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JAMES COOK UNIVERSITY AUSTRALIA

Broodstock Conditioning and Larval Rearing of the Marine Ornamental White-striped Cleaner Shrimp, *Lysmata amboinensis* (de Man, 1888)

Thesis submitted by

Vasiliki Tziouveli

For the degree of Doctor of Philosophy

In the Discipline of Aquaculture

Within the School of Marine and Tropical Biology

James Cook University, QLD, Australia

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Human (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1187).

_____ _____

Signature Date

Vasiliki Tziouveli

Name

Statement on the contribution of others

My sincere gratitude to V. Zupo and G. C. Fiedler for reviewing the thesis and helping me improve it through their corrections and suggestions. Publications from the thesis have been co-authored by my supervisors since they provided intellectual and technical guidance and editorial advice. Thanks to the many reviewers for the valuable feedback on the submitted manuscripts.

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This project has been as much a pleasure as a pain in the… neck. But I would not have had it any other way!

General Abstract

Lysmata amboinensis is a protandric simultaneous hermaphrodite (PSH); individuals develop as males (MPs) and then change to simultaneous hermaphrodites (SHs). PSHs exhibit functions of both males and females during each reproductive cycle. There are a number of important physiological reproductive features that have not been previously studies in this species, including male size at sexual maturity, fecundity of SHs based on body size differences and timing of the sex phase change. To answer the former two questions, MPs were paired with SHs to produce various size combinations. It was found that *L. amboinensis* MPs were sexually mature at a total length (TL) of 34.0 mm and larval production increased with increasing body length, with large SHs displaying 3 times the larval output of small and medium -sized SHs. To answer the latter question, MPs were exposed to different social situations by being paired with either another MP (of similar or larger size) or with an SH. Indeed, the timing of sex phase change was influenced by the social condition, indicating "environmental sex determination" (ESD). The "default" size at change was 37.1 mm (TL) when MPs were reared alone; when MPs were paired with a larger MP it was the larger MP that changed first to SH, while the partner shrimp changed at a larger size than the "default". If MPs were paired with similar-sized MPs or with SHs, phase change occurred at a smaller size than the "default". The observed patterns are discussed in terms of reproductive opportunities for a species occuring in small groups in the wild, referred to as a "low density" *spp*.

Aquaculture production of *L. amboinensis* has been problematic due to the prolonged duration of larval development, punctuated by periods of high mortality. Broodstock

maturation diets have been shown to affect fecundity and offspring quality. The primary aim of this component of the study was to identify a suitable maturation diet for *L. amboinensis* from a range of commonly available feeds. The six diets were squid, mussel, adult *Artemia*, a commercial feed, and combinations of the aforementioned and were fed to *L. amboinensis* over four reproductive cycles. Broodstock fed the squid-mussel diet lost > ½ of the egg mass during incubation, with decreased larval production, the lowest being 22 larvae. By contrast, broodstock fed adult *Artemia* retained > 80 % of their eggs; however, hatchability was poor, resulting in low numbers per hatch, ranging from 12 to 51 larvae. The commercial feed yielded high fecundity and larval output. In *L. amboinensis*, a maturation diet consisting of natural feeds alone results in poor reproductive performance; partial, or complete, replacement with an artificial feed is economically feasible.

Lipid enrichment of *Artemia* to boost their highly unsaturated fatty acid (HUFA) content is a standard procedure to improve diet performance and can be taylored to deliver key nutrients to culture animals. Frozen, lipid enriched adult *Artemia* were fed to *L. amboinensis* broodstock to investigate their suitability and elucidate the role of essential fatty acids (EFAs) in enhancing reproductive performance. Four lipid enrichment levels, un-enriched ("unenr"), 1/3 enriched ("1/3 enr"), 2/3 enriched ("2/3 enr") and enriched ("enr") *Artemia*, were fed to *L. amboinensis* over three reproductive cycles. Spawning was high for all diets. Viable fecundity (meaning the number of viable larvae produced) varied, however, and was significantly greater for broodstock fed the "enr" *Artemia*, with a mean 529 (± 77) larvae, as opposed to 49 (± 11) recorded for the "unenr". The increased larval production was attributed to better embryo hatchability and

was associated with an increase in the docosahexaenoic (DHA) content of the diet (11% of total fatty acids FAs). The roles of other dietary EFAs are also discussed.

