. ornatus*. The endopodite is rounded and well distinguishable in *S. verreauxi* while in *P. ornatus* the triangular endopodite and endites are fused at the base. Setation is present on the scaphognathite edges, and is richer in *S. verreauxi*, specifically at the posterior part of the scaphognathite. The elongated setae at the posterior tip, is longer in *P. ornatus* compared to *S. verreauxi*. First instar juveniles of both species had proportionally larger setation (Figure 3.5C and 3.5D), greater transparency of maxilla II (Figure 3.5E and 3.5F) and smaller endites than in adults, in relation to the overall size of the mouthpart (Figure 3.5C and 3.5D).

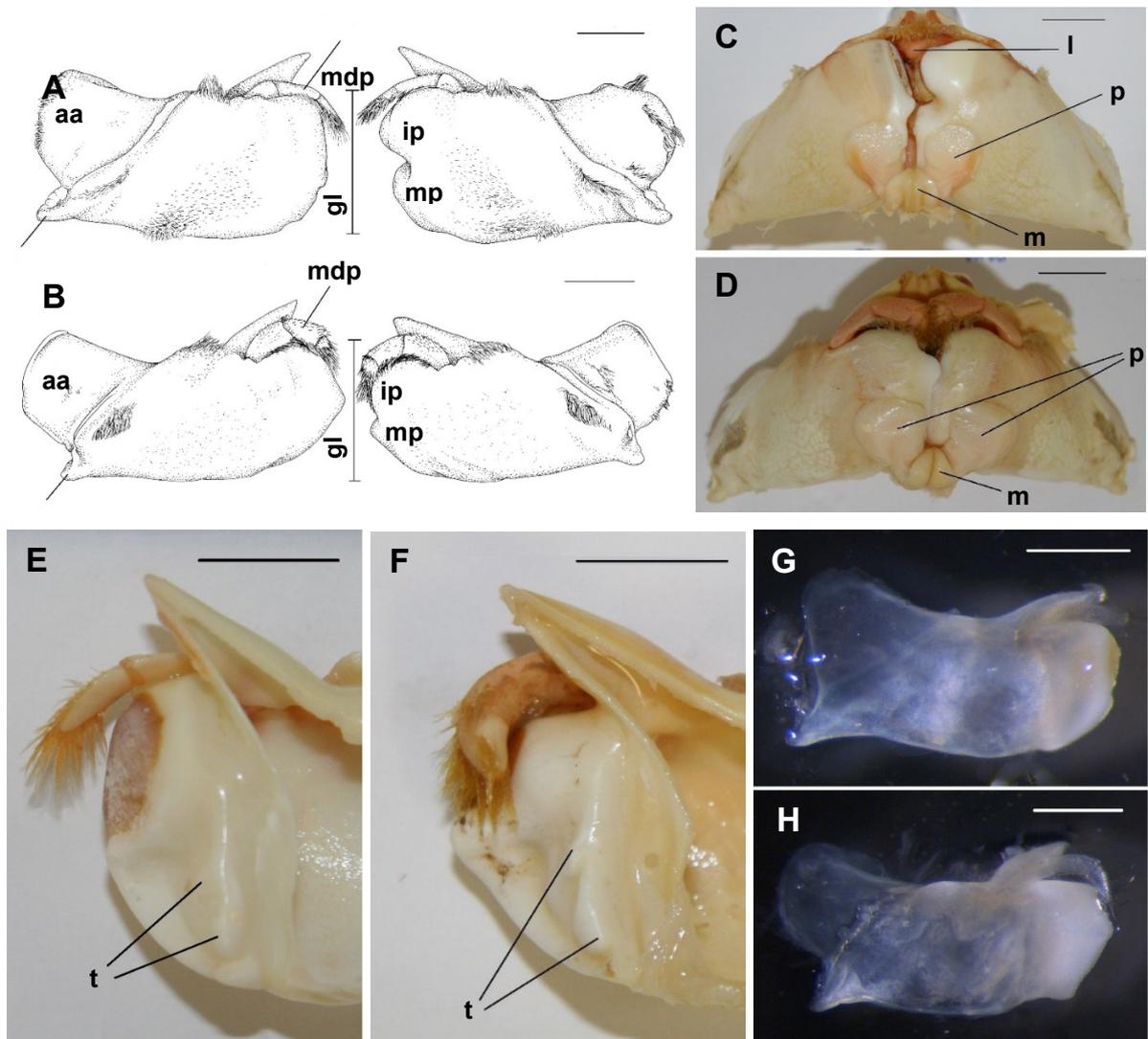


Figure 3.3. (A) *Panulirus ornatus*; and (B) *Sagmariasus verreauxi* mandibles; (C) adult *P. ornatus*; and (D) *S. verreauxi* mandibles; (E) adult *P. ornatus*; and (F) *S. verreauxi* dorsal side of right mandible; (G) first instar juvenile *P. ornatus*; and (H) *S. verreauxi* right mandible. aa, anterior apodeme; c, condyle; mdp, mandibular palp; gl, gnathal lobe; ip, incisor process; mp, molar process; l, labrum; m, metastoma; p, paragnaths; t, tubercles. Scale bar: A-F 10 mm; G-H 0.5 mm.

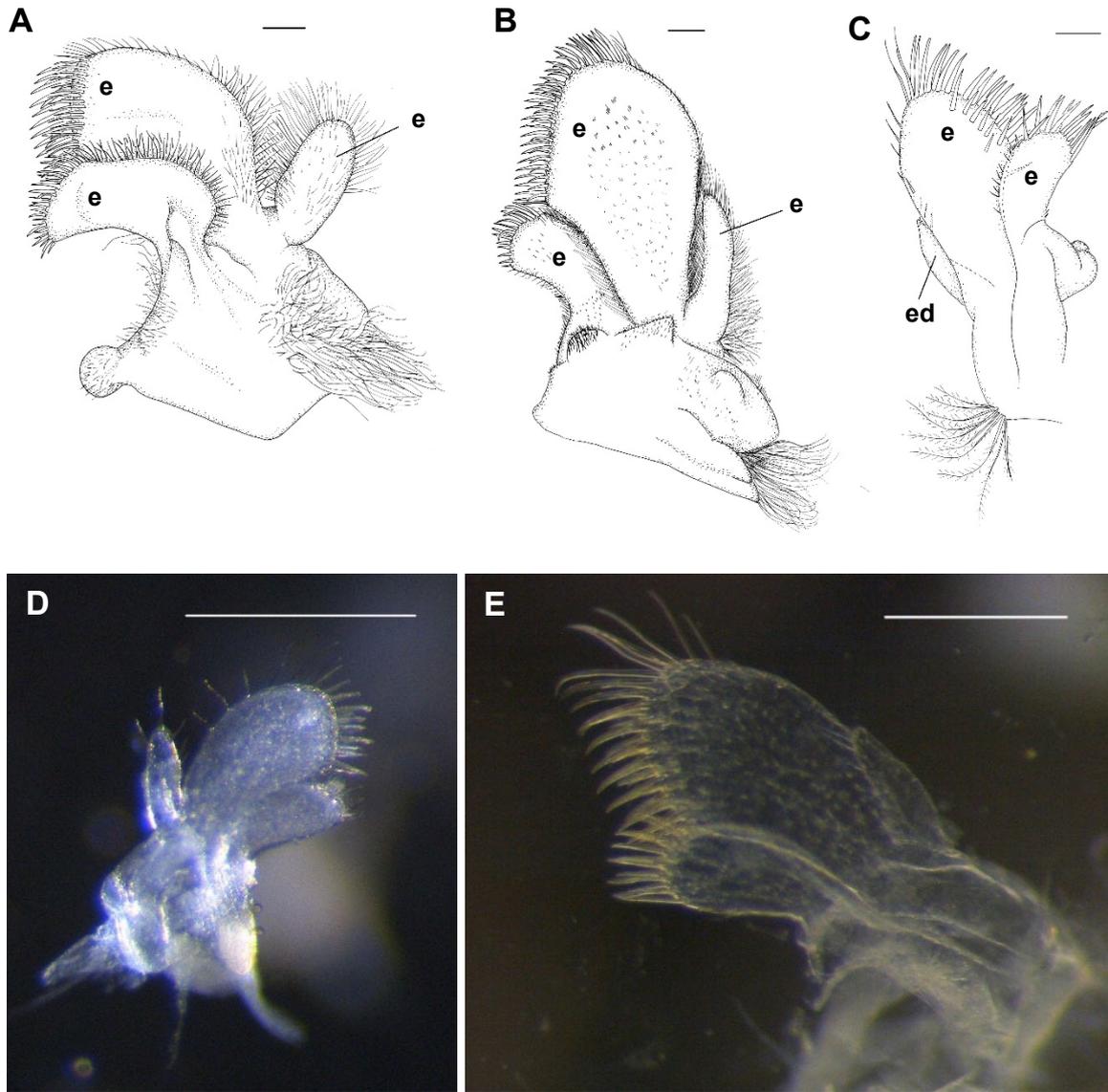


Figure 3.4. (A) *Panulirus ornatus*; and (B) *Sagmariasus verreauxi* left maxilla I; (C) first instar juvenile *S. verreauxi* right maxilla I; (D) first instar juvenile *P. ornatus* right; and (E) *S. verreauxi* (E) left maxilla I. e, endite; ed, endopodite. Scale bar: A-B 2 mm; C 0.2 mm; D-E 0.5 mm.

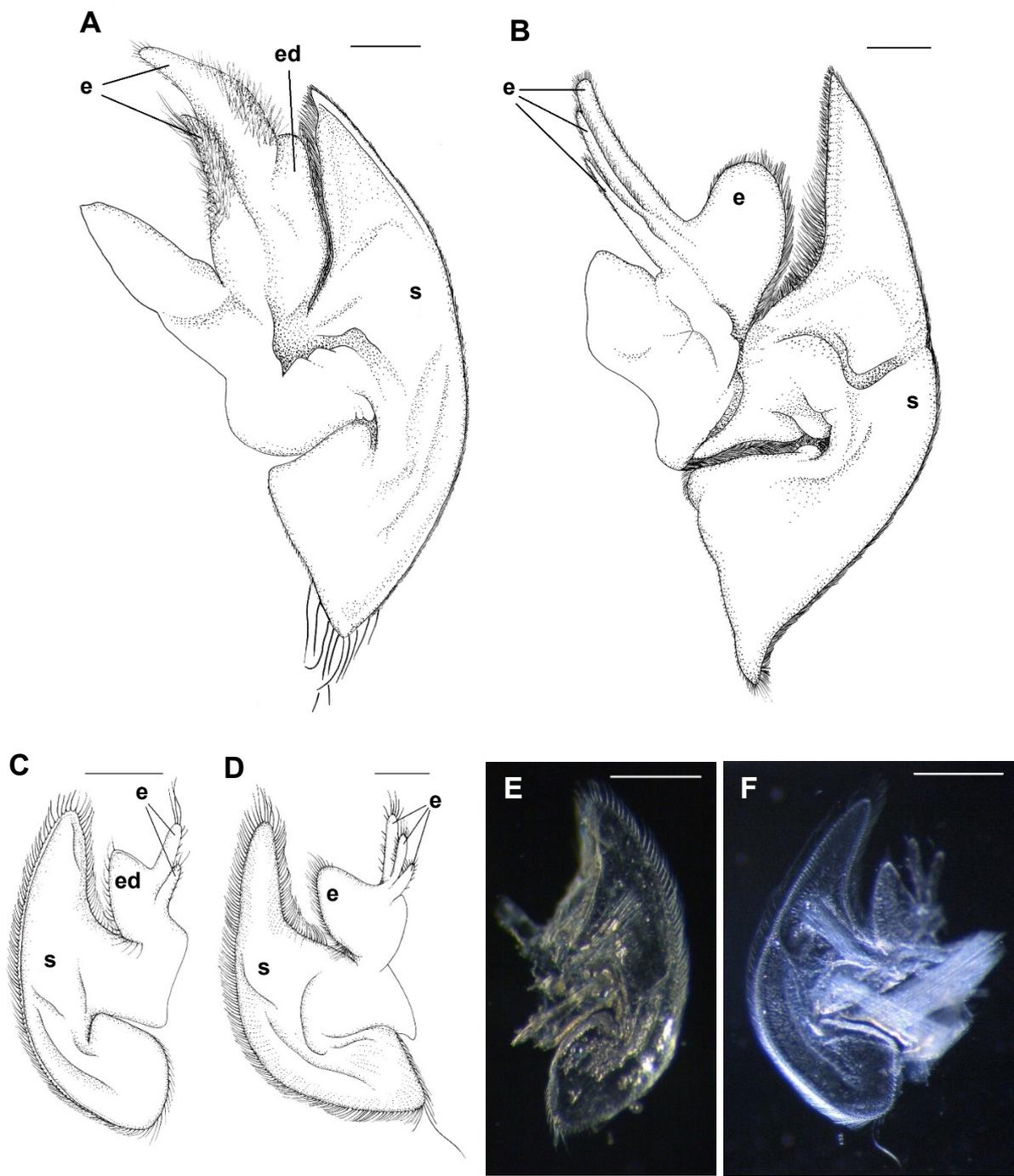


Figure 3.5. (A) *Panulirus ornatus*; and (B) *Sagmariasus verreauxi* left maxilla II; (C) first instar juvenile *P. ornatus*; and (D) *S. verreauxi* right maxilla II; (E) first instar juvenile *P. ornatus* left; and (F) *S. verreauxi* right maxilla II. e, endite; ed, endopodite; s, scaphognathite. Scale bar: A-B 5 mm; C-F 0.5 mm.

Maxilliped I

Maxilliped I consists of a basis, two fused endites, endopodite, exopodite and epipodite in both species (Figure 3.6A and 3.6B). The endites are fused with endopodite and bear setae on the medial side of endites and lateral side on endopodite. Exopodite bears a segmented flagellum with setation.

The differences observed through ontogeny included transparency in first instar juveniles in both species (Figure 3.6D and 3.6E) and morphology of flagellum which is not fully developed and bears little setation in first instar juvenile and large juvenile *S. verreauxi* (Figure 3.6C). In sub-adult to adult *S. verreauxi*, the flagellum is more developed and has more setation. This was not observed in first instar juvenile *P. ornatus* where the flagellum bears similar setation to adult lobsters (Figure 3.6D). The amount of setation on maxilliped I and flagellum, increases with ontogeny in both species.

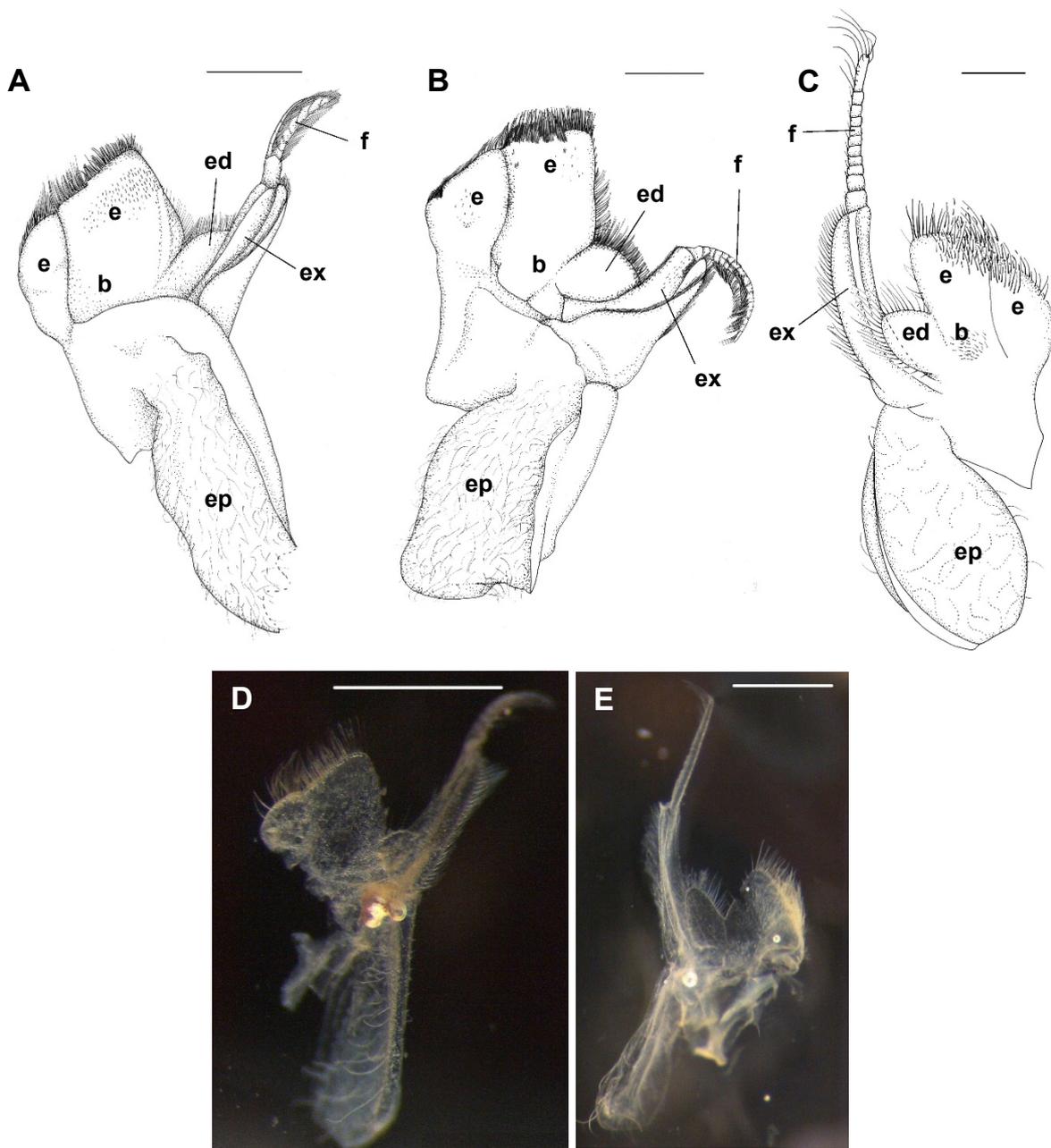


Figure 3.6. (A) *Panulirus ornatus*; and (B) *Sagmariasus verreauxi* left maxilliped I; (C) first instar juvenile *S. verreauxi* right maxilliped I; (D) first instar juvenile *P. ornatus* right; and (E) *S. verreauxi* right maxilliped I. e, endite; ed, endopodite; ex, exopodite; f, flagellum; b, basis; ep, epipodite. Scale bar: A-B 10 mm; C 0.5 mm; D-E 1 mm.

Maxilliped II

Maxilliped II in both species consists of a basis, endopodite and exopodite (Figure 3.7A and 3.7B). The endopodite consists of five segments; the ischiopodite, meropodite, carpopodite, propodite and dactylopodite. In *P. ornatus*, the ischiopodite is shorter and meropodite is longer in proportion to the size of mouthpart compared to *S. verreauxi*. Setation is present on the endopodite in both species, with spines located on the propodite and dactylopodite. The exopodite in *S. verreauxi* bears a segmented flagellum with setation, which is absent in *P. ornatus*.

Maxillipeds II of first instar juveniles were transparent and had lighter pigmentation compared to the juvenile and adult stage (Figure 3.7C and 3.7D). In the first instar juveniles, maxillipeds II carry spines on propodite and dactylopodite, and little amount of setation, which increases with growth. The flagellum present on the exopodites in the first instar juvenile and large juvenile *S. verreauxi* is less developed with limited setation compared to the later juvenile and adult stages (Figure 3.7D).

Maxilliped III

Maxilliped III consists of a basis, segmented endopodite in both species, and an exopodite with segmented flagellum in *S. verreauxi* (Figure 3.8A and 3.8B). The endopodite consists of five segments; ischiopodite, meropodite, carpopodite, propodite and dactylopodite. Setation is present on the median side of endopodite segments and spines are present on the dactylopodite. On the median side of the basipodite, ischiopodite and posterior half of the meropodite, there are crista dentata in both species.

Maxillipeds III are moderately transparent with light pigmentation in the first instar juveniles of both species (Figure 3.8C and 3.8D). In the first instar juvenile and large

juvenile *S. verreauxi*, flagellum present on the exopodite is not fully developed and bears little setation, which increases with development and growth (Figure 3.8D).

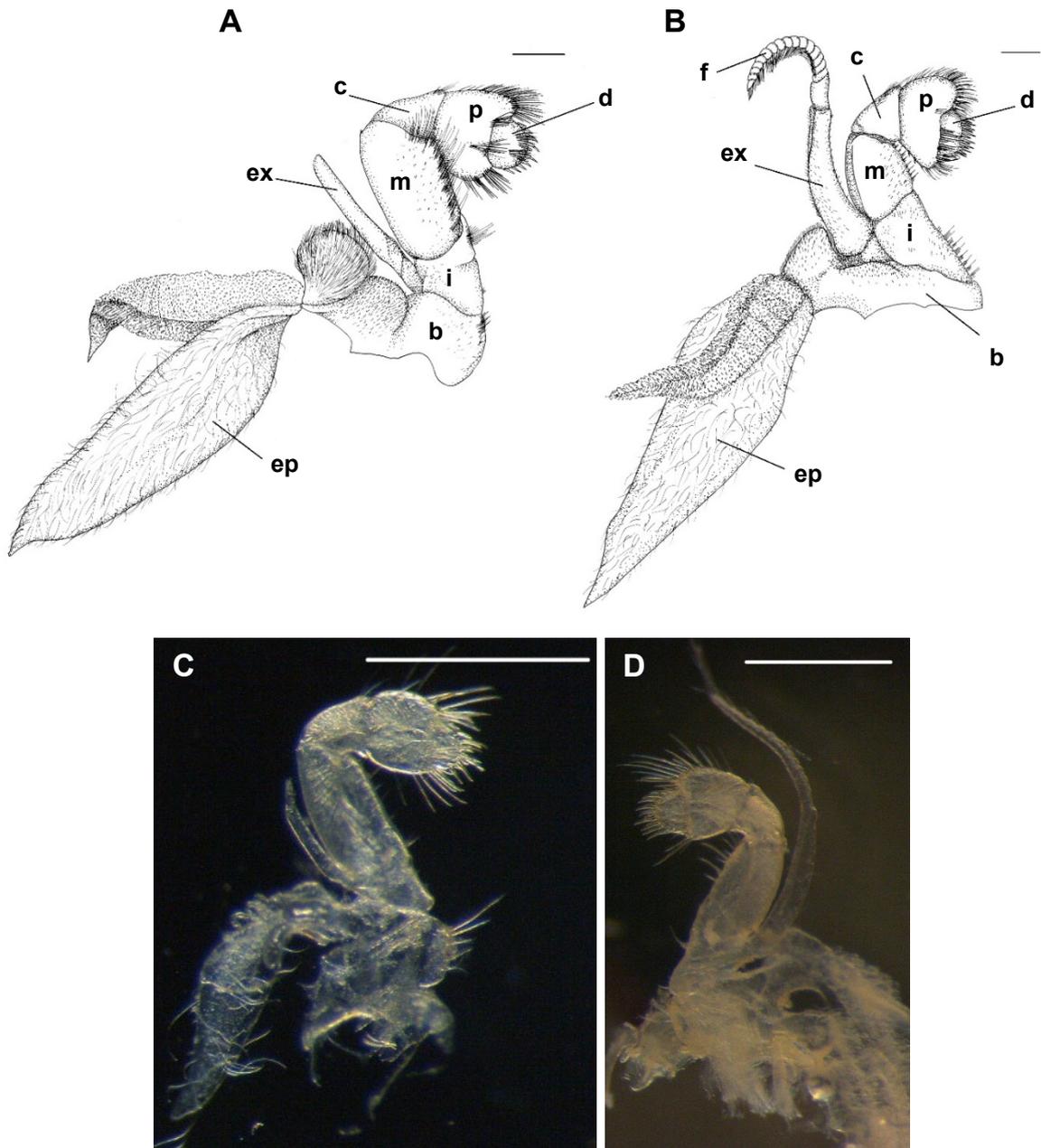


Figure 3.7. (A) *Panulirus ornatus*; and (B) *Sagmariasus verreauxi* right maxilliped II; (C) first instar juvenile *P. ornatus* right; and (D) *S. verreauxi* left maxilliped II. d, dactylopodite; p, propodite; c, carpopodite; m, meropodite; i, ischiopodite; b, basis; ex, exopodite; f, flagellum; ep, epipodite. Scale bar: A-B 5 mm; C-D 1 mm.

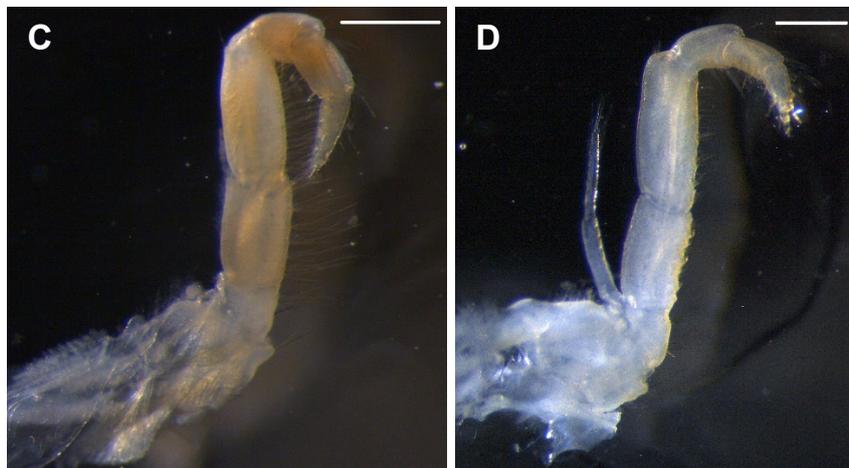
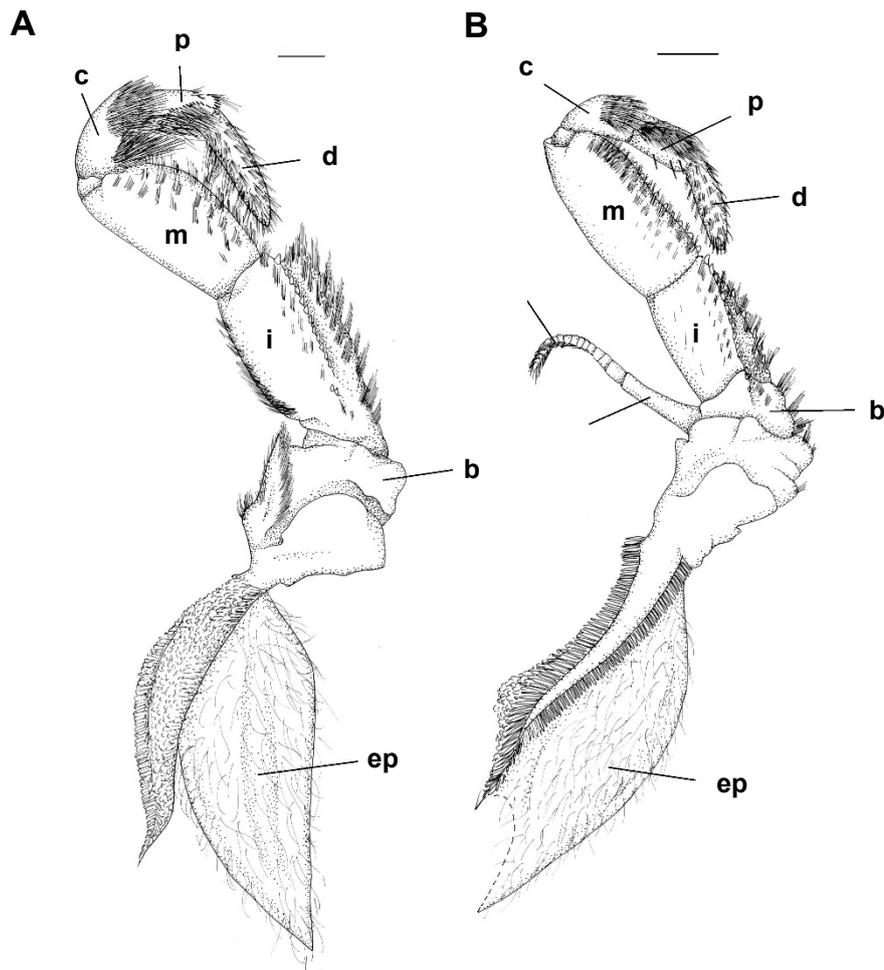


Figure 3.8. (A) *Panulirus ornatus*; and (B) *Sagmariasus verreauxi* right maxilliped III; (C) first instar juvenile *P. ornatus*; and (D) right maxilliped III. d, dactylopodite; p, propodite; c, carpopodite; m, meropodite; i, ischiopodite; b, basis; ex, exopodite; f, flagellum; ep, epipodite. Scale bar: A-B 5 mm; C-D 0.5 mm.

3.2 Discussion

This study showed that relative size of lobster mouth aperture is species specific and increases proportionally with growth. The mouth aperture size constitutes similar percentage of CL for large juvenile and sub-adult and adult lobsters demonstrating that it is possible to estimate this important feeding morphology trait throughout juvenile development using non-destructive measures. An understanding of mouth aperture size may be important for minimising feed waste in the development of efficient formulated feeds for lobsters. It has been previously demonstrated that feed wastage caused by fragmentation during ingestion can be decreased considerably by altering pellet dimensions in lobsters (Sheppard et al., 2002). However, these previous studies did not examine the relationship with lobster mouth aperture which should be used in future studies to define the optimal dimensions of pellets to minimise waste with lobsters. Once defined this could be used for modifying formulated feed diameter to fit mouth aperture of different sizes of lobsters and be useful as a management tool in routine husbandry of lobsters to minimize feeding waste. Differences in mouth aperture found between *P. ornatus* and *S. verreauxi*, indicate considerable species-specific differences which should be considered for the appropriate feeding of the different species. This finding agrees with the conclusion of Cox et al. (2008) that mouth aperture morphology may be not transferable between species. To minimise waste generated during ingestion, the formulated feed must fit the mouth aperture and allow for rapid and secure handling by the mouthparts. Inappropriate pellet sizes that lead to extended and inefficient handling, may also result in an increase of attractant leakage before ingestion and ultimately reduced feed intake. However, it is noted that the appropriate pellet sizes to suit mouth aperture is just one of several feed form aspects that may influence efficient feeding by lobster. Other aspects such as pellet

length, texture and stability also need to be considered in the development of effective formulated feeds for lobster aquaculture.

Panulirus ornatus and *S. verreauxi* mouthpart morphology remains quite similar through ontogeny from first instar juvenile to adult lobsters. However, key differences that should be considered in the development of formulated feeds included a gradual increase in calcification, pigmentation and changes in setation of the mouthparts from first instar juveniles to adults. Further, the maxilla II in first instar juveniles has larger setation and shorter endites than in adults, proportionally to the size of the mouthpart. Early juvenile spiny lobsters are solitary, they dwell and feed in the same location, usually macroalgae and seldom leave their habitat to feed compared to larger lobsters (Radhakrishnan et al., 2019a). Early juveniles feed best on soft and pulpy feeds, including clam tissue (Cox et al., 2008). Their mouthparts are predominantly transparent, suggesting limited capability to consume well armoured prey due to high risk of appendages damage, while soft prey allows for safe ingestion of feed pieces larger than mouth aperture (Cox et al., 2008; Juanes and Hartwick, 1990). This strategy maximises food intake and, as a consequence, increases growth rate and chance of survival. The physical properties of formulated feeds for early juveniles should be adapted to the lobsters needs and preferences at this stage of life. The soft texture allowing for ingestion of formulated feed pieces larger than mouth aperture may optimise feed intake and in consequence growth. To minimise waste the feed items should not disintegrate easily (Sheppard et al., 2002), indicating that relatively high viscosity may be required for early juveniles. The shift from solitary early juveniles to communal juveniles, sub-adults and adults is concurrent with change of habitat and the development of social hierarchies that include competition for feed (Radhakrishnan et al., 2019a). *Panulirus argus* is subject to ontogenetic shifts in habitat, daily activity

levels, foraging times and gregariousness resulting with concurrent changes in diet (Childress and Herrnkind, 1994). This leads to changes of available feed and feeding opportunities and, as a consequence, feeding behaviour and preferences. Throughout this time, the mouthpart functions remain similar (Cox et al., 2008) and the mouthparts allow for wide range of manipulations and ingestion methods. This indicates that increased mouthpart size is the predominant factor responsible for diet shifts and concurrent changes of habitat and social behaviour through ontogeny. Increasing calcification and robustness of mouthparts with lobsters development is likely to allow for consumption of firmer textured feeds as was suggested in Cox et al. (2008); and this is likely to remain relatively constant after the early juvenile stage.

The feeding behaviour and morphology is likely to be very different for lobsters feeding on formulated feeds in comparison to their natural diet. Lobsters collect and hold pellets with pereopods I before breaking them with the maxillipeds, maxillae and mandibles (Cox et al., 2008; Sheppard et al., 2002). This process involves multiple rotations and may include holding pellet with pereopods or maxillipeds III while the remaining mouthparts are used for breaking. Pellets of large sizes are cut or broken by mouthparts, to fit the mouth aperture, which causes considerable fragmentation and wastage, while the small pellets may be consumed whole (Sheppard et al., 2002). Behavioural studies show that lobsters tear pellets by holding them with mandibles and maxillipeds II and pulling the pellets with the maxillipeds III. Next, the maxillipeds III and II move the feed towards the mouth aperture and the mandibular palps position and push the feed in to mouth aperture for swallowing (Cox et al., 2008; Sheppard et al., 2002). Our morphological observations agree with the behavioural studies of Cox et al. (2008) and Sheppard et al. (2002) on spiny lobsters. Spines on distal segments of maxillipeds III and II in both species, indicate a function in holding and transporting

feed during ingestion. Moreover, we documented the spines on endites of maxilla I, indicating similar function for this mouthpart. Cox et al. (2008), showed that maxilla I spines were responsible for probing feed, holding in position during biting with mandibles and removing unwanted pieces by pushing them anteriorly in *P. argus*. This suggests that formulated feed should be homogenous to minimise risk of lobsters sifting through and rejecting unwanted pieces and should be soft enough to allow penetration of spines for efficient holding and ingestion.

This study showed that setae are prominent on most of the mouthparts in both *P. ornatus* and *S. verreauxi*, with subtle differences during ontogeny and between species. Many decapods use setae for filter feeding (Garm, 2004a). In the Hermit Crab *P. bernhardus*, filter feeding includes creation of currents by flagella on the exopods of maxillipeds III and II, the catching small prey with the maxillipeds III and both maxillae, brushing the maxillipeds III with the maxillipeds II and transporting prey towards mouth aperture for ingestion (Gerlach et al., 1976). Correspondingly, the exopodites with flagella on the maxillipeds III, II and I are used to create currents to carry feed to mouth aperture in *J. edwardsii* (Sheppard et al., 2002). The early juvenile life stages of the clawed lobster *H. americanus* have two feeding strategies, predation on small bivalves and crustacean meiofauna or filtering plankton suspended in water column, including copepods, molluscan and polychaetes larvae and invertebrates eggs (Lavalli and Barshaw, 1989; Sainte-Marie and Chabot, 2002). Our morphological observations show that *S. verreauxi* carries flagella on maxilliped III, II and I exopods, which may be an indication of ability to create currents and as a consequence facilitate filter feeding. This observation was absent from our study of *P. ornatus*, which does not carry exopod on their maxilliped III, is without flagellum on maxilliped II and only flagellum on exopod of maxilliped I. The reduction of exopodites in *P. ornatus*, may be

the consequence of fast growth of this species, which requires intensive feeding on the largest available prey (Kenway et al., 2009; Williams, 2009). In both species analysed, the maxilla I and II carry setation, which is more abundant in *S. verreauxi*, specifically on scaphognathite of maxilla II. The early juveniles in both species carry more abundant setation proportionally to the size of mouthpart, in comparison with adults, particularly on the maxilla II, which is important in filter feeding of *P. bernhardus* and was indicated as suitable for filtration and handling small particles in *P. argus* (Cox et al., 2008; Gerlach et al., 1976). This likely indicates utilisation of filter feeding by early juveniles, which potentially decreases in importance during ontogeny. This feeding technique is unlikely to be useful as major component in spiny lobster aquaculture; however, it may be considered as a delivery method during first feeding when it can be difficult to encourage feeding on to artificial feeds.

3.3 Conclusion

The mouthpart morphology and mouth aperture are likely important aspects for effective formulated feed development in the advancement of lobster aquaculture. The correlation of lobster mouth aperture with CL likely provides a species-specific tool for defining optimal dimensions and performing routine adjustments of pellet size optimal to the size of the lobster. However, further research is required to establish the relationship between mouth aperture and feeding efficiency expressed as waste produced during ingestion process, in lobsters. The ontogeny of mouthpart morphology highlights likely differences in feed texture requirements throughout lobster development, from soft feeds for early juveniles to firmer feeds for sub-adults and adults. The spines and setation on the mouthparts are likely important features affecting feeding effectiveness and differ considerably between species and during

ontogeny. The spines and crista dentata are likely important for the holding and the manipulation of feed, indicating that pellet texture should accommodate for this mode of puncturing and holding. Ontogenetic changes in setation, specifically on maxilla II, indicate shifts in functions of mouthparts at different life stages, which may be the evidence of filter feeding in early juveniles. This observation may have relevance to the first feeding juvenile stage. Overall, the mouth aperture and mouthparts morphology of two analysed lobster species indicate that formulated feeds need to be tailored to species and life stage, particularly regarding dimensions and texture.