The best feeding regime for the larvae in order to achieve high growth, survival and metamorphosis rates in culture and, eventually, production of new stock was also considered of prime importance. Therefore, larval prey items of varying shape, size and dietary value (specifically, rotifers, *Artemia* nauplii and *Artemia* metanauplii), and in different concentrations, were examined. Larval growth was not affected by any of the independent variables. Larval survival was influenced by absolute food availability rather than food concentration, given that feeding the early larvae 1 *Artemia* ml^{-1}, 3 *Artemia* ml^{-1}, 18 rotifers ml^{-1} or 54 rotifers ml^{-1} resulted in no difference. All prey types tested were suitable first feeds for zoea 1 (Z1) *L. amboinensis*, with survival > 90%, verifying the plasticity of *Lysmata spp*. However, from Z2 onwards *Artemia* may be a better feeding option than rotifers, since carry-over effects of the diet to the next larval stage were noted. The nutritional value of the prey affects development of *L. amboinensis* larvae, with those fed *Artemia* metanauplii enriched with Algamac-3000 showing high developmental rate and low zoeal stage dispersion. Exposing the larvae to starvation (as control for true diet effects), revealed that *L. amboinensis* larvae are facultative primary lecithotrophs (FPL), meaning they can moult to Z2 using only endogenous reserves.

Triplicate samples of the hepatopancreas, ovaries and tail muscle of *L. amboinensis* broodstock, and of newly hatched larvae and larvae subjected to a period of starvation or feeding (from hatch to Z2), were processed to generate their total lipid content and FA profiles. The hepatopancreas had a high lipid content, confirming its role as a process and storage organ in *L. amboinensis*. Lipids were also a major component of ovarian dry

weight (dw), in agreement with reports on other crustaceans during maturation. The tail muscle, being a functional rather than a storage organ, contained low total lipids, and was the tissue that closely resembled the FA profile of the newly hatched larvae. Saturated fatty acids (SFAs) and HUFAs were the most abundant components of the lipid profile in broodstock tissues and in larvae. Monounsaturated fatty acids (MUFAs) may have been preferentially catabolized to meet energetic and metabolic requirements. It appeared polyunsaturated fatty acid (PUFA) and HUFA requirements were met through the larval diet. DHA and eicosapentaenoic (EPA) were preferentially retained during nutritional stress, confirming their importance for *L. amboinensis* during early larval development.

Mouthparts and alimentary canal were examined in *L. amboinensis* larvae using scanning electron microscopy and histology. The morphology of mouthparts and digestive tract structures at different larval stages, indicate that ingestive and digestive capabilities are well developed in the species from early on. With increasing age of the larvae, the mouthpart appendages increased in size, the hepatopancreas in tubular density and the midgut in length. The density of setae, and robustness of teeth and spines of individual structures, increased. The most pronounced changes, from early to late stage larvae, involved the formation of pores on the paragnaths and labrum, the transformation of the mandibular spine-like teeth to molar cusps, the development of the filter press in the proventriculus, and of infoldings in the previously straight hindgut. The results suggest that early stage *L. amboinensis* larvae may benefit from soft, perhaps gelatinous prey, whereas later stages are better equipped to handle large, muscular or fibrous, foods.

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List of Abbreviations

A. franciscana = *Artemia franciscana*

AIMS = Australian Institute of Marine Science

ARA = arachidonic acid

B. rotundiformis = *Brachionus rotundiformis*

C. muelleri = *Chaetoceros muelleri*

CEF = controlled environment facilities

CMAF = Cairns Marine Aquarium Fish

DBH = day before hatch

DHA = docosahexaenoic acid

dw = dy weight

EFA = essential fatty acid

EPA = eicosapentaenoic acid

ESD = environmental sex determination

FA = fatty acid

FP = female phase

FPL = facultative primary lecithotrophy

HUFA = highly saturated fatty acid

I. galbana = *Isochrysis galbana*

JCU = James Cook University

J. edwardsii = *Jasus edwardsii*

J. verreauxi = *Jasus verreauxi*

LNA = linolenic

LOA = linoleic

L. opalescens = *Loligo opalescens*

L. ankeri = *Lysmata ankeri*

L. boggessi = *Lysmata boggessi*

L. debelius = *Lysmata debelius*

L. seticaudata = *Lysmata seticaudata*

L. wurdemanni = *Lysmata wurdemanni*

M. rosenbergii = *Macrobrachium rosenbergii*

MP = male phase

MUFA = monounsaturated fatty acid

P. borealis = *Pandalus borealis*

P. monodon = *Penaeus monodon*

P. semisulcatus = *Penaeus semisulcatus*

P. serratus = *Palaemon serratus*

P. vannamei = *Penaeus vannamei*

PL = postlarva

PSH = protandrous simultaneous hermaphroditism

PUFA = polyunsaturated fatty acid

S. serrata = *Scylla serrata*

SFA = saturated fatty acid

SH = simultaneous hermaphrodite

spp. = species

T. suecica = *Tetraselmis suecica*

TL = total length

wet wt = wet weight

Z = zoea

Chapter 1 - General Introduction

The marine aquarium trade and the aquaculture potential of the white-striped cleaner shrimp, *Lysmata amboinensis*

1.1 Trade profile of ornamental species

When the financial value of commercial fishing and that of product collected for the aquarium industry are compared, the aquarium product delivers a higher value per stock volume. In 2000, 1 kg of aquarium fish from coral reefs on the Maldives was valued at almost US$500, whereas 1 kg of reef fish harvested for food was worth US$6 (Wabnitz *et al*. 2003). Although fish, live coral and live rock dominate the aquarium market, the trade in ornamental crustaceans has been expanding (Gasparini *et al*. 2005). It is difficult to obtain accurate information on the market share of decapod crustaceans alone, as reports are either based on the entire ornamental trade or the invertebrate component of it. Accordingly, 20 million marine fish and another 9-10 million marine invertebrates are traded annually with a total invoice value estimated at US$330 million; excluding corals, invertebrates were worth US$30 million (Larkin and Degner 2001; Macfadyen *et al*. 2003).