Chapter 4 Effect of pellet texture and dimensions, on feed waste type and feeding efficiency in juvenile *Sagmariasus verreauxi*

Abstract

This study examined the relationship between feeding efficiency and feed waste by juvenile spiny lobster, *Sagmariasus verreauxi*, fed different formulated pellet diameters or pellet lengths across two separate experiments. Feed texture (hard and dry, HDP; soft and moist, SMP) was also examined in the pellet diameter experiment. The pellet length experiment examined seven lengths of HDP only. Juvenile lobsters (weight; $206.5 \text{ g} \pm 5.7 \text{ S.E.}$ and $224.6 \text{ g} \pm 6.8 \text{ S.E.}$, in diameter and length experiments, respectively) were fed experimental feeds at 0.5% BW for 6 h. Feed waste was then collected and categorised as either feeding related waste (FRW) or non-feeding related waste (NFRW). The FRW was resulting from manipulation and maceration when feeding, NFRW was uneaten/unmanipulated feed. Feeding efficiency and feed intake were also calculated. For all feed types the FRW increased with increasing pellet diameter and pellet length. The increase in FRW corresponded with a decrease in NFRW, particularly for HDP, resulting in no difference in total feed waste in any treatment investigated. Thus, even with improved feeding efficiency with small feed

dimensions, feed intake was not improved. Feed leaching rate decreased with increasing pellet size, suggesting a more rapid decline in feed attractiveness for smaller pellets. This finding suggests that currently a counteractive interaction exists between pellet size and feed attractiveness and suggests improving attractiveness would further enhance feeding. Future research should aim at optimising feed dimensions simultaneously to support efficient feeding whilst enhancing prolonged attraction/gustatory stimulation.

4.2 Introduction

The feeding behaviour of spiny lobsters remains an obstacle for formulated feed development, partly due to the inefficient format and consumption of feeds resulting from high feeding wastage (Sheppard et al., 2002). Spiny lobster feeding behaviour is often described as “messy” due to external fragmentation of feed caused by handling with multiple appendages and maceration of the formulated feeds prior to ingestion (Sheppard et al., 2002; Zoutendyk, 1988). Several studies on the feed intake mechanisms of spiny lobsters have enabled a clearer understanding of their feeding behaviour (Cox et al., 2008; Francis et al., 2014; Lau, 1987; Watling, 2013) and helped to set directions for research into minimising feed wastage, and indirectly maximising feed intake and feeding efficiency. Understanding the optimum feed dimensions relative to lobster size, particularly in relation to the size of the mouthparts, is one approach to reduce feeding related waste (FRW). Such an approach has been investigated with *Panulirus argus* (Cox et al., 2008), *Jasus edwardsii* (Sheppard et al., 2002) and *Panulirus ornatus* (Smith et al., 2009a).

Feed dimensions relative to lobster size is an important factor when investigating FRW, however other factors such as feed texture and species are additional

considerations. For practical reasons feed texture is often imprecisely defined, commonly being referred to as “soft, moist” and “hard, dry” pellets (Conklin et al., 1977; Smith et al., 2009a). For *P. ornatus*, it was found that soft, semi-moist pellets (1 mm diameter) are less efficiently consumed (more waste produced) by small size lobsters (~2 g) in comparison to hard, dry pellets (1 mm diameter), however there was a preference for small lobsters to consume soft, semi-moist pellets (Smith et al., 2009a). For larger *P. ornatus* (≥ 50 g), feed diameter and length appeared to be more critical to feed consumption efficiency than feed texture. In *P. argus*, first instar juveniles feed most efficiently on soft and pulpy feeds and change their preferences to firmer feeds with growth (Cox et al., 2008). This shift of texture selectivity may be aligned with the decreasing risk of appendages damage during growth (Juanes and Hartwick, 1990). Hence, feeding appendages morphology and animal size is an important factor to consider when investigating optimal feed pellet dimension and texture. In Chapter 3 the mouthpart morphology and mouth aperture of *Sagmariasus verreauxi* and *P. ornatus* from first instar to adult were documented. There was an indication that mouth aperture in *S. verreauxi* may be an important parameter to consider when optimising formulated feed dimensions. While feed texture and feed dimensions influence the feeding of spiny lobster, Cox et al. (2008) highlighted that feeding behaviour differences exist between spiny lobster species, indicating that formulated feed development for spiny lobsters may be species specific.

Studying the feed waste when spiny lobsters eat is critical to understanding the feeding behaviour of spiny lobsters (Smith et al., 2009a), however to date the feed waste for *S. verreauxi* has not been studied. Feed waste can either be qualified as uneaten/unmanipulated feed, termed as non-feeding related waste (NFRW), which is unbroken, unfragmented and without any obvious manipulation or waste resulting from

manipulation and maceration when feeding, termed FRW, which consists of partially eaten, fragmented or macerated pellets and fine feed particles. Thus, distinguishing between the two types of feed waste would be highly beneficial to understand feeding behaviour, such as feed preferences and feeding efficiency. For instance, if the feed waste is composed of a high percentage of NFRW, feed attractiveness and/or gustatory stimulation may be sub-optimal. On the contrary, a high percentage of FRW in feed waste may indicate active feeding but with poor feeding efficiency. The ratios or amounts of the two types of feed waste may vary depending on feed size and/or texture. For example, is there an optimal feed dimension/texture which maximise feeding efficiency (low FRW) or enhance attraction/gustatory stimulation (low NFRW) or both? Thus, in the present study, we aim to investigate the type of feed waste produced by juvenile *S. verreauxi* when fed varied feed dimensions and textures in two separate experiments. In the first experiment (expt. 1), the effect of pellet diameter was investigated with two types of feed textures: hard and dry pellet (HDP) or soft and moist pellet (SMP). Based on expt. 1, in the second experiment (expt. 2), an appropriate feed diameter and texture were selected to investigate the effect of pellet length.

4.3 Materials and methods

4.3.1 Experimental animals and systems

Two feeding experiments (expt. 1 and 2) were conducted, with a total of 24 juvenile *S. verreauxi* lobsters used in each experiment. In expt. 1, feed waste type was examined when animals were fed different diameter HDP or SMP feeds, lobsters were an average wet weight (WW) of 206.5 g \pm 5.7 S.E., and carapace length (CL) of 74.3 mm \pm 0.8 S.E. In expt. 2, the effect of pellet length on feed waste type was examined with

lobsters $224.6 \text{ g} \pm 6.8 \text{ S.E.}$, WW and CL $76.4 \text{ mm} \pm 0.8 \text{ S.E.}$. A HDP of diameter of 4.3 mm with varied lengths was selected based on results from expt. 1. Prior to experimentation, lobsters were held in a communal tank and co-fed to excess with half shell blue mussels and a commercial shrimp pellet (2 mm diameter). At the start of each experiment lobsters were randomly allocated to eight rectangular 18 L vessels (38 cm length x 24 cm width x 24.8 cm height), with three individuals per vessel, to utilise available lobsters in size group and to optimise replication for further analysis (Blainey et al., 2014; Briceño et al., 2020). Each vessel was covered with a mesh lid above the water's surface to prevent lobsters from escaping (16 mm oyster mesh). Half of the lid was covered with additional mesh (1.6 mm oyster mesh) to provide additional shading. Two oyster mesh rectangles (15 cm long, 20 cm wide) were suspended along the long walls of the vessels to provide climbing substrate. Water was obtained from river Derwent (Taroona, Tasmania) and exchange rate was kept at six exchanges $\text{h}^{-1} \text{ vessel}^{-1}$ with aeration provided to each vessel. Water quality was maintained at a temperature of $20.7 \text{ }^{\circ}\text{C} \pm 0.0 \text{ S.E.}$, salinity $34.5 \text{ ppt} \pm 0.0 \text{ S.E.}$ and dissolved oxygen $9.5 \text{ mg L}^{-1} \pm 0.0 \text{ S.E.}$ (106.0% sat. $\pm 0.2 \text{ S.E.}$). The photoperiod was set at 9:15 L:D and all work during the dark-phase was done with the use of a red-light torch to minimise disturbance on nocturnal activity of lobsters (Fitzgibbon and Battaglione, 2012). The photoperiod at 9:15 L:D was set to accommodate experimental work, including feeding and collection of feed waste during dark phase and maintenance during light phase. The length of photoperiod within the used range does not negatively affect juvenile spiny lobsters feed intake, growth and survival (Chittleborough, 1975; Crear et al., 2003; Simon and James, 2007).

4.3.2 Experimental feeds

Feed composition for both expt. 1 and 2 were identical except for their physical characteristics and were made according to the formulation utilised in Shu-Chien et al. (2017). The manufacturing of the feed was performed as described by Landman et al. (2021). Briefly, after mixing the dry and wet feed components, the resultant feed dough was cold extruded with La Monferrina Dolly II pasta extruder. The feed strands produced after extrusion were designated as SMP and contained 51.55 ± 0.22 % moisture. Different dies were used to produce different SMP feed strands which were then further processed to meet the feed characteristics defined in expt. 1. and expt. 2. The pellet characteristics for expt. 1 and expt. 2 were as follows:

- 1) Expt. 1 – SMP feed strands of diameters, 1.5, 2.8, 3.7, 5.0, 7.0, 8.7, 10.2 mm were manufactured and cut to a standard length of 20 mm regardless of the diameter to produce SMP. Half of the manufactured SMP were dried from 6 to 24 h depending on the diameter, in a Steridium DS500 dryer, at 45 °C to 9.90 ± 0.61 % moisture to produce HDP. After drying of SMP to HDP, the pellet diameters shrunk and measured at 1.3, 2.3, 3.3, 4.3, 5.8, 7.8, 9.5 mm. Therefore, in total, 14 different feeds were produced from one batch, 7 SMP and 7 HDP.
- 2) Expt. 2 – one batch of HDP standard diameter of 4.3 mm were made as above but cut to lengths 5, 8, 13, 21, 33, 53, 84 mm. A standard 4.3 mm diameter HDP was selected as it represented the first equal or lower (\leq) available point where levels of FRW and NFRW were closest in HDP diameter tested in expt. 1.

In expt. 1, the eight vessels containing three lobsters each were randomized over a fourteen-day period to allocate feed treatment such that every individual vessel of lobsters was supplied with each of the fourteen different pellets (Table 4.1). Thus,

there were eight replicates for each pellet diameter and texture tested (HDP, n = 8; SMP, n = 8). Similarly, in expt. 2, eight vessels containing three lobsters were randomly supplied one of the seven different feed lengths (HDP, n = 8) to each individual tub (Table 4.2) each day of the seven days of testing.

4.2.1 Feeding and feed waste collection

Lobsters were acclimated to the experimental feeds for two days after allocation to the culture vessels. The acclimation to the pellet diameter test (expt. 1) included feeding with an equal mix of seven diameters of HDP on one day and an equal mix of seven diameters of SMP on the other, with a random order of feeding with HDP or SMP. The acclimation to the pellet length test (expt. 2) included feeding lobsters with an equal mix of pellets of seven lengths for two days after allocation. Each vessel was supplied with a ration at 0.5% lobster WW, on a feed dry matter (DM) basis. The daily feed ration of 0.5% lobster WW was set below the optimal feeding ration of 0.8 – 1.2% (Simon, 2009) to encourage complete feed consumption. In expt. 1, the feeding rations consisted of ~150, ~50, ~26, ~16, ~9, ~5 and 4 pellets from lowest to highest diameter respectively in either HDP or SMP. In expt. 2, the rations consisted of ~44, ~33, ~20, 14, 10, 6 and 4 pellets from lowest to highest length respectively. The feeding ration was added to vessels 5 min after the beginning of the dark phase. Lobsters were fed for six hours after which the feed waste was collected by siphoning. The siphoned material was captured on a 124 µm mesh screen (Landman et al., 2021), additionally an identical screen was used on the water outlets continuously, to trap any fine feed particles. The collected samples were gently rinsed with deionised water to remove salts and all NFRW were carefully separated from the total feed waste after careful visual inspection. The feed texture and water stability allowed for precise differentiation

between FRW and NFRW for both SMP and HDP as the pellets remained intact unless manipulated by the feeding behaviour of the lobsters. All feed wastes were kept frozen at -20 °C until DM determination. Additionally, feed DM loss due to nutrient leaching was examined in all feeds tested in both the diameter and length experiments at the equivalent feed ration amount of 0.5% of experimental lobsters WW. Each feed was added to the same culture vessels in triplicate, without lobsters. Each of the triplicate samples were collected after six hours of exposure to the experimental conditions and DM loss was determined. All feeds and feed waste collected were dried at 105 °C for 24 h (AOAC, 1999) to determine DM. After correction for leachate loss, NFRW and FRW were expressed as a percentage of the delivered feed ration. The feed intake (FI) was calculated as = Feed delivered – (NFRW + FRW). The feeding efficiency was expressed as $100\% - ((100\% \times \text{FRW}) / (\text{FI} + \text{FRW}))$, and was calculated to investigate how pellet dimensions affected FRW and its correlation with FI. NFRW was excluded from calculations, to focus solely on the ingestion related aspects.

Table 4.1. Expt. 1 feeding randomisation for fourteen consecutive days; experimental feeds included seven diameters (Ø) HDP (hard dry pellet) and seven diameters (Ø) SMP (soft moist pellet).

Feed type	Feed Ø (mm)	Feeding day number							
		Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8
HDP	1.3	13	14	7	7	5	10	10	4
HDP	2.3	10	10	6	9	7	9	2	6
HDP	3.3	5	8	1	1	13	6	4	9
HDP	4.3	2	6	10	11	3	1	14	7
HDP	5.8	3	3	8	3	8	5	1	13
HDP	7.8	14	12	13	13	2	12	12	2
HDP	9.5	7	9	5	10	14	4	8	3
SMP	1.5	9	7	9	5	12	7	6	8
SMP	2.8	8	5	11	8	11	13	11	12
SMP	3.7	4	13	4	12	10	8	13	1
SMP	5.0	12	11	12	6	6	3	3	11
SMP	7.0	6	4	2	4	9	2	9	5
SMP	8.7	1	2	14	14	4	14	5	14
SMP	10.2	11	1	3	2	1	11	7	10

Table 4.2. Expt. 2 feeding randomisation for seven consecutive days; experimental feeds included seven lengths HDP (hard dry pellet).

Feed type	Feed length (mm)	Feeding day number							
		Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8
HDP	5	6	3	4	7	2	3	1	6
HDP	8	4	4	6	2	3	2	3	7
HDP	13	2	6	5	5	4	7	5	5
HDP	21	7	7	7	1	6	5	6	1
HDP	33	1	1	2	6	1	1	4	4
HDP	53	3	2	3	3	5	4	7	2
HDP	84	5	5	1	4	7	6	2	3

4.2.2 Data analysis

All tanks were exposed to each of pellet diameters and textures in expt. 1 (Table 4.1.) and each pellet length in expt. 2 (Table 4.2.) in randomised order. The used method allowed for collection of 112 (8 per diameter) replications in expt. 1 and 56 (8 per length) in expt. 2. Linear regression models were used to analyse the relationship between pellet diameter or length and feed waste (NFRW and FRW) expressed as a percentage of feed delivered for HDP and SMP in expt. 1 and HDP in expt. 2. The feeding efficiency data was fitted to linear and logarithmic regression, for expt. 1 and expt. 2, respectively. Feed intake and feed leaching rate were analysed with linear regressions and ANOVA was used for means comparison. Data were tested for homogeneity of variance with the Shapiro-Wilk W test and arcsine or Log10 transformed when normality was not met. In cases where transformation was not effective, non-parametric Kruskal-Wallis test was used for data comparison and the raw data was fitted to the regressions. An ANCOVA using texture as fixed factor and pellet diameter as covariate was used to compare slopes and intercepts of regression lines of average feeding efficiency and pellet diameters between HDP and SMP.

4.3 Results

4.3.1 Diameter experiment

With all the measures performed except for DM leaching, the data were highly variable within treatments. The NFRW and FRW measured for HDP resulted in significant linear regressions (Figure 4.1). The amount of NFRW decreased with an increase in pellet diameter, whereas the reverse pattern was observed for FRW. The point of intersection between the two regression lines denoted the pellet diameter at which

equal amounts of NFRW and FRW existed. Like HDP, the FRW for SMP increased linearly with larger pellet diameter, however there was no significant pattern for NFRW with changes in pellet diameter (Figure 4.2). The increase in FRW with larger pellet diameter for both HDP and SMP meant that the feeding efficiencies linearly decreased for both feed textures (Figure 4.3). There was no significant interaction between texture and diameter (ANCOVA regression lines; d.f. 1,13, $F = 0.4$, $P = 0.545$), indicating that there were no differences in the rates at which feeding efficiencies decreased between HDP and SMP. The FI of lobsters did not differ when fed different pellet diameters (Table 4.3), indicating that on average the same total amounts of feed waste were produced irrespective of pellet diameters. Likewise, irrespective of pellet diameter and feeding efficiency, the FI of lobsters fed SMP did not differ, indicating feed waste production did not differ. In general, DM leaching decreased with increasing pellet diameters for both HDP and SMP, although the pairwise comparison showed significant differences, with highest leaching in diameters 4.3 and 1.5 mm, and lowest in 9.5 and 7.0 mm for HDP and SMP, respectively. A significant linear regression was obtained for SMP only (Table 4.3).

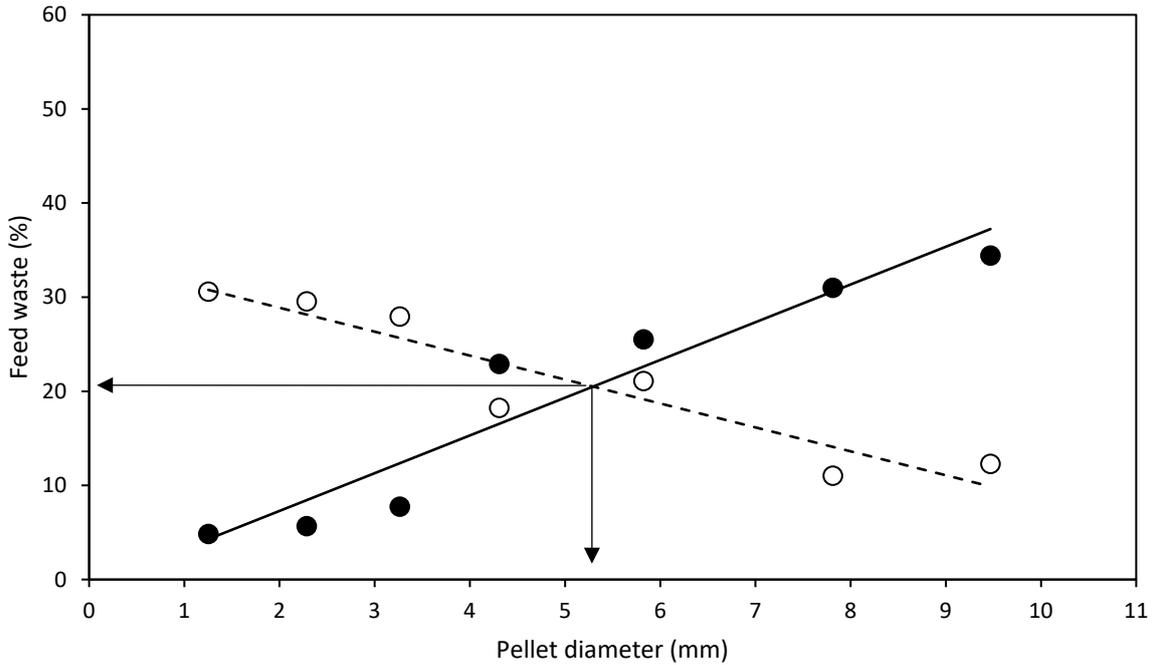


Figure 4.1. Effect of pellet diameter on hard and dry pellet (HDP); 1) non-feeding related waste (NFRW), white symbols; and 2) feeding related waste (FRW), black symbols. The dashed line shows linear regression for NFRW $y = -2.5x + 34.0$, $R^2 = 0.105$, $P = 0.015$; the solid line shows linear regression for FRW $y = 4.0x - 0.7$, $R^2 = 0.32$, $P = 0.000$. Intercept of regressions, $x = 5.3$, $y = 20.5$. Values are average, error bars denote S.E.

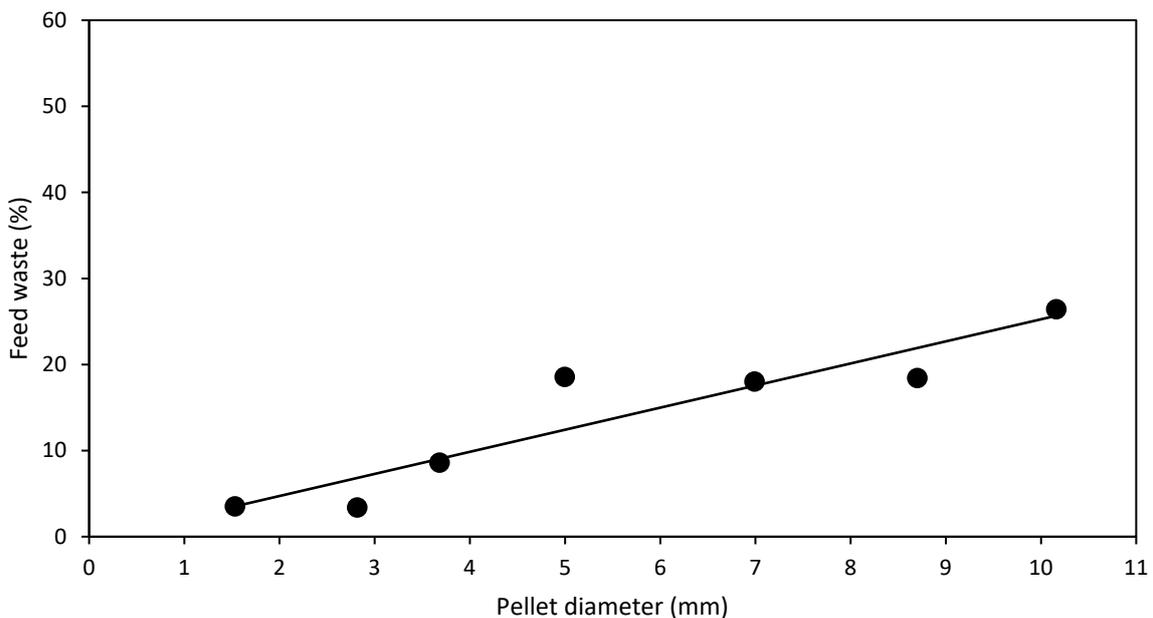


Figure 4.2. Effect of pellet diameter on soft and moist pellet (SMP) feeding related waste (FRW). The line shows linear regression for FRW $y = 0.26x - 0.4$, $R^2 = 0.26$, $P = 0.000$. Values are average, error bars denote S.E.

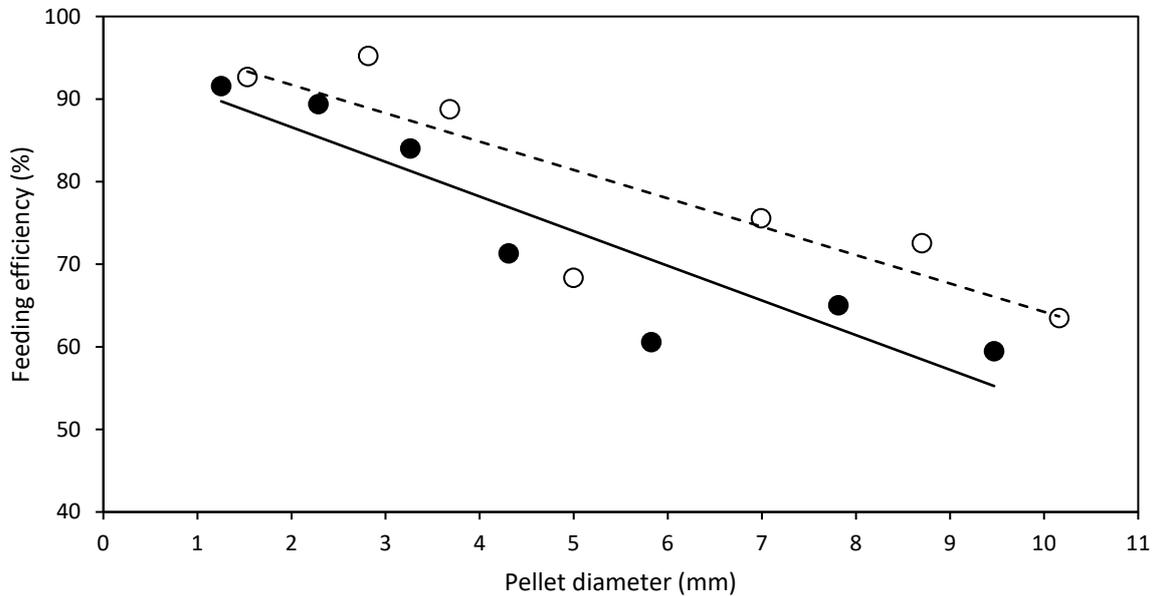


Figure 4.3. Effect of pellet diameter on feeding efficiency of 1) soft and moist pellet (SMP), white symbols, the dashed line shows linear regression $y = -3.4x + 98.6$, $R^2 = 0.193$, $P = 0.001$ and 2) hard and dry pellet (HDP), black symbols, the solid line shows linear regression $y = -4.2x + 95.0$, $R^2 = 0.222$, $P = 0.000$. Values are average, error bars denote S.E.

Table 4.3. Feed intake (FI) and feed leaching rate in juvenile *Sagmariasus verreauxi* when supplied varied feed diameters of HDP (hard dry pellet) and FI, NFRW (non-feeding related waste) and leaching rate when supplied varied feed diameters of SMP (soft moist pellet). Data represents mean \pm S.E. (n = 8 for FI and NFRW; n = 3 for leaching rate), significant differences between means (ANOVA or Kruskal-Wallis (K-W), P < 0.05) are marked with superscript and significant linear regressions (P < 0.05) are marked with asterisk (*). Survival was at 100 %.

Parameter	Texture	Diameter (mm)							Test	a	b	R ²	df	F/Chi-Sq	P
		1.3	2.3	3.3	4.3	5.8	7.8	9.5							
FI (% DW)	HDP	64.6 \pm 8.8	64.8 \pm 10.8	64.3 \pm 11.3	58.9 \pm 9.9	53.4 \pm 12.6	58.0 \pm 10	53.3 \pm 8.2	ANOVA				6,49	0.290	0.939
										Regression	-1.5	66.8	0.021	1,54	1.179
Leaching rate (% DW*6 h ⁻¹)	HDP	8 \pm 0.2 ^a	6.9 \pm 0.8 ^{ab}	8.6 \pm 0.9 ^a	8.7 \pm 0.3 ^a	7.3 \pm 0.4 ^{ab}	8.6 \pm 0.0 ^a	5.2 \pm 0.1 ^b	ANOVA				6,49	6.273	0.002*
										Regression	-0.2	8.6	0.149	1,19	3.321
Parameter	Texture	Diameter (mm)							Test	a	b	R ²	df	F/Chi-Sq	P
		1.5	2.8	3.7	5.0	7.0	8.7	10.2							
FI (% DW)	SMP	67.1 \pm 11.1	76.3 \pm 6.2	73.4 \pm 6.6	49.4 \pm 10.7	66.5 \pm 9.6	59.0 \pm 11.0	58.4 \pm 13.1	ANOVA				6,49	0.687	0.661
										Regression	-1.5	72.9	0.026	1,54	1.465
NFRW (% DW)	SMP	29.4 \pm 10.5	20.3 \pm 5.6	18.0 \pm 5.0	32.0 \pm 8.6	15.5 \pm 5.0	22.6 \pm 7.0	15.2 \pm 6.3	Regression	-1.0	27.5	0.023	1,54	1.275	0.264
Leaching rate (% DW*6 h ⁻¹)	SMP	12.3 \pm 0.2 ^a	10.0 \pm 0.3 ^{ac}	9.1 \pm 0.6 ^{abc}	8.2 \pm 0.2 ^{abc}	6.1 \pm 0.4 ^b	7.6 \pm 0.3 ^{bc}	6.7 \pm 1.6 ^{bc}	K-W				6,20	14.615	0.023*
										Regression	-0.6	11.7	0.601	1,19	28.611

4.3.2 Length experiment

With all the measures performed except for DM leaching, the data were highly variable within treatments. The NFRW and FRW measured for HDP showed significant linear regressions (Figure 4.4). The NFRW decreased with increase in pellet diameter, whereas the reverse pattern was observed for FRW. The point of intersection between the two regression lines denoted the pellet length at which equal amounts of NFRW and FRW existed.

The feeding efficiency was highest at the smallest length (Figure 4.5). Like pellet diameter and despite the significant effects of pellet length on feed waste type and feeding efficiency, the FI was not significantly different (Table 4.4), indicating similar levels of the total waste produced. As with pellet diameter, the leaching of DM decreased with pellet length and showed significant differences in pairwise comparison, with the highest leaching in 5 and lowest in 84 mm length. The leaching data showed significant linear regression (Table 4.4).

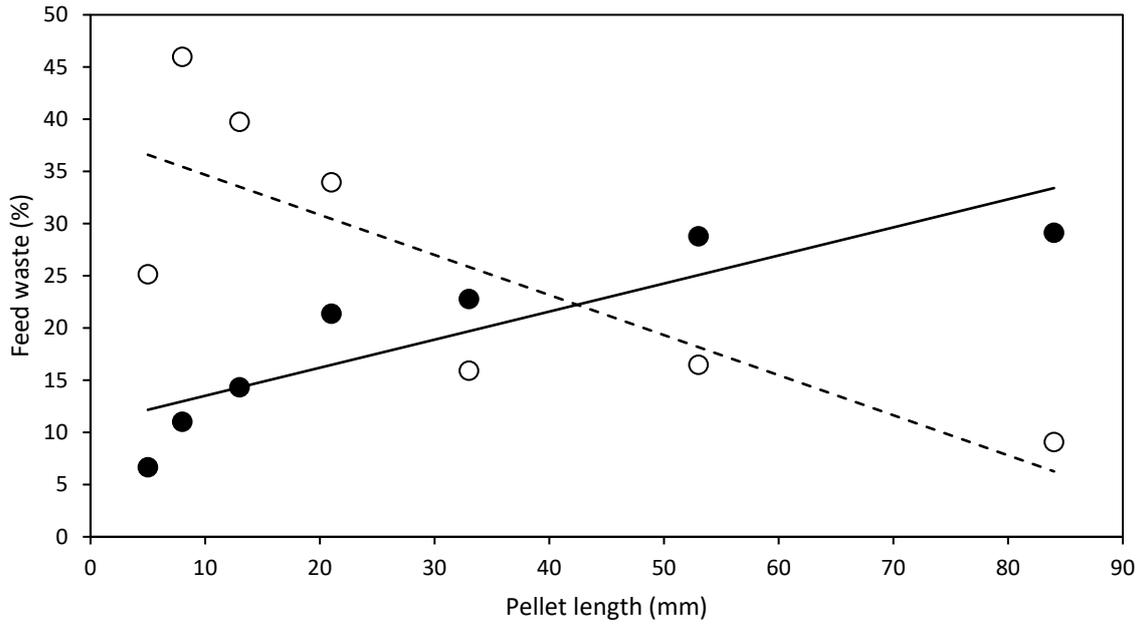


Figure 4.4. Effect of pellet length on hard and dry pellet (HDP) 1) non-feeding related waste (NFRW), white symbols and 2) feeding related waste (FRW), black symbols. The dashed line shows linear regression for NFRW $y = - 0.38x + 38.5$, $R^2 = 0.197$, $P = 0.001$; the solid line shows linear regression for FRW $y = 0.26x + 10.8$, $R^2 = 0.207$, $P = 0.000$. Intercept of regressions, $x = 42.4$, $y = 22.2$. Values are average, error bars denote S.E.

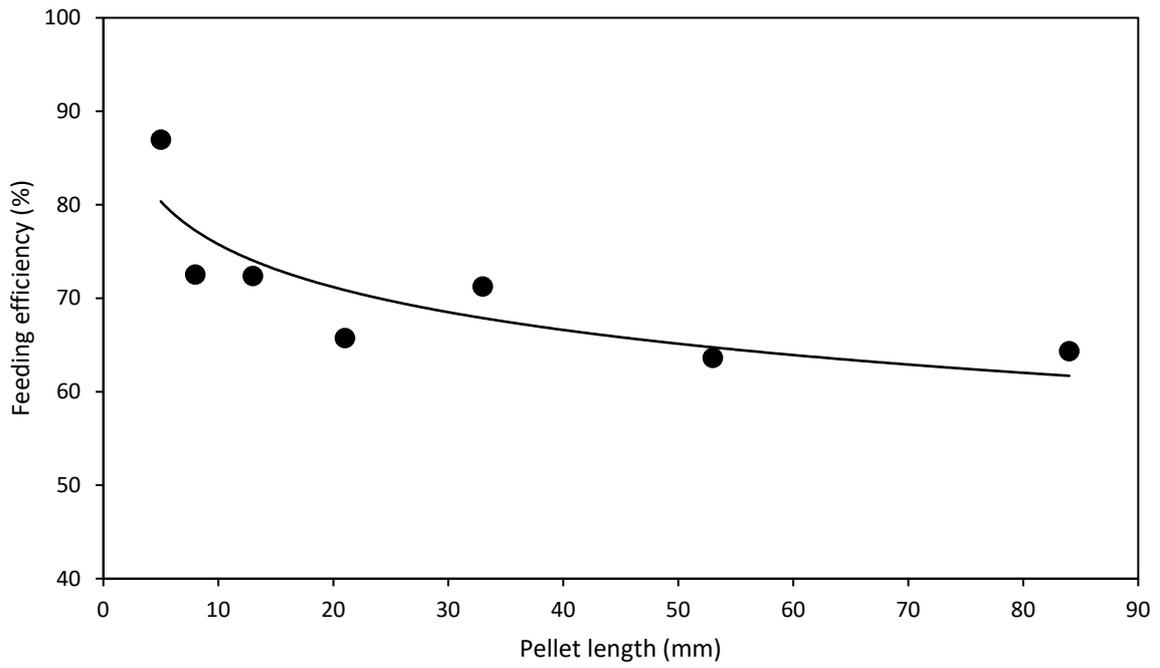


Figure 4.5. Effect of pellet length on hard and dry pellet (HDP) feeding efficiency, the line shows logarithmic regression $y = - 6.6\ln(x) + 91.0$, $R^2 = 0.075$, $P = 0.041$. Values are average, error bars denote S.E.

Table 4.4. Feed intake (FI) and feed leaching rate in juvenile *Sagmariasus verreauxi* when supplied varied feed lengths of HDP (hard dry pellet). Data represents mean \pm S.E. (n = 8 for AFI; n = 3 for leaching rate), significant differences between means (ANOVA, P < 0.05) are marked with superscript and significant linear regressions (P < 0.05) are marked with asterisk (*). Survival was at 100 %.

Parameter	Texture	Length (mm)							Test	a	b	R ²	df	F/Chi-Sq	P
		5	8	13	21	33	53	84							
FI (% DW)	HDP	71.4 \pm 7.8	53.1 \pm 8.1	56.2 \pm 6.7	55.9 \pm 7.0	68.7 \pm 6.8	64.5 \pm 6.5	70.1 \pm 9.0	Anova				6,49	1.044	0.409
									Regression	0.1	58.9	0.026	1,54	1.427	0.238
Leaching rate (% DW*6 h ⁻¹)	HDP	10.0 \pm 0.3 ^a	8.9 \pm 0.4 ^{ac}	7.7 \pm 0.4 ^{bcd}	7.7 \pm 0.3 ^{bcd}	8.1 \pm 0.4 ^{abc}	6.2 \pm 0.5 ^{bd}	6.1 \pm 0.4 ^d	Anova				6,49	12.365	0.000*
									Regression	-0.04	9.1	0.605	1,19	29.109	0.000*

4.4 Discussion

The feed intake of formulated feeds by spiny lobsters has been described as “messy” due to the breakdown of feed particles while manipulating and macerating pellets (Daning Tuzan, 2018; McGaw and Penney, 2014; Zoutendyk, 1988), leading to high amounts of feeding related waste (FRW). For spiny lobsters, particularly *J. edwardsii*, it was estimated that up to 50% of formulated feed was wasted by inefficient feeding (Sheppard et al., 2002). However, there were some indications that FRW may be reduced by optimizing feed dimensions for *P. argus* (Cox et al., 2008), *P. ornatus* (Smith et al., 2009a), and *J. edwardsii* (Sheppard et al., 2002). Thus, optimizing feed dimensions, may improve feeding efficiency and perhaps increase feed intake in spiny lobsters. The results of the present study showed high variability in feed waste, which in turn affected all the other dependent measures when juvenile *S. verreauxi* were supplied with HDP and SMP of different diameters and HDP of different lengths. The high variability highlights the complex nature of the feeding behaviour of spiny lobsters in general. Several intrinsic factors may be responsible for the observed variations such as the moult cycle status of the animals, hierarchy and gender. Despite the variable dataset, it was clear that feed diameter and feed length of HDP affected the type of feed waste produced. An increase in FRW irrespective of texture, suggested a reduced feeding efficiency as pellet diameter increased, as was observed for feed length with HDP. However, the increase in feeding efficiency due to low FRW for small diameter pellets did not improve feed intake as the total amount of feed waste was not reduced; i.e. improved FRW was offset by a corresponding increase of NFRW. Overall feed waste varied from 32 to 57% irrespective of tested dimension (diameter or length) or texture. The minimal FRW for the smallest pellet diameters (SMP and HDP) and lengths (HDP) indicated that lobsters were efficiently consuming whole pellets after

locating them. Similarly, for *J. edwardsii* it was observed that lobsters could also sweep multiple small pellets dispersed on the tank floor and scoop with pereopods and pass to mouthparts, in attempt to consume as many as possible (Sheppard et al., 2002). For *P. ornatus*, feeding related waste was a function of pellet diameter and less FRW was produced when juvenile lobsters (50-60 g) were fed 3 mm diameter HDP, when compared to 9 mm diameter HDP (Smith et al., 2009). At the lowest diameter (1.3 mm - HDP and 1.5 mm - SMP) investigated, the set ration of 0.5% BW consisted of ~150 pellets and the highest diameter (9.5 mm - HDP and 10.2 mm - SMP) consisted of 4 pellets. In the expt. 2, the 0.5% BW rations consisted of ~ 43 pellets for lowest length (5 mm) and 4 for highest (84 mm). The expectation that the higher number of pellets for the smallest feed diameter or length would favour encounter and therefore more feeding, did not occur. The effect of feed encounter was probably offset by the fact that small pellets lose attraction at a faster rate than larger pellets because of the higher relative surface area exposed to leaching. In addition, the smaller pellets would require more handling, specifically picking up and holding with first pair of walking legs and III maxillipeds for ingestion (Francis et al., 2014; Lau, 1987). Thus, it is highly likely that the combined effect of extended feed handling time and rapid attractant leaching in small diameter/length pellets may have led to decrease of motivation to feed and in consequence to an increase in NFRW. The significant increase for HDP NFRW in diameter and length experiments, supports the argument that while feed encounter is higher with small pellets, the duration of attraction to the feed is shorter. High numbers of small pellets to consume in a limited time frame of attraction, inevitably leads to more NFRW. However, there were no clear pattern for the NFRW for SMP of different diameters, indicating that feed diameter did not influence the amounts of NFRW produced with SMP. The less pronounced results for NFRW in SMP may be the

outcome of different SMP pellets characteristics. For example, it may be possible that the leaching rates of attractants in SMP is different to HDP. However, all these characteristics need further investigations. For both SMP and HDP, it was clear that optimising feed diameter and for HDP, optimising feed length, improves feeding efficiency, however, it did not result in improvement of feed intake due to an increase in uneaten feed (NFRW). Lobsters were fed at 0.5% lobsters BW to exclude the possibility of overfeeding; thus, it is highly unlikely that lobsters were fully satiated (Simon, 2009). Loss of attraction to feed and/or poor gustatory stimulation provides a strong valid explanation for the lack of improvement in feed intake when feeding efficiency was optimised. Thus, future research in feed development for juvenile lobsters should investigate solutions to improve feed intake. To reduce feed waste and ultimately improve feed intake, innovative solutions need to be employed because the present feed manufacturing technique does not allow the production of a feed which is either attractive enough for extended periods or has the suitable format to maximise feed intake.