Approximately 85% of all invertebrates in the aquarium trade originate from the Indo-Pacific reefs, with the Western Atlantic a close second (Green 2003). The major exporters are the Pacific Island nations, the Philippines and Indonesia, followed by Florida and

Hawaii. North America imports approximately two thirds of all traded organisms and the remainder goes to the European Union and Japan (Corbin *et al*. 2003; Olivier 2003).

The criteria for ornamental status have been defined as: 1) Appearance, such as dazzling colouration and unusual morphological patterns (e.g. spots and stripes), 2) Ease of care and 3) Hardiness in captivity (Calado *et al*. 2003a, b). Furthermore, species with notable behaviour, such as removal of fish ectoparasites and control of nuisance organisms (e.g. of the glass anemone), or displaying symbiotic associations with vertebrates or invertebrates, may be of interest to the aquarium hobbyists (Rhyne *et al*. 2004).

Several shrimp in the infraorder Caridea satisfy the above criteria and occupy approximately 40% of the trade in ornamental decapods. Caridean shrimp found in both public and private aquaria come from the following families: Hippolytidae (e.g. *Thor amboinensis*, the sexy shrimp), Rhynchocinetidae (the hingebeak shrimp, e.g. *Rhynchocinetes durbanensis*), Alpheidae (the snapping shrimp, e.g. *Alpheus bellulus*), Gnathophyllidae (the bumblebee shrimp, e.g. *Gnathophyllum americanum*), Hymenoceridae (the harlequin shrimp, e.g. *Hymenocera picta*) and Palaemonidae (e.g. *Periclimenes pedersoni*, the anemone shrimp) (Calado 2008). Approximately 49 caridean species are traded, 17 more than the next decapod group, anomuran crabs. About a third of the traded carideans come from the family Hippolytidae, with *Lysmata* species accounting for the majority (Calado *et al*. 2003a). The fire shrimp, *Lysmata debelius*, and the white-striped cleaner shrimp, *L. amboinensis* (Plates 1.1a, b), are amongst the most popular marine ornamentals and are of high retail value (Moe 2001). Current efforts to

regulate the marine ornamental trade in the European Union have already highlighted these two shrimp as species whose imports should be closely monitored (Calado 2008).

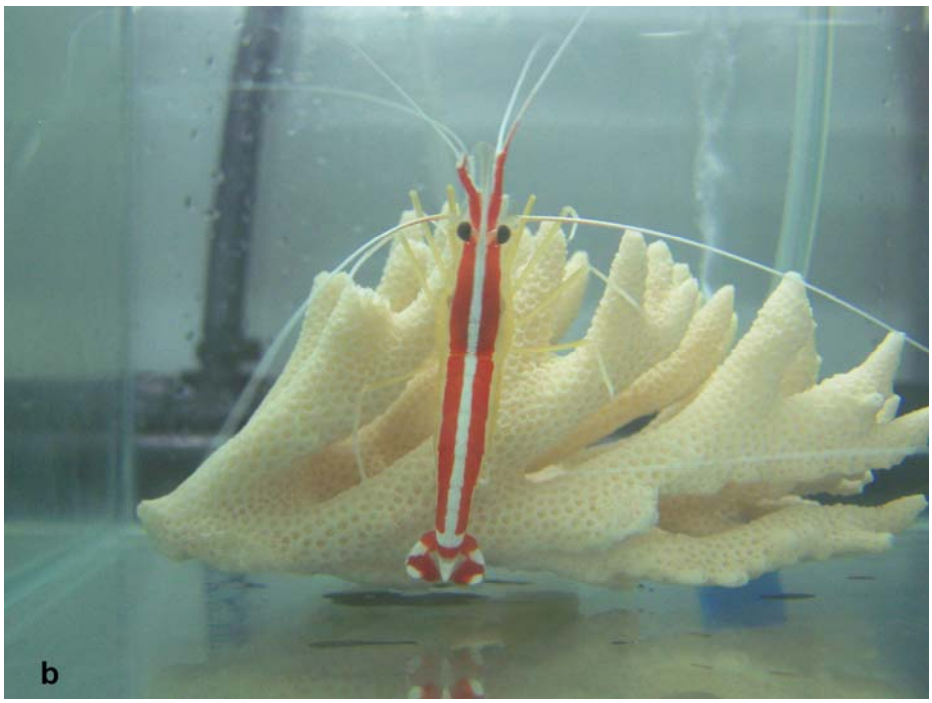

PLATE 1.1 Popular in the aquarium trade *Lysmata* species, a) the fire shrimp, *L. debelius* (http://www.freshmarine.com) and b) the white-striped cleaner shrimp, *L. amboinensis*.

1.2 Reproductive biology and ecology of *Lysmata amboinensis*

Lysmata amboinensis is found in coral reefs of the Indo-Pacific, and has been observed to clean numerous fish, from groupers to moray eels (Calado 2008). Despite being one of the most collected shrimp species in the aquarium industry, its population ecology has never been addressed. Most recent research explores the development of culture protocols (Calado *et al*. 2007; Calado *et al*. 2008a, b; Cunha *et al*. 2008), while earlier studies focused on the fundamentals of reproduction of captive broodstock (Fletcher *et al*. 1995; Fiedler 1998; Simoes *et al*. 1998).

The cleaner shrimp, *L. amboinensis*, is a PSH, which is the sexual system characteristic of the genus (Bauer and Holt 1998; Udekem d' Acoz 2003; Bauer and Newman 2004; Baeza and Anker 2008). Accordingly, all individuals pass through an early male phase (MP), which later evolves to include a female phase (FP). Protandry is not uncommon within carideans, although gonochory, meaning separate sexes, is the dominant sexual system (Bauer 2004). In protandric species, during the transition from the MP to the FP external male characters (e.g. the *appendix masculina*) are lost and those of the FP (e.g. the "breeding dress") are attained. The gonads of sex changers function as testes in MPs and then ovaries in FPs.

In *L. amboinensis*, while the MP is similar to that of other protandric carideans, sex change to the FP is never complete, as the testicular tissue and male ducts are retained and sperm is produced (Bauer 2004). Thus *Lysmata* individuals are defined as PSHs or SHs. Generally, the size at which MPs change sex phase (to FP or SH) appears to be a flexible trait in carideans, resulting in the development of theories and models to predict

the timing of change (Hannah and Jones 1991; Bergstrom 1997; Bauer and Holt 1998; Lin and Zhang 2001a; Baeza and Bauer 2004; Zhang and Lin 2005a, b).

Although PSH appears to be consistent among *Lysmata*, the population structure, in terms of sociality and possibly the mating strategies, may differ between species. In "low density" species, like *L. amboinensis*, wild shrimp are found in small groups or in pairs (Criales 1979; Wirtz 1997; Bauer 2006). On the contrary, in "high density" or "crowd" species (e.g. *Lysmata wurdemanni*, *Lysmata seticaudata*) individuals occur in large aggregations and show minimum aggression even when in breeding status (Bauer and Holt 1998; Bauer 2006).

Maturation of ovaries, moulting, mating, spawning, embryonic development and hatching are inter-related processes in breeding *L. amboinensis*. Following the hatching of larvae, the FP undergoes ecdysis (moult) to renew the "breeding dress" and shed any unhatched embryos. Mating occurs soon after the parturial (pre-spawning) moult and the MP hermaphrodite attaches a pair of spermatophores under the FP hermaphrodite abdomen. Spawning takes place a few minutes to a few hours after copulation, even if fertilization has not been successful, and the new embryo incubation cycle is initiated (Fiedler 1998; Bauer 2004).

In *L. amboinensis* incubation of eggs (Plate 1.2) lasts approximately two weeks (Fletcher *et al*. 1995). Incubation period duration is related to the reproductive ecology of species; tropical and warm temperate carideans, which are usually successive breeders, have a short incubation period, whereas incubation of embryos lasts several months in high latitude species, which may spawn only once a year (e.g. *Pandalus borealis*, Bergstrom 2000). Because of the cost of brooding the embryos, fecundity in carideans is

significantly reduced, for example 172-418 eggs for *Palaemon northropi* and 170-8,960 eggs for *Macrobrachium olfersii* (Anger and Moreira 1998) as opposed to 353,834-884,708 eggs for *Penaeus plebejus* (Courtney *et al*. 1995) or 100,000-1,000,000 eggs for *Penaeus vannamei* (Sunden and Davis 1991). The principle also applies to *L. amboinensis* with a hatch usually ranging from 300 to 1650 larvae (Fletcher *et al*. 1995; Calado *et al*. 2008b). The aforementioned variation in *Lysmata* could be attributed to the size of spawners, since fecundity is positively correlated to female body length (Corey and Reid 1991; Bauer 2002a), but parasitism, stress, temperature, and diet are also known to affect reproductive output in decapods (Kuris 1991; Smith and Ritar 2005; Calado *et al*. 2008b).

PLATE 1.2 *Lysmata amboinensis* adult with freshly spawned eggs attached under the abdomen (green area).