4.5 Conclusion

This study has highlighted the large variability in feeding efficiency for juvenile *S. verreauxi*, irrespective of the feed dimensions and texture investigated. This highlights the complex feeding behaviour of spiny lobsters and therefore the difficulty to assess feed waste and ultimately feed intake. However, supported with significant patterns from regression analyses, we found some evidence to suggest that feeding efficiency may be optimised through manipulating feed dimensions, although feed intake was unaffected. This finding suggests that currently a counteractive interaction exists between pellet size and feed attractiveness and suggests improving attractiveness

would further enhance feeding. Future research should aim at optimising feed dimensions simultaneously to support efficient feeding whilst enhancing prolonged attraction/gustatory stimulation.

Chapter 5 The effect of feed frequency on growth, survival and behaviour of juvenile spiny lobster *Panulirus ornatus*

Abstract

Spiny lobsters have a range of complex chemical communication pathways that contribute to feeding behaviour. Feed detection, localisation and consumption are primarily coordinated by specialised chemical sensors located on the antennae, pereopods and mouthparts, and are complemented by visual, acoustic and tactile stimuli. Consequently, feed intake is modulated by physical traits such as food attractiveness and availability, and behavioural factors such as social hierarchy and circadian rhythm. This study investigated the effect of feed frequency on survival and growth of early-stage (instar 2-6) juvenile *Palinurus ornatus*. In addition, we verified that response to feed was an interactive effect of feed frequency and circadian rhythm. Lobsters were fed a frequency of either one, two, four, eight, sixteen or thirty-two times per day to supply their daily ration over 49 days. The effect of feed frequency on growth and survival was determined. Circadian feeding activity under these feeding treatments was assessed by time-lapse photography. Increased feed frequency from once to sixteen feeds daily improved growth by increasing apparent feed intake (AFI) and feed attraction, confirmed by the increased presence of lobsters in the feeding area. The leaching of feed attractant was modulated by manipulating feed frequency

with subsequent impacts of feed intake and growth. More than sixteen feeds daily, resulted in decreased feed intake and a subsequent reduction in growth thought to be associated with saturation of the culture environment with attractants and reduced the behavioural response to feed supplies. This may indicate the need for depletion of attractants to retrigger a feeding response. As lobsters were grown communally, faster growth at sixteen rations per day was also coupled with increased cannibalism, driven entirely by vulnerability at ecdysis. While circadian rhythm indicated more activity at night, the interaction of daytime and feed frequency was not found.

5.1 Introduction

Effective nutrient intake is key to the nutrition of any organism and is critical for achieving optimal fitness and growth, in the wild and in aquaculture. Lobster feed intake involves several key aspects, including feed perception, identification, location of chemical signal, attractiveness, motivation, feed capture and ingestion (Derby and Atema, 1982b; Derby et al., 2001). These steps are predominantly stimulated and administered by chemical pathways, which utilise two types of chemosensillar, the olfactory, located on antennules, and distributed chemoreception located on all body areas (Derby et al., 2001; Kozma et al., 2018). The antennular chemoreceptors are used in searching and locating feed, while chemoreceptors located on legs and mouthparts provide information that allows the lobster to catch and, consume or reject feed (Derby and Atema, 1982b; Derby et al., 2001). Chemoreceptors in crustaceans are often specialised to detect specific substances and allow detection of a key component from complex mixtures (Derby and Atema, 1988). The behavioural response to presence of attractants is usually stronger for complex mixtures, indicating a synergistic effect, however single chemicals such as glycine provide strong attraction for *Panulirus interruptus* (Derby and Atema, 1988; Zimmer-Faust et al., 1984). Feeding

can be accompanied by learned behaviours, which can influence the decision as to whether a feed search should be initiated (Major et al., 2017). In the wild, spiny lobsters exhibit omnivorous and detritivore feeding behaviour, consuming a range of foods including, crustaceans, gastropods and marine plants (Boudreau and Worm, 2012; Radhakrishnan and Kizhakudan, 2019; Radhakrishnan et al., 2019b). The presence of feed also triggers agonistic behaviours, including competition and dominance and as a consequence may lead to variable growth rate within an aquaculture cohort (Atema and Cobb, 1980; Thomas et al., 2003).

At the Institute for Marine and Antarctic Studies (IMAS) hatchery technologies have been developed for tropical rock lobster, *Panulirus ornatus* as a result, regular production of seedstock is possible (Fitzgibbon and Battaglione, 2012; Fitzgibbon et al., 2012; Fitzgibbon et al., 2017). To support the growth of a lobster grow-out industry the development of a formulated feed is key. Feed attractiveness is a major component of feed intake and subsequently the development of a formulated feed for spiny lobsters. Low molecular weight of water soluble compounds such as free amino acids (e.g. betaine) and organic acids are known to provide attraction to feeds (Derby and Atema, 1982a; Williams, 2007). However, these same compounds are water soluble, leach rapidly from the feed when submerged providing a short window where feeds are most attractive. The presence and concentration of these substances can be investigated using ninhydrin (Moore and Stein, 1948; West, 1965). Marchese et al. (2019) demonstrated that formulated feeds were attractive to lobsters for 2-3 hours after supply, while mussels remained attractive throughout the daily feeding cycle. Increasing the feed frequency of set rations to extend the time over which feeds remain attractive is one strategy that has been investigated for several crustacean species. The exposure of juvenile prawns *Penaeus merguensis*, *Penaeus vannamei* and

Penaeus monodon to different feeding frequencies showed highest growth in groups fed most frequently, which was four (for *P. merguensis* and *P. vannamei*) or six (for *P. monodon*) times per day (Arnold et al., 2016; Robertson et al., 1993; Sedgwick, 1979). However, there have been a number of studies with spiny lobsters that have demonstrated that increased feed frequency did not improve growth (Cox and Davis, 2006; Simon and Jeffs, 2008; Syafrizal et al., 2018; Thomas et al., 2003). Syafrizal et al. (2018), found inconclusive results with increased feeding frequency on the growth in juvenile *Panulirus versicolor*, while Thomas et al. (2003) noted a decreased competitive and agonistic behaviours in *Jasus edwardsii*, with increased feeding frequency. Cox and Davis (2006), found that feeding *P. argus* juveniles once a day to excess at the start of the dark phase was beneficial. Thus, in the present study, our primary aim was to investigate an optimal feed frequency for maximising growth and survival. In addition, we verified whether response to feed is an interactive effect between feed frequency and circadian rhythm.

5.2 Materials and methods

5.2.1 Experimental animals, design and culture system

Juvenile lobsters were hatchery reared from egg at the Institute for Marine and Antarctic Studies (IMAS) Taroona, University of Tasmania, based on previously described methods (Fitzgibbon and Battaglione, 2012; Fitzgibbon et al., 2012; Ikeda et al., 2011). Before experimental allocation, juvenile lobsters were co-fed with shucked blue mussel flesh, moist feed and experimental dry pellet (0.8 mm diameter) with similar composition to that described by Shu-Chien et al. (2017) except that defatted green shell mussel meal (GSM) was replaced with non-defatted GSM.

Juvenile lobsters (instars 2 to 6) of average initial carapace length (CL_0) $9.8 \text{ mm} \pm 0.18$ and initial wet weight (WW_0) $0.93 \text{ g} \pm 0.05$, were randomly stocked into the experiment vessels. Eleven lobsters were allocated to each replicate; the average initial biomass (B_0) per replicate was $10.6 \text{ g} \pm 0.01$. Six feeding frequencies were tested: one (F1), two (F2), four (F4), eight (F8), sixteen (F16) and thirty-two (F32) feed portions per day. The daily feeding regime extended over twenty-two hours per day with the remaining two hours per day reserved for waste removal (siphoning) and system maintenance. Treatments had six replicates ($n=6$), however one replicate from the F8 was lost due to system failure ($n=5$). The replicate vessels were 18-L blue polypropylene tanks 38 cm long, 24 cm wide, 24.8 cm high, they were equipped with sixteen small hides per vessel (PVC tubes; 2.5 cm diameter \times 5 cm length) with eight shelters located along one short wall of the vessel and eight along the long wall of the vessel plus two 10 cm mini-Mills collectors (Mills and Crear, 2004), hanging off the adjacent long wall. After nine days, four medium hides (PVC tubes; 3 cm diameter \times 6 cm length), were added to accommodate larger animals as they moulted. For the last nineteen days, hides consisted of three small, four medium, eight large (PVC tubes; 4 cm diameter \times 8 cm length) hides and two mini-Mills collectors. All tube hides used in the experiment were lined with 2 mm fibreglass fly mesh. The water supplied to vessels was filtered and ozonated, the water quality was maintained at $27.7 \text{ }^\circ\text{C} \pm 0.03$, pH 8.10 ± 0.01 , salinity $33.7 \text{ ppt} \pm 0.03$, and dissolved oxygen $117.4\% \text{ sat.} \pm 0.58$. Water exchange was maintained at six turnovers by volume h^{-1} . The photoperiod was 12:12 L:D, light was supplied with Fluval Aquasky 2.0 LED lights. The red light was on for 24 h day^{-1} and during the light-phase blue light was supplied for 12 h day^{-1} . The red light was used for better visibility during time-lapse recordings, it is not visible for lobsters and does not disturb their night activity (Endler, 1978; Fitzgibbon and Battaglione, 2012; Mills,

2005). The photoperiod at 12:12 L:D was in the range occurring in the natural environment and was indicated as not negatively affecting feed intake, growth and survival (Chittleborough, 1975; Crear et al., 2003; Simon and James, 2007). Moulting events and mortality were recorded every morning and prior to siphoning before the start of the next feeding cycle, with the work order randomised daily.

5.2.2 Experimental feed and feeding

Throughout the duration of the experiment, lobsters were fed solely with the previously described experimental dry pellet. The feed was manufactured weekly according to established methods (Landman et al., 2021). Briefly, after mixing all the dry and wet ingredients, the resultant dough was cold extruded with La Monferrina Dolly II extruder, into feed strands of diameter 0.8 mm. The feed strands were left to set in a refrigerator (4 °C) overnight, cut into 2 cm length pieces and dried at 40 °C for four h (Steridium DS500 dryer), to achieve a moisture content of 10%. The size of daily feed ration was set at 25% of lobster body weight. On day 23, lobsters from each replicate were bulk weighed for group WW to adjust feed ration. Daily rations were divided into equal portions, among the tested feed frequencies. Feed portions for each frequency were supplied by FIAP belt feeders, which were calibrated to supply feed portions in set time intervals corresponding with assigned frequency. Belt feeders were equipped with funnels attached with tape below the belt drop, which were transferring feeds to PVC pipes to provide a consistent delivery of the feed below the water surface and 10 cm above the vessel base. The feeding cycle began after commencement of the dark phase. Feed was delivered to a feeding area on the vessel floor. The feeding area was defined by one long and one short wall of the vessel and 10 mm distance from rows of shelters located alongside the opposite walls (Figure 5.1).

5.2.3 Apparent feed intake

The apparent feed intake (AFI) was recorded during the sixth week of the experiment (Fitzgibbon et al., 2017). For 7 days, feed waste was collected from all replicates after the twenty-two hours of feeding cycle, by siphoning into separate 124- μm sieves, rinsed with deionised water, and frozen for later analysis. After completion of the experiment, feed rations were supplied to vessels with no lobsters present to account for the percentage dry matter loss in water. Samples of feed, before and after submersion, were dried at 105 °C, for 24 h to determine dry weight (DW). The AFI was obtained by the difference between DW of feed supplied and DW waste feed after correcting for dry matter loss to leaching:

$$AFI = Fi(g) - Fo(g) - (leaching(\%) * (Fi(g) - Fo(g)))$$

where:

Fi (g) – dry weight of feed supplied to the vessels (feed in)

Fo (g) – dry weight of feed waste from vessels (feed out)

$$leaching = \frac{(100\%)*(Fo(g))}{Fi(g)}$$

The AFI was presented as feed consumed per head per day (g DW*head⁻¹*day⁻¹).

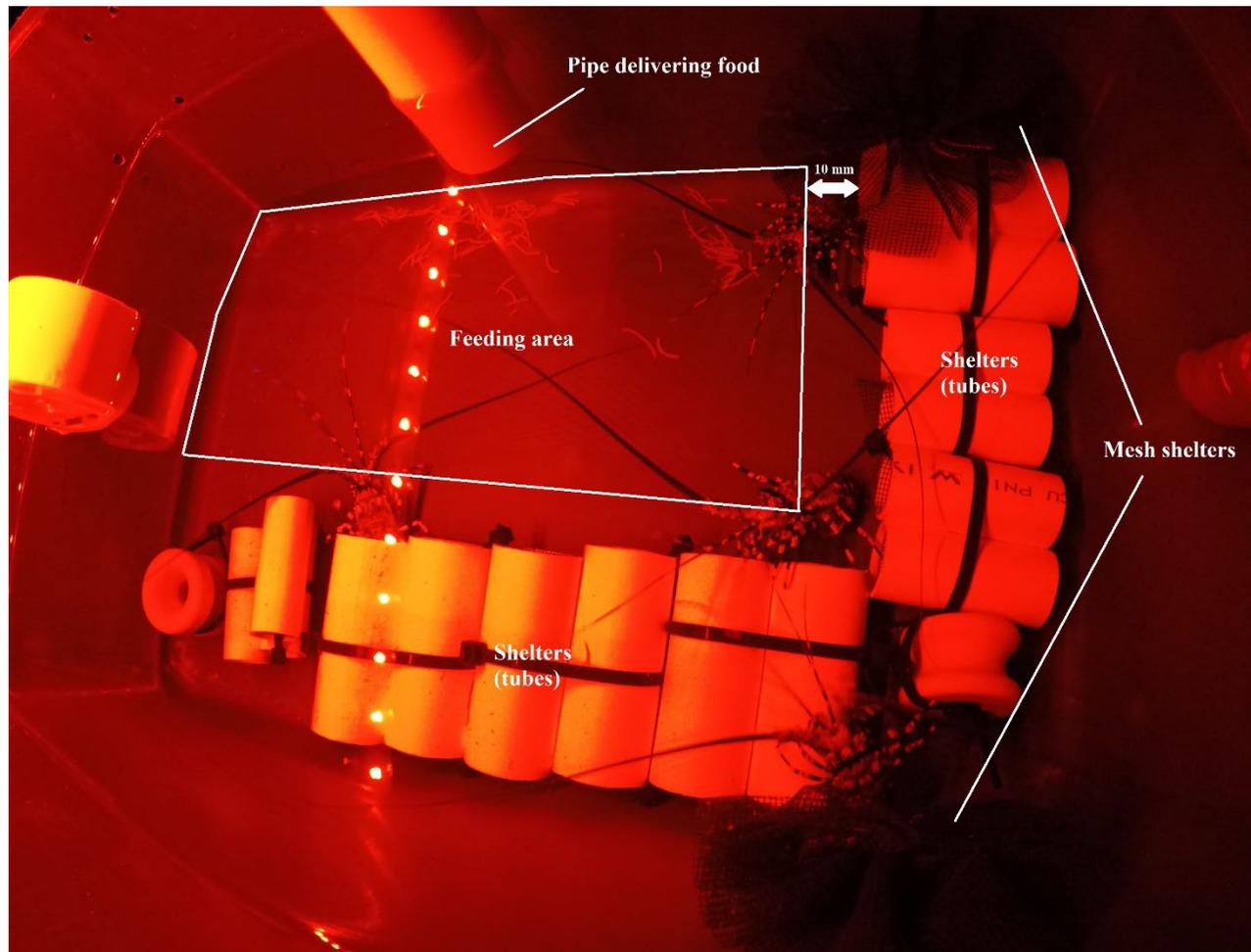


Figure 5.1. Plan of experimental vessel floor, showing distribution of shelters, location of pipe delivering food and feeding area.

5.2.4 Analysis of leaching of free amino-acids (FAA) and ninhydrin positive substances

The leaching of free amino-acids (FAA) and ninhydrin positive substances from experimental feed was determined using a modified ninhydrin assay (Jones et al., 2002; Moore and Stein, 1954). Briefly, experimental feeds were immersed in seawater at five time points (0.5, 1, 3, 6 and 24 h), under same operating condition as the experiment in triplicate (n=3). After immersion, the feeds were siphoned into the separate 124 µm sieves, rinsed with deionised water and frozen for later analysis. The immersed feeds and a non-immersed feed samples (t = 0) were freeze-dried until constant weight and ground to a homogenous powder using a hand mortar and pestle. 100 mg samples with 50 ml of deionised (DI) water were homogenised with hand homogeniser (Cat x120) for one min each before sonication (Sonics Vibra-Cell CV334) three times for one min and centrifuged (Heraeus Multifuge X1R) for 15 min. Then 10 µL of supernatant was added to microwell plates along with 90 µL of DI and 75 µL of Ninhydrin Reagent (2%). The microwell plates were placed in the oven at 80 °C, for 30 min before cooling and the addition of 100 µL of 50% ethanol to stabilise the reaction. Absorbance of samples was measured at 570 nm using Biotek Synergy HT plate reader. A glycine standard curve was constructed to estimate molar concentrations of FAA and ninhydrin positive substances in immersed and non-immersed feed samples.