Lysmata embryos hatch as planktonic larvae termed zoeae. The number of stages a larva undergoes prior to settling varies amongst species of the genus, for example *L. seticaudata* has nine zoeal stages (Calado *et al*. 2004), *Lysmata rafa* has 11 (Rhyne and Anker 2007) and *L. amboinensis* has 14 (Wunsch 1996; also current study, see Appendix A). The time it takes to complete the larval cycle varies not only among *Lysmata* species but among individuals of the same species or even the same batch. Genetics and environmental conditions (e.g. physical parameters and prey availability) may influence the rate of development. Under unsuitable rearing conditions, *Lysmata* larvae are known to display "mark-time moulting", whereby larvae continue to moult and grow but not progress to the next zoeal stage (Gore 1985; Calado 2008). Indeed, older *L. amboinensis* larvae several millimetres bigger than the "average" last stage have been recorded in culture (pers. obs.). *Lysmata amboinensis* larvae may take as little as 3 months or as long as 5 to settle (pers. obs.), with the latter time period being more common (Fletcher *et al*. 1995; Wunsch 1996).

1.3 Culturing marine ornamental shrimp: Problems and solutions

Marine shrimp for the aquarium industry are largely sourced from the wild, a practise that has long been regarded as problematic (Tlusty 2002; Wabnitz *et al*. 2003). Many have argued that removal of certain species of shrimp that display associations with various sea creatures could result in long-term ecological imbalances; for example, collection of *L. amboinensis* or other cleaner shrimp from the reefs could potentially

diminish the health of client fish and reduce their abundance (Limbaugh *et al*. 1961; Jonasson 1987; Bunkley-Williams and Williams 1998). Although damaging fishing techniques (e.g. sodium cyanide or quinaldine) are not commonly employed for the collection of marine ornamental shrimp, other problems associated with wild collection remain. Such are the unreliability of supply, which raises the number to be collected beyond what would be necessary to meet market demand, and the inadvertent introduction of parasites or disease from wild animals (Wabnitz *et al*. 2003). In addition, wild shrimp may adapt poorly to the captive environment and suffer high mortality during acclimation, requiring the collection of more animals to satisfy demand (Baquero 1999; Wabnitz *et al*. 2003). When combined with the absence of data on population fluctuations in nature, the possibility of overexploitation of wild stocks becomes alarming (Wood 2001; Bunting *et al*. 2003).

As so little is known about marine ornamental shrimp ecology and the impact of harvesting these organisms from the wild, aquaculture has been suggested as the alternative (Zhang *et al*. 1998a, b; Lin *et al*. 1999; Calado 2008). Aquaculture could meet the demands of the aquarium trade by year-round, domesticated species availability and quarantine safety. Consequently, development of appropriate husbandry has become a priority, with much research focus on *L. amboinensis* and other sought after species (Fletcher *et al*. 1995; Palmtag and Holt 2001; Calado *et al*. 2003a; Parks *et al*. 2003). For mass production of ornamental shrimp to become a reality, their life cycle must be consistently completed in captivity. The steps involved would include: 1) Captive spawning of the broodstock, 2) Mass rearing and settlement of the larvae, 3) Grow out of the juveniles and 4) Subsequent mating and spawning of the captive raised stock. Each

step has its own challenges but larval rearing has been the major complication in the successful culture of *L. amboinensis*, the main issue being the particularly long larval duration and the low larval survival (Lin *et al*. 2002; Calado *et al*. 2003a; Cunha *et al*. 2008).

Efforts to increase survival rate and shorten the larval cycle of ornamental shrimp have focused on the provision of appropriate larval rearing systems and larval feeds (Zhang *et al*. 1998a, b; Simoes *et al*. 2002; Rhyne and Lin 2004; Calado *et al*. 2003c; Calado *et al*. 2005a, b). Palmtag and Holt (2001) described a cylindrical rearing chamber for *L. debelius* and Calado *et al*. (2003c) a "planktonkreisel" type system for *L. seticaudata* and *L. wurdemanni*, suitable for other ornamental species as well. Captive larval rearing has been based almost exclusively on live feeds, occasionally supplemented with fresh or fresh-frozen animal tissue in late stage larvae and postlarvae (PL) (Palmtag and Holt 2001; 2007). Despite the differences in feeding habits of penaeid and caridean larvae (herbivores/omnivores and strict carnivores, respectively, Jones 1998), the use of *Artemia* has been uniform throughout shrimp larviculture (Samocha *et al*. 1989; Fletcher *et al*. 1995; Zhang *et al*. 1998a, b; Narciso and Morais 2001; Cunha *et al*. 2008). Rotifers are a common live first feed due to their small size (Fletcher *et al*. 1995; Mourente *et al*. 1995; Dhert *et al*. 1997; Zhang *et al*. 1998b; Cunha *et al*. 2008). Although of lower nutritional value, the relative ease of production of rotifers and *Artemia* continues to ensure their predominance over more suitable but less readily available preys, such as copepods (Stottrup 2000). Enriching *Artemia* and rotifers with HUFA, carotenoids and vitamins may improve their nutritional profile, and result in increased survival, growth and

synchronous metamorphosis of the larvae of several commercial shrimp species (Coutteau and Sorgeloos 1997; Sorgeloos *et al*. 1998).