5.2.5 Growth and survival

The initial biomass (B_0) of lobsters was measured for each replicate at stocking of the experiment. Biomass was measured again on day 22, 42 and at termination of the experiment (day 49). Biomass measurements involved low impact handling, by bulk

weighing lobsters in a jug of seawater, the measurements after three and six weeks, were used to check progress and adjust feed ration. At the termination of the experiment individual lobsters were wet weighed (WW_t) and carapace length (CL_t) measured (Vernier calliper). The sum of WW_t of all survivors in the replicate was used to calculate final biomass (B_t). Prior to the commencement of the study individual initial carapace length (CL_0) and wet weight WW_0 was measured on three groups of eleven lobsters from the same pool used to stock the experiment by the same procedure. Culture performance parameters were calculated: specific growth rate (SGR), survival (S), average moults per day (AMD), biomass gain (BG), productivity (P), carapace length gain (CLG), wet weight gain (WWG) (Fitzgibbon et al., 2017; Kropielnicka-Kruk et al., 2019; Simon and James, 2007). The analysed parameters were calculated following formulae:

- 1) $WWG = WW_t - WW_0$
- 2) $CLG \text{ (mm)} = CL_t - CL_0$
- 3) $BG \text{ (\%)} = (B_t - B_0)/B_0 \times 100$
- 4) $SGR \text{ (\% WW d}^{-1}\text{)} = (\ln WW_t - \ln WW_0)/t \times 100$
- 5) $AMD \text{ (average number of moults *lobster}^{-1}\text{*day}^{-1}\text{)}$

$$AMD = \frac{X_{R1} + X_{R2} + \dots + X_{RN}}{R_N}$$

$$X_R = \frac{\left(\frac{N_R}{S_R}\right)_{Day\ 1} + \left(\frac{N_R}{S_R}\right)_{Day\ 2} + \dots + \left(\frac{N_R}{S_R}\right)_{Day\ 49}}{t}$$

- 6) $P \text{ (g m}^{-3} \text{ day}^{-1}\text{)} = (B_t - B_0) \times n_t / (V \times t)$
- 7) $S \text{ (\%)} = (n_t/n_0) \times 100$

Where X_R is the average number of moults *animal⁻¹ *replicate⁻¹ *day⁻¹ (N_R - number of moults in replicate, S_R - number of live animals in replicate); R_N is the number of

replicates; t is the duration of experiment (49 d); n is the number of animals; V is the water volume *lobster^{-1} ($1.55\text{-L}=0.00155\text{ m}^3$).

5.2.6 Behavioural observations

Behavioural observations were digitally recorded using time-series photography (GoPro Hero 5 Black cameras) (Kropielnicka-Kruk et al., 2019; Marchese et al., 2019) during the fifth and sixth week of the experiment. Observations were recorded at all tested feeding frequencies and all replicates within each frequency. For each replicate, an image was recorded every 5 sec over a 22 h period. To exclude risk of discrepancies in the analysis of behavioural observations, replicates where cannibalism occurred during observations were excluded from analysis (two in F16, one in F1, F4, F8, F32) due to the associated increase in lobster activity associated with cannibalism. For equal comparison, analysis was conducted on four random replicates from each tested frequency. Images captured the complete floor area of the experimental tank. The observed area was divided into two sections, the shelter and feed areas. Time-series photographs were analysed for all lobsters present in the feeding area. Presence in the feed area was defined as a lobster present in the feed area where food was available, with no physical contact with shelters. The nominal score of one was given to each observed lobster present in the feeding area within each recorded photograph. The time that animals spent in the feeding area, was calculated in min, with each min of observation comprised of twelve images. Image analysis was performed with custom created MATLAB code. The data for the graphic presentation of behavioural observations was summed into 30 min intervals (preceding 30 min) with the observations of four replicates from one frequency averaged into the 22 h feed presentation timeframe.

5.2.7 MATLAB code details

Image data were analysed to detect juvenile lobsters within the designated feeding area for each replicate tank. Object detection was performed using an adaptive weighted background subtraction routine to detect areas of movement, or significant difference from the generated mean background image. Potential candidate detections were then filtered based on basic pixel intensity statistics of a 37 x 37-pixel neighbourhood centred at the candidate centroid.

Multiple stages of vetting were performed to account for situations where false positives were likely – surface reflections, movement of antennae and antennules of lobsters in hides, in the water column directly above the feed pile, or feed pellets floating on the tank water surface. Finally, the centroid coordinates of confirmed positive detections were returned (Figure 5.2).

Although not strictly detecting specific objects, the performance of the detection routine was evaluated using standard metrics of recall and precision on a sample of 250 frames of data. Of the 274 ‘ground truth’ lobster instances, 244 true positives (*TP*) were detected, with 5 false positives (*FP*), and 35 false negatives (*FN*) giving a recall of 87% and a precision of 98%, where $recall = TP / (TP + FN)$, and $precision = TP / (TP + FP)$.

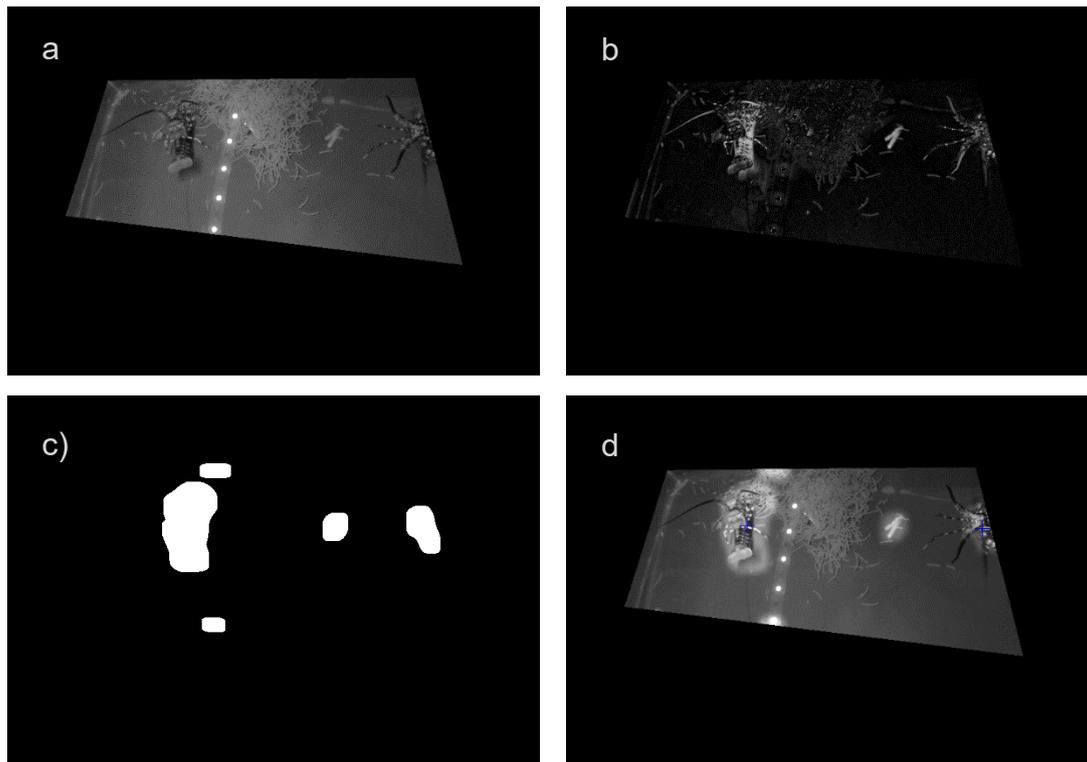


Figure 5.2. Detection of juvenile lobsters within an aquaculture test tank using adaptive background subtraction. a) Original image with masked region of interest (ROI), b) Following background subtraction, c) Output of morphological operations identifying candidate detections, and d) Identification of confirmed positive detections (blue markers) following a basic statistical vetting of candidates, overlaid on to the original image with all potential detections highlighted.

5.2.8 Statistical analysis

Statistical analysis of culture performance parameters of lobsters was using the six replicate vessels except for the F8, which was analysed using five replicates (as described in 2.1. *Experimental animals, design and culture system*). All data was tested for homogeneity of variance with Shapiro-Wilk W test. When normality was not met, the data was square root transformed. The statistical analysis included fitting quadratic polynomial regressions to the data, to describe relationship between feed frequency and growth (WW_t , WWG , CL_t , CLG , AMD , B and SGR), S and AFI .

Behavioural observations expressed as presence in the feeding area were fitted to quadratic polynomial regression and were analysed with two-way ANOVA (phase of photoperiod (Light/Dark) and feed frequency). When significant difference was found, the Tukey HSD test was performed for post hoc analysis of means. The Levene's test was used for analysis of homogeneity of variances in two-way ANOVA. To meet the homogeneity of variances condition, the data was square root transformed prior to analysis. First derivatives of significant quadratic regressions were optimal solutions. The FAA and ninhydrin positive substances data was fitted to the logarithmic regression. The immersion times of samples tested for FAA included zero values (samples not immersed in water). To account for zero values in the logarithmic regression, all immersion times were increased by 0.0001 h. All data presented in text shows mean value \pm S.E., unless otherwise indicated.

5.3 Results

5.3.1 Growth and survival

All growth parameters displayed positive significant quadratic regressions relative to feed frequency (Table 5.1, Figure 5.3a – f). According to the quadratic regressions, the highest WW_t , WWG , CL_t , CLG , SGR and AMD were found in F16, and lowest in F1. Calculated optimal feed frequency for WW_t , WWG , CL_t , CLG , SGR and AMD ranged between 17.7 and 19.3 feed day⁻¹ (Figure 5.3a – f)

Survival (S) demonstrated a significant negative quadratic regression with feed frequency (Table 5.1, Figure 5.3g). According to the quadratic regression, the highest S was found in F1 and lowest in F16. Calculated minimum S for feed frequency was 16.7 feed day⁻¹. Based on the significant quadratic regressions, B_t , BG and P did not

display significant quadratic relationships relative to feed frequency (Table 5.1). The highest B_t and BG was found in F4.

5.3.2 Apparent feed intake (AFI)

Apparent feed intake (AFI) displayed a significant positive quadratic regression relative to feed frequency (Table 5.1, Figure 5.3h). According to the quadratic regression, the highest AFI was obtained in F16. The calculated maximum AFI based on the significant quadratic regression was estimated at feed frequency 15.7 feed day⁻¹.

Table 5.1. Quadratic regression details ($y = ax^2 + bx + c$). Quadratic regressions are describing the influence of feed frequency on parameters; final wet weight (WW_t), wet weight gain (WWG), final carapace length (CL_t), carapace length gain (CLG), final biomass (B_t), biomass gain (BG), specific growth rate (SGR), moulting frequency (AMD), productivity (P), survival (S) and apparent feed intake (AFI). The significant (ANOVA, $p < 0.05$) regressions are marked with asterisk (*).

Parameter	a	b	c	R ²	df	F	P
WW_t (g)	-0.006	0.225	5.474	0.236	2,32	4.934	0.014*
WWG (g)	-0.006	0.225	4.541	0.236	2,32	4.933	0.014*
CL_t (mm)	-0.006	0.219	17.845	0.226	2,32	4.678	0.017*
CLG (mm)	-0.006	0.219	8.051	0.226	2,32	4.678	0.017*
B_t (g)	0.011	-0.335	29.197	0.020	2,32	0.333	0.719
BG (%)	0.096	-2.955	175.371	0.018	2,32	0.297	0.745
SGR (% BW d ⁻¹)	-0.002	0.071	3.601	0.239	2,32	5.031	0.013*
AMD (moults*lobster ⁻¹ *day ⁻¹)	-7.78E-05	0.003	0.058	0.359	2,32	8.973	0.001*
P (g m ⁻³ day ⁻¹)	0.031	-1.002	35.588	0.077	2,32	1.327	0.280
S (%)	0.053	-1.767	47.700	0.198	2,32	3.950	0.029*
AFI (g DW*head ⁻¹ *day ⁻¹)	-0.001	0.019	0.301	0.297	2,32	6.759	0.004*

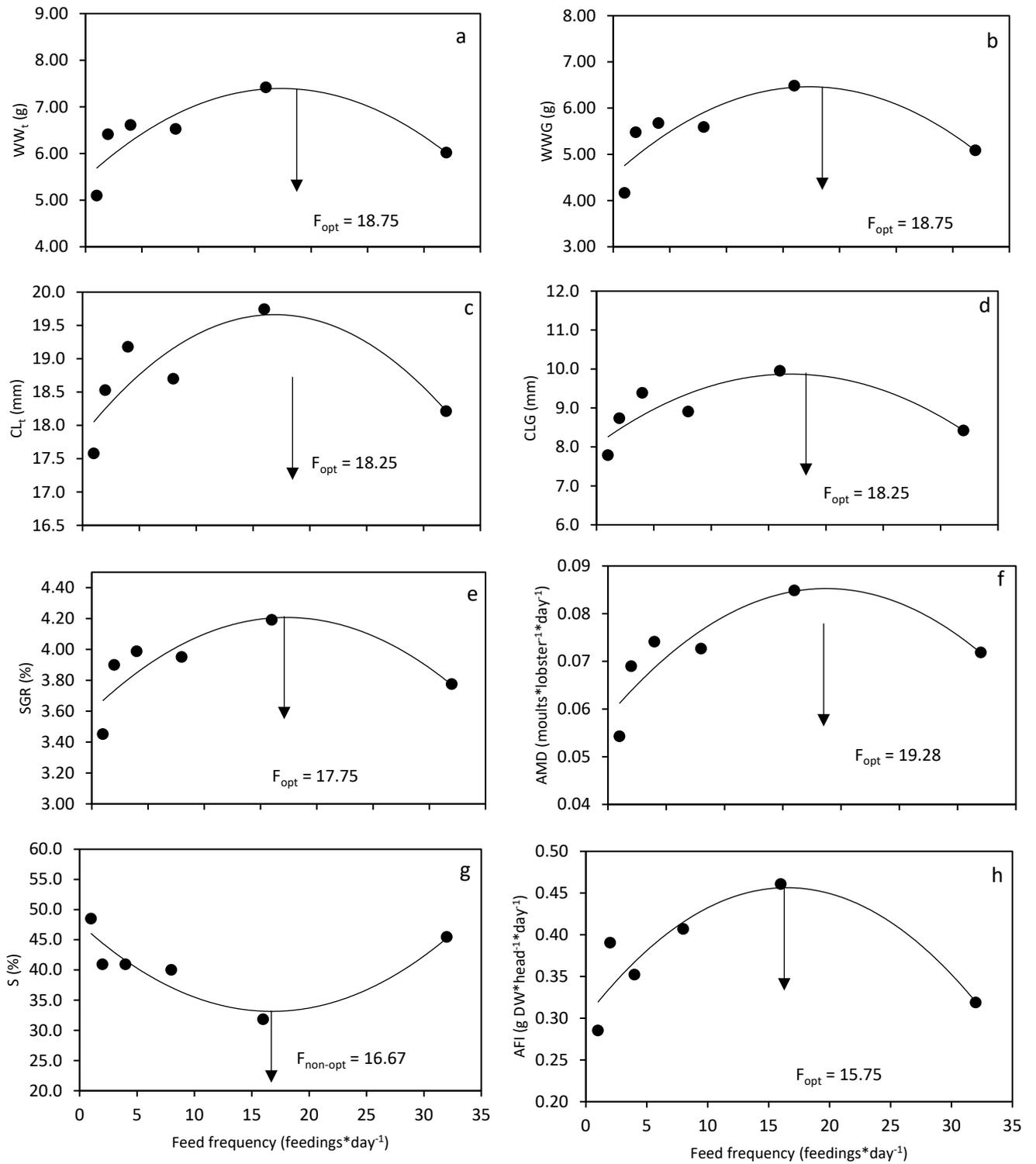


Figure 5.3. Effect of feed frequency on *Panulirus ornatus* a) final wet weight (WW_t), b) wet weight gain (WWG), c) final carapace length (CL_t), d) carapace length gain (CLG), e) final biomass (B_t), f) biomass gain (BG), g) specific growth rate (SGR), h) average moults per day (AMD), i) productivity (P), j) survival (S). The graphs show mean values ± S.E. The arrows show optimal feed frequency (F_{opt}) for WW_t, WWG, CL_t, CLG, SGR and AMD. For S the arrow shows non-optimal feed frequency (F_{non-opt}).

5.3.3 Behavioural observations

In all feed frequency treatments, lobsters displayed a general pattern of increased nocturnal activity in the feeding area. (Figure 5.4). Overall observed lobster presence in the feeding area during both the day and night were lowest for F1. Lobster presence in the feeding area appeared to increase through feeding frequencies of F8 and F16 and dropped in F32. The presence of lobsters in the feeding area during daylight was relatively low in all tested frequencies.

The total sum of lobster presence in the feeding area during both the day and night showed significant quadratic regression (ANOVA, $F = 3.782$, d.f. = 2,21, $p = 0.04$) (Figure 5.5). The highest time in the feeding area was shown for F16, and the lowest in F1. The calculated optimal solution based on the significant quadratic regression was at $19.1 \text{ feed} \cdot \text{day}^{-1}$.

In the two-way ANOVA analysis of feeding behaviour, a significant difference was found for feed frequency on lobster presence in the feeding area ($F = 2.655$, d.f. = 5,36, $p = 0.038$). Tukey HSD comparisons showed significant differences between treatments of F1 and F16 feedings (Figure 5.6). Day/night feeding was also significantly different ($F = 68.763$, d.f. = 1,36, $p = 0.000$). There were no significant interactions between feed frequency and day/night ($F = 1.070$, d.f. = 5,36, $p = 0.393$).

5.3.4 Free amino-acids (FAA) and ninhydrin positive substances

The FAA and ninhydrin positive substances in the feed displayed a significant negative logarithmic ($y = b \ln x + a$) correlation with immersion time (ANOVA, $F = 173.592$, d.f. = 1,16, $p = 0.000$) (Figure 5.7). The regression describes influence of immersion time on the concentration of FAA and ninhydrin positive substances in the experimental feed, with approximately 53% (from $6,786.9 \pm 217.0$ to $3,639.3 \pm 66.2 \mu\text{M/g}$ of the feed,

respectively) leached from the feed within the first hour of immersion. Subsequent concentrations of FAA and ninhydrin positive substances remained relatively constant at, and after, three hours of immersion (Figure 5.7).