Studies have shown that late stage larvae and PL quality is associated with larval culture conditions, whereas egg and, to an extent, early larvae quality depends on the physiological condition of the broodstock (Anger 1989; Palacios *et al*. 1998; Racotta *et al*. 2003). It is possible to manipulate the dietary regime of caridean and penaeid broodstock to positively alter the fecundity, fertilization rate, egg biochemical composition, hatchability and larval stress tolerance (Harrison 1990; Bray and Lawrence 1992; Cahu *et al*. 1995; Wehrtmann and Graeve 1998; Cavalli *et al*. 1999; Palacios *et al*. 2001; Racotta *et al*. 2004). For example, adding carotenoids to the diet of *P. vannamei* improved the condition of the broodstock showing symptoms of reproductive exhaustion and, by providing better nutrient reserves to the embryos, resulted in increased larval survival (Wyban *et al*. 1997). In *Penaeus monodon* the diet levels of arachidonic (ARA), EPA and DHA were positively correlated with greater spawning frequency, fertilisation and hatching rate (Millamena 1989). These HUFAs have been repeatedly linked to the reproductive performance of prawns (Middleditch *et al*. 1980; Xu *et al*. 1994; Cavalli *et al*. 1999; Wouters *et al*. 1999). Improving broodstock nutrition has also been suggested as an approach to enhance larval quality in ornamental shrimp (Simoes *et al*. 1998; Lin and Zhang 2001b; Lin and Shi 2002; Calado *et al*. 2008b).

Standard practise in penaeid hatcheries has been to feed the broodstock fresh or fresh-frozen foods (e.g. polychaetes, squid, mussel), to obtain optimal reproductive output (Wouters *et al*. 2000; 2002); frozen *Artemia* biomass has also been included in the maturation diet of some species (Naessens *et al*. 1997; Simoes *et al*. 1998; Wouters *et al*.

1999; Lin and Zhang 2001b; Calado *et al*. 2008b). To account for the variability in the nutritional value of *Artemia* batches, which may cause discrepancy in the reproductive performance of prawns, *Artemia* enrichment has become common practise (Leger *et al*. 1985; Browdy *et al*. 1989; Sorgeloos and Leger 1992; Coutteau and Sorgeloos 1997). Furthermore, different processing techniques of *Artemia* have been tested (e.g. freeze-drying), due to a preference of hatchery managers for dry diets, instead of fresh moist ingredients (Browdy 1998; Wouters *et al*. 2002). Generally, the relatively high cost, inconsistency in nutritional quality and risk of disease introduction via fresh frozen feeds have caused a heightened interest in supplementing or replacing natural feeds with artificial diets (Marsden *et al*. 1997; Denece *et al*. 1999; Calado *et al*. 2008b).

Comparisons amongst the biochemical profile of maturation diets, female organs, eggs and larvae has been a useful tool in assessing the role of various nutrients in the reproductive process and subsequent larval quality. Lipids help sustain continuous spawning and play an important structural and metabolic role, producing over 60% of the total energy expenditure of the developing embryos (Middleditch *et al*. 1980; Wehrtmann and Graeve 1998; Racotta *et al*. 2003). As a result, many studies have focused on generating and comparing the lipid and FA profiles of diets, broodstock and larvae (Bray *et al*. 1990; Millamena and Pascual 1990; Tidwell *et al*. 1998; Cavalli *et al*. 2001; Palacios *et al*. 2001; Calado *et al*. 2005a, b; Palmtag and Holt 2007). These data sets may allow aquaculturists to adjust maturation and larviculture protocols to ensure the provision of dietary nutrients required for larval production and development.

Another method to gain an understanding of the feed requirements of larvae is through studying the morphology of the mouthparts and gut, to allow the provision of

suitably sized and textured prey items, which are compatible with the ingestive and digestive capabilities of different larval stages (Anger, 2001). Scanning electron micrographs of the mouthparts and histological sections of the alimentary canal have revealed the tools decapods are equipped with to manipulate food items (Hunt *et al*. 1992; Sangha 1996; Johnston and Ritar 2001; Sousa and Petriella 2006). Using this knowledge to detect shifts in dietary preference (e.g. for different-sized preys or food texture) would enhance the aquaculture potential of the studied organisms.