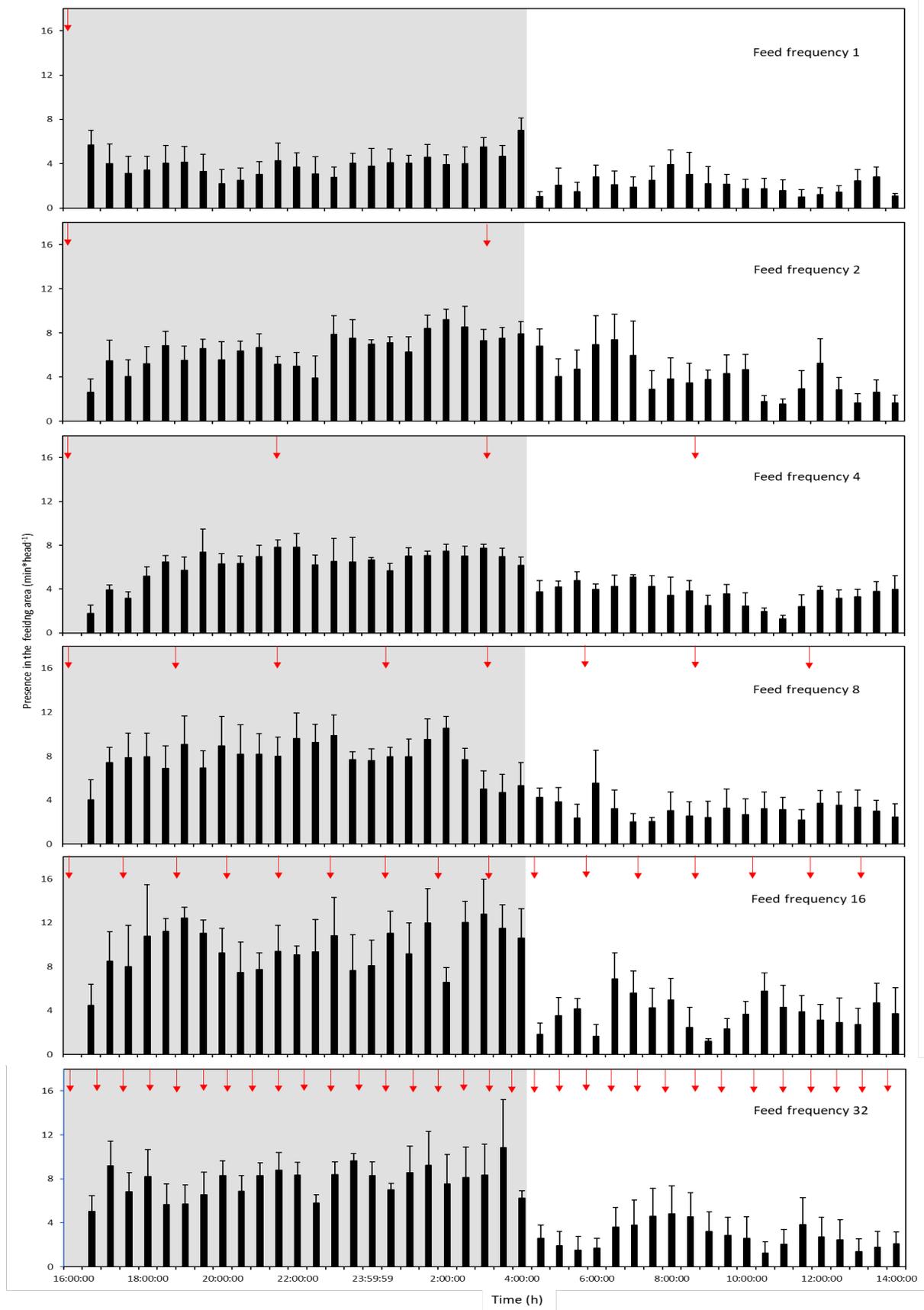


Figure 5.4. Lobsters presence in the feeding area ($\text{min} \cdot \text{head}^{-1}$), shown for all tested feeding frequencies, average 22 h, error bars denote s.e.

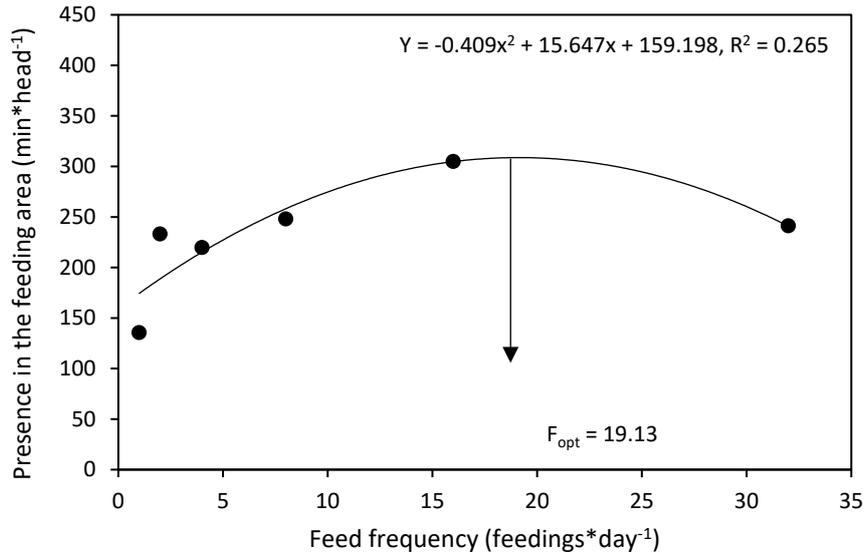


Figure 5.5. Effect of feed frequency on sum of *Panulirus ornatus* presence in the feeding area. The graph shows mean values \pm S.E. The arrow shows optimal feed frequency (F_{opt}) for highest sum of lobsters presence in the feeding area.

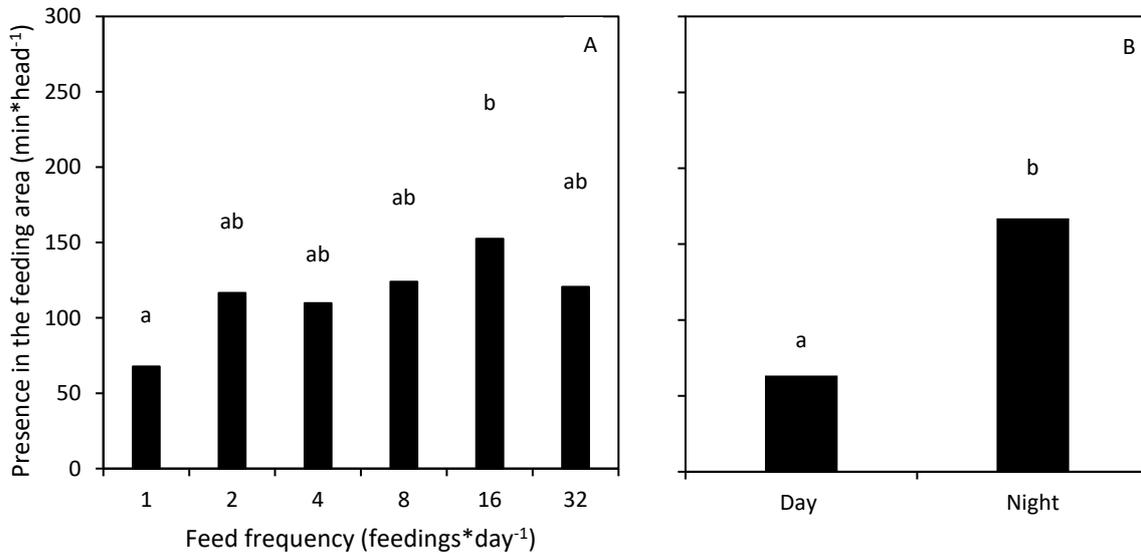


Figure 5.6. Effect of feed frequency (A) and daytime (B) on *Panulirus ornatus* presence in the feeding area (min*head⁻¹). The graphs show mean values \pm S.E., significant differences are indicated with superscripts.

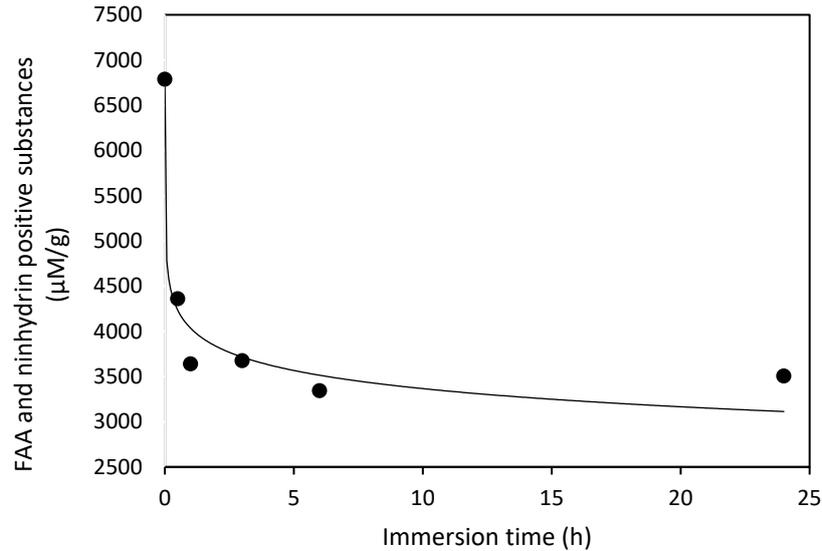


Figure 5.7. Effect of immersion time on free amino-acids (FAA) and ninhydrin positive substances concentration in the feed. The graph shows mean values \pm S.E.

5.4 Discussion

A strong relationship was demonstrated between feed frequency and growth, survival, feeding behaviour and feed intake in juvenile *P. ornatus*. The current research indicated the importance of identifying the optimum feed frequency to maximise growth, as well as the negative consequence of some frequencies augmenting cannibalistic behaviour of lobsters at moult and therefore, reducing survival. In the context of circadian activity feed frequency influenced lobster dark phase activity but not light phase activity.

There was a significant effect of feed frequency on juvenile *P. ornatus* growth, which was highest in F16 for all growth parameters. The optimal feeding frequency for growth parameters was found between 17.7 and 19.3 feed day⁻¹ suggesting that frequent feeding is optimal to satisfy juvenile *P. ornatus* nutritional needs, this was also demonstrated for other spiny lobster species (Syafrizal et al., 2018), penaeids

(Robertson et al., 1993; Sedgwick, 1979) and freshwater crayfish (Arnold et al., 2016; Cortes-Jacinto et al., 2003). The present study is the first in lobsters to show increasing growth, concurrent with increasing formulated feed frequency up to peak at F16 and drop in F32. The peak observed at F16 may be the consequence of spiny lobsters omnivorous dietary preferences, motivating them to be constantly seek feed in the wild consuming a range of invertebrates, fish and marine plants (Boudreau and Worm, 2012; Radhakrishnan and Kizhakudan, 2019; Radhakrishnan et al., 2019b). This multi trophic feeding indicates that lobsters utilise frequent opportunities to forage occurring in their habitat (Boudreau and Worm, 2012). The decreased growth at F32 indicates a limit to growth optimisation through the provision of a specific number of feeds per day. Previous studies on spiny lobsters, including *J. edwardsii* and *Panulirus cygnus*, have demonstrated low growth and survival on formulated feeds, when compared with control groups fed with mussels (Johnston et al., 2007; Perera and Simon, 2015; Simon and James, 2007). The impediments to effected feed utilisation include feed characteristics such as attraction, soak times, particle size, manipulation by lobsters, and attractant leaching (Simon and Jeffs, 2008; Thomas et al., 2003) as well as lobster biological characteristics, including small foregut capacity, slow filling times and long appetite revival (Perera and Simon, 2015; Simon and Jeffs, 2008). Some of these dietary restraints were addressed by the provision of increased feed frequency compensating for feed attractiveness and leaching properties as well as potentially compensating for foregut size and filling periods.

Our study confirmed the use of a formulated feed to provide the nutritional requirements for spiny lobsters over a prolonged period of feeding, similar to that used in a prior study by Shu-Chien et al. (2017). It was noted in our study that AFI was high, especially when provided at a high frequency (F16). The consistency in our results

and outcomes of Shu-Chien et al. (2017) study, suggest that feed as defined above sufficiently satisfied lobsters nutritional needs. The daily feeding ration in our experiment was set considerably above lobster nutritional needs, at an amount of 25% of their WW, while their daily feed intake was reported at 0.8 – 1.2% of WW (Simon, 2009). This approach was taken to provide feeding opportunity for all lobsters and to minimise cannibalism, despite feeding to excess feed frequency significantly affected growth, survival, feed intake and behaviour. This suggests that frequently motivating lobsters to forage and the regular provision of attractive feed are vital aspect of feed intake optimisation. In this study, the FAA emitted from experimental feed were attractants stimulating intake (Williams, 2007; Williams et al., 2005). The ninhydrin method shows positive results for range of substances, including FAA, peptides and proteins (Moore and Stein, 1948) and demonstrated that FAA leach quickly from feed under typical aquaculture conditions (Holm and Walther, 1988). The release of the FAA concentrations in the first hour provided sufficient attraction to stimulate lobsters to feed, after that time the other ninhydrin positive substances were stable within the feed were not substantially released and did not enhance feeding behaviour. This suggests that lobsters react readily to presence of attractive feed and lose interest quickly after depletion of FAA confirming that attractants are a crucial component of formulated feeds to optimise feed intake and growth in spiny lobster aquaculture (Aaqillah-Amr et al., 2021; Kamio and Derby, 2017).

Important aspects of feeding optimisation are feed detection and discrimination, orientation, approach, start of foraging, continuation or cessation of foraging (Fine-Levy and Derby, 1992; Lee, 1996). In this study, the feed attractants stimulated feeding response until leaching limited their effect. With continual exposure to attractants under the F32 frequency lobsters showed decreased AFI and growth. The

doubling of feed frequency from the optimal treatment (F16) may have established a weak but constant background concentration of FAA in the culture water resulting a failure to provide a discrete cue for the provision of fresh feed, either through an insufficient amount or through constant exposure causing habituation. Daniel and Derby (1988) have noted that lobsters can be habituated to attractants, decreasing their responsivity which is counterproductive to sustained feed intake. The provision of small feed rations can also be a stimulant for aggressive competition, stress and or subordination resulting in decreased feeding activity (Thomas et al., 2003).

In contrast to the positive effects of the feed frequency of F16 on growth, survival was negatively impacted. This result is intrinsically linked to AMD with increased moulting frequency providing more opportunities for lobsters to be cannibalised. At and post ecdysis soft shelled lobsters are readily cannibalised by conspecifics, with this complex behaviour being defined as killing and eating of conspecifics (Claessen et al., 2004). In this study, all moults and deceased specimens were at least partly consumed by conspecifics, only remains were found during recording moulting and survival. The factors underlying cannibalism include size dependence, aggressive encounters, competition, hierarchy establishment, prolonged moulting (Claessen et al., 2004; Romano and Zeng, 2017) or as we observed stimulated by the moulting process, commencing immediately before and during ecdysis (cannibalism frequency chapter Vid 13). The increased growth and moulting frequency resulting from higher AFI, created more opportunities for cannibalism, which were readily used by conspecifics. It is acknowledged that increased growth may be related to nutrients supplementation from cannibalism. The experimental conditions used in this study may have contributed to the possible causes of cannibalism, notably high lobster density, vessel size and animal size distribution. In the wild cannibalism is a mechanism regulating

density of social groups and handling food scarcity (Getto et al., 2005). It is clear, that further research is required to investigate methods of decreasing cannibalism to optimise growth.

Lobsters exposed to F16 were most often present in the feeding area, indicating that *P. ornatus* are likely to forage frequently on attractive feed. This study is the first to demonstrate the effect of feed frequency on the duration of feeding time in spiny lobsters, previously demonstrated in omnivorous crabs (*Pachygrapsus transversus*) where in their natural environment they foraged 10-fold longer than fish in the same area (Christofoletti et al., 2010). During our study lobsters spent considerable time in the feed area, with maximum and minimal times of 5 h and 2.3 h for F16 and F1, equalling 10 and 23% of the day (22 h feeding day⁻¹), respectively. The remaining frequencies showed lobster presence in the feeding area between 3.7 and 4.2 h, equalling 17.8% of the day. These results are similar to that observed by Do Nascimento et al. (2020) with freshwater crabs (*Kingsleya attenboroughi*), where they fed for approximately 17% of the day. These observations demonstrate the frequent feeding behaviour of these omnivores, and their interaction and selection of a preferred prey when it is available (Greenwood, 1984). The frequent feeding behaviour of lobsters is a trait that should be utilised in aquaculture.

This study showed similar presence in the feeding area of *P. ornatus* juveniles during daylight across all treatments. This may be the result of the predator free environment and learning abilities of spiny lobsters. This observation corresponds with study on *P. argus* juveniles. Lobsters caught from natural environment, remained in shelters during daylight hours for 2 weeks. After this time, lobsters increased their daytime activity, including feeding and climbing on cages (Lozano-Alvarez, 1996). In juvenile *P. ornatus*, the daylight activity may increase through ontogeny in a laboratory

environment (Kropielnicka-Kruk et al., 2019). This may be the example of ability to validate feeding against potential risk of leaving shelter during daylight.

During the experiment lobsters were observed to be waiting for the start of feeding cycle near or under the pipe supplying feed. This behaviour was often accompanied by chasing away other lobsters from the feed after delivery, suggesting feed competition and dominance. Feeding competition and agonistic behaviours were observed in *J. edwardsii*, exposed to low feed ration and were decreased through increasing feed ration (Thomas et al., 2003). In this study, the increasing feed frequency may have acted to provide attractive feed to all lobsters present in the experimental vessel. After dominant lobsters were satiated first, the submissive specimens had opportunity to feed on attractive feeds delivered later.

5.5 Conclusion

Feed frequency is an important production factor and influences AFI, growth, survival and behaviour in *P. ornatus*. The best feed intake and growth occurred at F16 and indicated that frequent feedings are essential to stimulate foraging and improve growth. The increased growth in F16 in consequence led to increased frequency of susceptibility to cannibalism at ecdysis, due to higher AMD. Further research is required to resolve the problem of cannibalism in aquaculture of *P. ornatus*. The behavioural observations indicated that lobsters start feeding quickly after feed delivery and during time when feed remains attractive (about 1 h). The constant concentration of attractants maintained in the F32 led to decrease of lobster presence in the feeding area, suggesting a need to reduce attractants concentration to retrigger a feeding response.

Supplementary material

The detection routine flowchart is presented in Figure 5.8 (left). For each image set, following image pre-processing and re-sizing of images down to 750 x 1000 pixels (0-255 grayscale), the feeding area was manually defined by user input to create a region-of-interest (ROI) mask. Edges of the ROI that bordered hide structures were identified and shifted a set distance of 10 mm to eliminate possible detections of lobsters that were in hides but not active within the feeding area. The resulting ROI mask was applied to the entire image set (Figure 5.2a). A background image was generated for each image within the set according to the following equation:

$$BI_i = (1-\alpha) \cdot I_{i-1} + \alpha \cdot BI_{i-1}$$

where BI_i is the background image for the i^{th} image, α is a fixed weighting ($\alpha = 0.6$), I_{i-1} is the previous image frame, and BI_{i-1} is the previous background image where BI_0 is the pixel-wise mean image of the entire image set. As such the background image is adaptive to account for slight temporal changes within the scene such as the addition or movement of feed pellets. A background subtraction ($I_{i_BS} = I_i - BI_i$) was then performed (Figure 5.2b), followed by image binarization, and a series of morphological operations (dilations and erosions) to identify contours of candidate detections (Figure 5.2c). For each candidate detection the centroid coordinates were calculated and a 37 x 37 pixel neighbourhood was extracted from the original and background images centred at the candidate centroid (N_C and N_B , respectively). Filtering of potential detections was then performed based on contour size, as well as the mean and standard deviation (std) of neighbourhood pixel intensities for both original and background images (Figure 5.8, right). In reference to Figure 5.8 (right), fixed values

for minimum area (A_{min}), mean (p), standard deviation (q), and ratio of standard deviation values (R) were used for all data (where $A_{min} = 300$ px, $p = 75$, $q = 10$, $R = 4$). Multiple stages of vetting were performed to account for situations where false positives were likely – surface reflections, movement directly above the feed pile, or feed pellets floating on the tank water surface. Finally, the centroid coordinates of confirmed positive detections were returned (Figure 5.2d).