1.4 Objectives of the project

For the commercial production of *L. amboinensis* to become feasible, improvements in larviculture efficiency need to be made. This is a particular challenge for a species with a prolonged larval cycle, with the ability to further delay metamorphosis in captivity, and with poor survival to the last stages. Experiments conducted during this study were designed to, directly or indirectly, address these limitations and to advance knowledge and capability for the successful and reliable completion of the planktonic larval phase of *L. amboinensis* in captivity.

The initial focus was on the reproductive behaviour of *L. amboinensis* broodstock to improve reproductive success and ensure a constant supply of good quality larvae. Given that *L. amboinensis* is a PSH, it is important to determine the minimum size of male rerproduction and ability to fertilize an SH, as well as the size the males would then become SHs themselves. An understanding of these parameters would ascertain the optimal time to establish reproductive pairs to maximize larval production. In addition,

discovering which, if any, factors affect the timing of sex phase change in *L. amboinensis* means these could be potentially used to influence the sex of individuals, so as to serve the needs of captive breeding programmes. Whereas such information exists for *L. wurdemanni* and *L. seticaudata*, this knowledge is lacking for *L. amboinensis*. Chapter 2 deals with the reproductive history of the species.

Maturation diets have a bearing on the quality of the broodstock, which in turn influences early larval quality. To further improve hatchery production, the dietary regime of *L. amboinensis* broodstock should thus be assessed. To address the nutritional aspect of broodstock maturation, crustacean aquaculture operations have relied on fresh frozen diets and, as a supplement, commercial mixed diets. The decisions on administered broodstock diets for marine ornamental shrimp have been based on available information on other commercial decapods, and have not been robustly substantiated. Chapter 3 compares the effectiveness of various maturation diets on the reproductive performance of *L. amboinensis*, and discusses the option of a commercial artificial diet. Providing cultured crustaceans with a dietary source of EFAs is considered crucial for ovary maturation and embryo development. The role of EFAs in the maturation of *L. amboinensis* has not been investigated comprehensively. Chapter 4 examines the FA requirements of the broodstock, using *Artemia* as the delivery medium.

Chapters 5, 6 and 7 investigate different aspects of larval rearing to improve the feeding regime in culture and achieve higher survival, sustained growth and development of *L. amboinensis*. Chapter 5 evaluates the suitability of prevalent commercial live feeds, rotifers and *Artemia*. Although both preys are used in the rearing of marine larvae, to date, there has been no comparative study of their effectiveness in *L. amboinensis*

larviculture. Additionally, a variety of *Artemia* lipid enrichments were used to obtain an indication of EFA requirements of *L. amboinensis* larvae. In Chapter 6, a comparison of the FA profile of adult *L. amboinensis* and Z1 larvae, subjected to different dietary treatments, is presented. The information allows an assessment of both the maternal and dietary contributions to the nutrient reserves of early larval stages,, and provides an insight into the metabolic processes that occur during development. Finally, the description of the feeding structures of early and late *L. amboinensis* larvae in Chapter 7, improves our understanding of prey processing ability at various developmental stages. Changes in the functional morphology of mouthparts and gut may predict, or justify, changes in dietary preferences. Such knowledge may be applied in the development and selection of appropriate diets and diet characteristics, for use in the larviculture of *L. amboinensis*.

In summary, the aims of this project were:

- To elucidate the reproductive history of *L. amboinensis*, by identifying MP size at sexual maturity, timing of sex phase change and SH size effect on fecundity - Chapter 2
- To identify suitable broodstock maturation diets amongst fresh frozen or dry commercial items - Chapter 3
- To assess the role of EFAs in broodstock nutrition, using frozen adult *Artemia* as the delivery medium - Chapter 4

- To compare the suitability of live preys, such as rotifers and *Artemia* nauplii or metanauplii, for early larval stages and assess the role of EFAs in larval nutrition - Chapter 5
- To provide the qualitative scope of FAs present in broodstock and larvae and evaluate the maternal, as opposed to dietary, contribution to the lipid profile of early larval stages - Chapter 6
- To describe the developmental changes in feeding and digestive structures of early and late larval stages - Chapter 7

The culture of marine ornamental shrimp, particularly valuable species such as *L. amboinensis*, will likely experience substantial growth in the future, as natural resources management and conservation efforts become more prominent. The value of the current study is that it deals with various culture parameters in both the broodstock and larvae of *L. amboinensis*. It links reproductive performance and maturation diets with the larval nutritional profile, and the larval feeding regime with the functional morphology of ingestive and digestive structures. This information is vital for the realisation of commercial production of *L. amboinensis*.

Chapter 2

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Sexual Maturity and Environmental Sex Determination in the white-striped cleaner shrimp, *Lysmata amboinensis*

2.1 Introduction

Many reproductive studies have focused on caridean shrimp and the diversity of sexual systems exhibited in these decapods (Gherardi and Calloni 1993; Bauer 2000; Bergstrom 2000; Lin and Zhang 2001a; Chockley and St Mary 2003; Zhang and Lin 2005a; Baeza 2006; Zupo *et al*. 2008). The most common sexual system in carideans is gonochorism (Correa and Thiel 2003), meaning separate sexes in the population (e.g. *Thor floridanus* in Bauer and VanHoy 1996). Other species such as *Thor manningi* are protandric (Bauer 1986), a form of sequential hermaphroditism, whereby individuals develop first as males then change to females at a certain size/age (Shapiro 1987). The gonads of sex changers function as testes in the male and then as ovaries in the female.