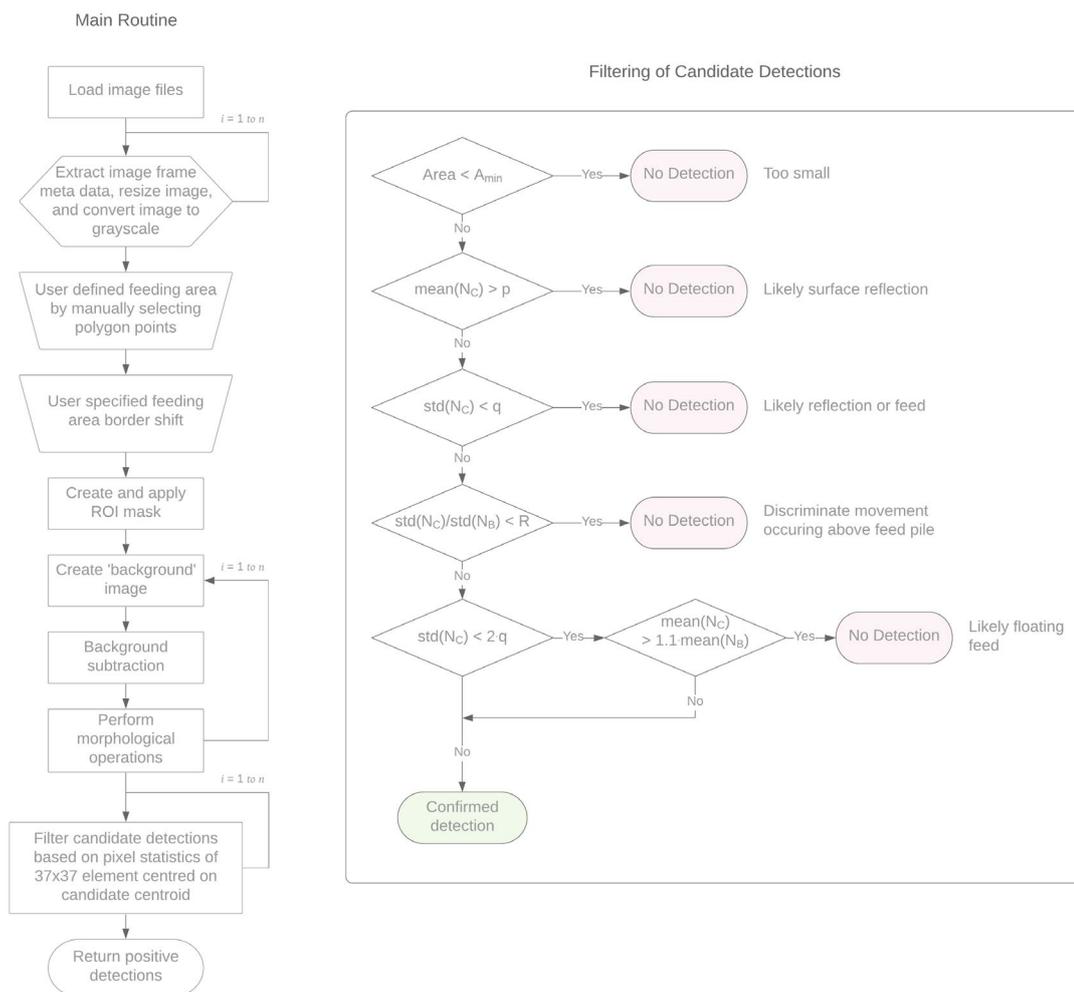


Figure 5.8. Flowchart of main image analysis routine (left), and filtering of potential candidate detections based on pixel intensity statistics of a 37 x 37 pixel region centred at the candidate centroid (right).

Chapter 6 General discussion

Feed is a major cost of lobster aquaculture production, its effective delivery and consumption is critical to the development of successful aquaculture. There are several major components to the optimisation of feed intake and growth in lobsters, of these, the nutritional composition of the feed and the optimisation of feed intake are critical. Effective formulated feeds do not exist for spiny lobsters, their development to date has been hampered by a poor understanding of lobster nutritional requirements, mouthpart morphology, and scheduling of feed presentation. It is these latter physical and behavioural properties that are addressed in this thesis. Ineffective lobster feeding behaviour and resultant growth is strongly influenced by animal feeding patterns, feed attraction and developmental mouthpart morphology. An improved understanding of the unique feeding mechanisms and habits of lobster is essential in the development of more effective feeds and feeding strategies towards the successful development of spiny lobster aquaculture. This thesis employed a holistic approach to investigate feeding behaviour complemented by morphological analysis to understand feed intake in spiny lobsters. Insights were provided into the characteristics required for the development of formulated feeds and associated feeding strategies for lobster aquaculture.

6.1 Behavioural interaction and cannibalism

This study demonstrated that interaction between conspecific lobsters reared communally was an essential aspect of optimisation of feed intake and had a significant effect on lobster growth and moulting frequency (Chapter 2). This finding agrees with studies by Irvin and Williams (2009a), Ratunil Jr (2017) and Marchese et al. (2019) which all demonstrated improved growth of *P. ornatus* juveniles in

communal culture. Likewise, for *P. homarus*, the best growth and moulting frequency was also found in communal culture, a result which was reversible when lobsters were transferred into an individual culture (Vijayakumaran et al., 2010). It is suggested that close tactile contact and/or competition driven feeding stimulation contribute significantly to growth in spiny lobsters. In previous studies, communal culture was compared to individual adjacent cages (Irvin and Williams, 2009a; Ratunil Jr, 2017; Vijayakumaran et al., 2010) or individual unconnected vessels (Marchese et al., 2019). Our study compared all three culture types (unconnected vessels, adjacent cages and communal), which allowed for differentiation of behaviour characteristics between the three tested culture types. The most outstanding behavioural observation was increased activity of lobsters held in adjacent cages, which was not associated with increased feed intake, potentially indicating searching for interaction with conspecifics instead of feeding. Similar behaviour was found in the crab *C. maenas*, which travelled longer distances when held in solitary, and had decreased activity in presence of conspecifics (Fürtbauer and Fry, 2018). This social conformity effect occurs in social animals and is an adaptation of an individual to a group (Fürtbauer and Fry, 2018). The observed correlation of feed intake with gregariousness in lobsters provides justification for the importance of behavioural observations in nutritional research for this species.

During this study, survival was not impacted by culture method, despite the presence of cannibalism in communal culture. Likewise, other studies by Irvin and Williams (2009a); Ratunil Jr (2017) and Vijayakumaran et al. (2010) have found that holding method and hence cannibalism often is not the primary cause of mortality. Indicating the need to investigate the other aspects that may lead to mortality and/or cannibalism, including moult failure and/or nutritional deficiency in lobster culture (Claessen et al.,

2004; Irvin and Williams, 2009a; Thomas et al., 2003). As it has been previously noted that cannibalism does occur in communal culture of juvenile *P. ornatus*, feed frequency was examined as a modulator of this behaviour (Chapter 5). Greatest mortality and associated cannibalism, was observed in the group with the highest growth and moulting frequency, suggesting that cannibalism is inextricably linked to opportunities for predation when lobsters are vulnerable during the moult. Interestingly, behavioural observations showed that agonistic behaviour and cannibalism commenced immediately before and during ecdysis with lobster being killed quickly during the very early stages of moult process (Chapter 5). These observations do not support the theory that cannibalism is related to impaired moulting and suggest that olfactory cues are related to the incidence of cannibalism in *P. ornatus* culture. Similar survival across communal and individual culture suggests overarching suboptimal culture influencing mortality and hence survival.

Further research is required to better determine the nature of cannibalism in *P. ornatus* culture which should include the influence of biological factors such as lobster size, sex and moult stage status. The influence of olfactory stimulation related to cues emitted from moulting individual also requires further consideration. These may include the investigation of mechanisms to limit the incidence of cannibalism by masking olfactory stimulation or the provision of cues similar to those emitted by moulting lobsters, including proline, sarcosine and tryptophan (Juneta-Nor et al., 2020).

It is acknowledged that in communal culture (Chapter 2 and 5) cannibalism and exuviae consumption may supplement lobster nutrition and in consequence, be associated with increased growth. In the crab *Portunis pelagicus*, cannibalism makes a significant contribution to the growth of the large, dominant specimens through the

consumption of smaller, submissive conspecifics (Møller et al., 2008). Therefore, future studies examining the contribution of cannibalised conspecifics to nutrition and growth performance should be undertaken.

In juvenile *P. ornatus*, movement, feeding, and cannibalism, increased with the onset of the dark-phase and the provision of feed, after a period there was a decrease in activity for the remainder of the dark-phase (Chapter 2). Similarly, other spiny lobsters including *P. argus*, *P. cygnus* and clawed lobsters, including *H. americanus* and *H. gammarus* display high levels of nocturnal activity (Cox et al., 1997; Golet et al., 2006; Jernakoff, 1987; Karnofsky et al., 1989; Moland et al., 2011). In this thesis, lobsters were also observed to increase their light-phase activity in a laboratory environment (Chapter 2 and 5), indicating the ability to adapt to a predator free environment. This agrees with Lozano-Alvarez (1996) and Marchese et al. (2019), where captive lobsters also displayed feeding and extended their activity during the light-phase. It is likely that feed attractiveness was a leading motivator supporting daytime feeding, possibly allowing submissive lobsters an opportunity to feed after dominant conspecifics are satiated overnight. Interestingly, the light-phase activity was constant across all feed frequencies (Chapter 5), in contrast to the peaks in dark-phase activity, supporting the theory that submissive lobsters, with higher motivation to feed, are leaving shelters in similar numbers during the light-phase.

6.2 Feed format

Feed intake regulates nutrient consumption and ultimately growth, it is closely related to a lobster's feed processing capacity and mouthpart morphology, including mouth aperture size. This study found a correlation between lobster mouth aperture size and carapace length making it easy to assess an optimal pellet diameter with regards to

lobster mouth aperture capacity (Chapter 3), noting this is species specific. The ability to manipulate feed diameter is beneficial in minimising waste caused by fragmentation during feed ingestion (Sheppard et al., 2002) (Chapter 3 and 4). This study noted, that with increasing pellet diameter, non-feeding related waste (NFRW) decreased concurrently with an increase in feeding related waste (FRW). The low FRW found with small pellet diameters indicated that lobsters were consuming whole pellets with minimal waste. This supports previous conclusions where lobsters were also found to feed relatively efficient on small pellets (Sheppard et al., 2002; Smith et al., 2009a). In this study, improved feeding efficiency on smaller pellet diameters did not correlate with increased feed intake, likely due to the rapid leaching of attractants from small pellets making them less attractive and reducing consumption (Francis et al., 2014; Lau, 1987), (Chapter 5).

Designing feed to meet the mastication ability of lobsters as they develop is possible when feed manufacturers are equipped with pellet specifications of width and length, texture, viscosity and stability. The ontogenetic changes in morphology, including increase of calcification and robustness, indicated that optimal feed textures (softness/hardness) may be different for the growth stages. Cox et al. (2008), concluded similarly for *P. argus*, that mouthparts size, calcification and robustness increased progressively with growth, and pellet texture should be adjusted to animal size. Further research is needed to optimise formulated feed intake however, findings from this thesis (Chapter 3 and 4) indicate that defining the optimal feed characteristics will be a necessary component and that a multifaceted approach may be required. The use of videography of the feeding mechanism and general behaviour could be used to help determine optimal feed formats.

A novel variable was developed in this thesis, whereby feed waste is categorised into NFRW and FRW (Chapter 4), this is a valuable tool that can be used in understanding complex response of spiny lobsters to variable feed characteristics.

The assessment of lobster mouthparts showed variable setation across *S. verreauxi* and *P. ornatus* elucidating a differential for filtration of particles as a feeding mechanism. The greater richness of setation in *S. verreauxi* compared to *P. ornatus* may reflect a higher affinity for this type of feeding behaviour. Similarly, appendages allowing for filter feeding were found in *J. edwardsii* and *P. argus* (Cox et al., 2008; Sheppard et al., 2002). Filter feeding is used by many decapods however its role may decrease during ontogeny as demonstrated for *H. americanus* (Garm, 2004a; Gerlach et al., 1976; Lavalli and Barshaw, 1989). This indicates that filter feeding could be used by spiny lobsters, particularly for the early juvenile stage. The dispersed feed particles may be consumed by early juveniles and may motivate them to search for larger feed pieces to consume. This is not something that has previously been considered and further research is required to analyse value of this method in context of nutrition and motivating to feed.

Collectively, this thesis demonstrates the complexity of spiny lobster feed ingestion in relation to morphological and behavioural factors. Through a holistic approach, including ontogenetic morphology (Chapter 3) and effect of pellet dimensions and texture on feed waste (Chapter 4), this thesis provides additional knowledge to enable the deciphering of feed ingestion and waste production in spiny lobsters. Formulated feeds characteristics, including diameter, length and texture should be tailored to the mouth aperture size, mouthpart morphology and growth stage. In context of pellet consumption, the optimal pellet diameter is governed by mouth aperture size. The optimal pellet length is governed by mouthparts morphology, in context of pellet

handling. In addition, the pellet texture is linked to mouthpart morphology (spines) and ontogeny. The morphological investigation indicates that mouth aperture size and mouthparts morphology should be considered individually in each spiny lobster species in context of formulated feed development in agreement with Cox et al. (2008). Video observations have proven to be invaluable in deciphering general feeding behaviour and intake (Chapter 2 and 5) however, the exact mechanisms of feed waste production remain unclear. Video analysis of the moving mouthparts whilst feeding on feeds of different formats would likely provide useful insight into the mechanism of feeding and waste production.

6.3 Feeding behaviour and attraction

The feeding behaviour in spiny lobsters, includes feed search, detection, identification, capture and ingestion or rejection (Major et al., 2017). These steps are determined by chemosensory systems and mouthpart morphology. In addition, feeding behaviour is affected by social behaviours, including gregariousness and hierarchy. This study indicated a strong relationship between feed frequency and growth, survival, feeding behaviour and feed intake in juvenile *P. ornatus* (Chapter 5). Lobster feed intake and growth was maximised at a feed frequency of 16 times per day (F16), indicating that frequent feeding is optimal to satisfy juvenile *P. ornatus* nutritional needs. Similarly, frequent feeding was suggested as potentially useful for growth improvement in *P. versicolor* and other decapods (Arnold et al., 2016; Cortes-Jacinto et al., 2003; Robertson et al., 1993; Sedgwick, 1979; Syafrizal et al., 2018). Spiny lobsters are omnivorous and consume a wide range of feeds on multi trophic levels, which is correlated with persistent feed seeking behaviour in the wild (Boudreau and Worm,

2012; Radhakrishnan and Kizhakudan, 2019; Radhakrishnan et al., 2019b), and suggests the benefit of extended foraging time.

In this study, lobsters were feeding for approximately 10 to 23% of the day in lowest (F1) to optimal (F16) feed frequency, respectively, with an average 17.8%, similar to that of the freshwater crab *Kingsleya attenboroughi* (Do Nascimento et al., 2020). This confirms a similar proportion of time spent on feeding for omnivorous decapods, and indicates a prolonged feeding period, which allows feeding preference based on availability (Chubatý et al., 2014; Greenwood, 1984). This suggests that lobsters in culture should forage on the feed available in the tank, however if they lose interest in feed concurrently with depletion of attractants, feed intake is reduced. In culture, feed attraction/exposure is the primary aspect in the motivation to feed for lobsters, and prolonging attractiveness is key to feed intake. Free amino acids (FAA) are crucial in feed attraction but leach quickly from formulated feeds after water immersion (Aaqillah-Amr et al., 2021; Holm and Walther, 1988; Kamio and Derby, 2017; Williams, 2007; Williams et al., 2005). In this study (Chapter 5), adequate amounts of FAA were emitted from the feed within the first hour after supply to stimulate feeding behaviour. There was also a trend for decreased AFI and growth when feed was provided in very small amounts and with high frequency, which may have been due to insufficient, or alternatively, a constant low concentration of FAA in the culture water (Daniel and Derby, 1988). The habituation to attractants leads to decreasing responsivity, which acts counterproductively to feed intake (Daniel and Derby, 1988). The provision of small feed rations to the hierarchical group can also trigger aggressive competition, subordination and stress resulting in feeding decrease (Thomas et al., 2003).

There was also some visual stimulation associated with the provision of feed (Chapter 2 and 5), indicating that visual exposure to feeds may play an important role in the

attraction. Further research should investigate visual aspect of exposure to feeds either by movement (feeds movements) or supplying feeds in the lobster field of view. Extending sinking time of pellets and in consequence increasing visual exposure may strengthen motivation to feed. Future research should include observations of individual lobsters and the effect of their social position on feed intake. Analysis of effect of social behaviours on feeding, may indicate that multitrophic levels are required for feeding optimisation in hierarchical group of lobsters.

6.4 Conclusion

The findings of this thesis have revealed complex interactions between behavioural and morphological aspects of feeding and growth in juvenile spiny lobsters. The gregariousness and physical interactions were found to be essential in feed intake and growth optimisation but also highlighted the complex interaction with cannibalism. Understanding some of the triggers for cannibalism may assist in mitigating the negative consequences for spiny lobster aquaculture. It was noted that there was some plasticity in lobster behaviour, with feeding extending into daylight hours in the absence of predators. This indicates that *P. ornatus* has the ability, to alter established behaviours, adapt to alternate feeding patterns providing additional methods to enhance feed intake and optimise growth for some members of a cohort.

Feed intake was impacted by the physical properties of pellets, including dimensions, texture and attractiveness, suggesting that pellet dimensions and texture should be adapted to the lobster mouth aperture and ontogenetic shifts in mouthpart morphology. This was confirmed by the analysis of feed waste composition (FRW and NFRW), as a method to optimise feeding efficiency in spiny lobsters. The species specific correlation between lobster mouth aperture and CL noted in this thesis provides a non-

invasive method to adjust pellet dimensions for lobster size. Mouthpart morphology additionally highlighted a filter feeding capacity in juvenile spiny lobsters, which may play a role in early feeding and, or a feed stimulatory response. Feed attractiveness drives a stimulatory response and determines optimal intake in relation to feed frequency, with up to 16 feedings proving optimal for the feeds provided in this study. AFI, growth, survival and behaviour in spiny lobsters are highly dependent upon feed intake and the minimisation of cannibalism. Further research is required to investigate attraction/gustatory and visual aspects of exposure to feeds, whereby the analysis of different attractants on feeding response and behavioural observations of individual lobsters may add important knowledge towards improving feed intake, growth and survival in captive reared spiny lobsters.

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