A unique modification of protandry exists in the family Hippolytidae, in the genus *Lysmata* (Fletcher *et al*. 1995; Fiedler 1998; Bauer and Holt 1998; d' Udekem d' Acoz 2003; Bauer and Newman 2004; Zhang and Lin 2005a; Baeza *et al*. 2007), and is termed PSH (Bauer, 2000). While the MP is similar to that of other protandric carideans, the

female differs in that sex change is not complete, since testes are retained and continue producing sperm (Baldwin and Bauer 2003). Thus, individuals function as both sexes during the reproductive cycle; each SH functions as a male mating partner when the other SH has moulted, but functions as a female after moulting itself (Bauer and Holt 1998; Fiedler 1998).

Accordingly, *L. amboinensis*, the white-striped cleaner shrimp, is an "out-crossing functional PSH" (Fiedler 1998; Bauer 2000). It is a high-value ornamental species (Moe 2001; Calado *et al*. 2003a, b) found in coral reef systems of the Indo-West Pacific. In Australia its range extends from the Coral Sea (East Coast of North Queensland) to Seal Rocks in northern New South Wales. To assist in preserving numbers of the species in the wild and also to satisfy increasing demand by the aquarium trade, the interest in breeding this species in captivity has grown.

Breeding *L. amboinensis* adults are found as SH pairs in the wild and as documented in laboratory studies can produce broods continuously, with an incubation time of approximately 13 days. Following the hatching of larvae and prior to the new spawning, the SH undergoes ecdysis to shed any unhatched eggs debris. Mating takes place after the parturial moult, with the partner shrimp attaching a pair of spermatophores under the abdomen. Spawning occurs regardless of whether spermatophore attachment has been successful (Bauer 2002b).

Fecundity is an important measure of reproductive potential. In carideans, a positive linear relationship exists between female size and brood size (Corey and Reid 1991). The "Size Advantage" model (Ghiselin 1969) suggests that sex change is adaptive in those species that reproduce more efficiently as one sex when younger/smaller, but the opposite

sex when older/bigger. Protandry, then, predicts greater male reproductive advantage at small body size. Although in *Lysmata* sex change is incomplete, the same principle may apply. A small MP would produce adequate sperm to fertilize the relatively low number of eggs a *Lysmata* SH produces, with larger SHs producing more eggs and offspring (Bauer 2004).

Various models have been applied to predict timing of sex change in carideans and explain why in some protandrous species certain individuals either never change or change is delayed (Bauer 1986; Bauer and Holt 1998; Bergstrom 2000). In *Pandalus danae*, the frequency of sexual morphs in the population was shown to be dependent on between-generation genetic selection (Marliave *et al*. 1993), as opposed to within-generation demographic control proposed by Charnov and Anderson (1989) for *P. borealis.* Seasonal variations in temperature, photoperiod and food availability have also been shown to affect sex differentiation in carideans; for example in the protandric *Hippolyte inermis* an abundance of diatoms would result in the development of primary females (Zupo 2001).

Thus, it has been hypothesized that size/age at change is: a) genetically predetermined and under selective pressure (Warner 1975; Marliave *et al*. 1993; Bergstrom 1997), b) based on the seasonal variation of abiotic factors (Bauer 2002a; Zupo and Messina 2007), or c) dependent on the demographic situation and mediated by social interactions, termed ESD (Charnov 1982; Hannah and Jones 1991; Lin and Zhang 2001a; Fiedler 2002; Baeza and Bauer 2004). The factors affecting the proportion of MPs and SHs within a population have only been investigated in *L. wurdemanni* and *L. seticaudata* from the genus (Baldwin and Bauer 2003; Baeza and Bauer 2004; Calado and Dinis 2007).

The objectives of the study were: a) to identify the size at which *L. amboinensis* MPs successfully copulate with SHs, b) to compare viable fecundity of a size range of SHs, c) to determine the size at which MPs undergo sex phase change and d) to examine whether social factors may influence this change.

2.2 Materials and Methods

Broodstock maintenance

The shrimp were collected in the Coral Sea, by divers from CMAF, using hand-nets. During acclimation at the Controlled Environment Facilities (CEF) at AIMS (Plate 2.1a), they were held in 2 x 270 L PVC holding tanks in individual 500 ml plastic containers perforated with 4 mm holes to allow water exchange. The shrimp were fed chopped squid or mussel *ad libitum*, once daily, on alternating days. Uneaten food and waste were siphoned before the next feeding, and moults and mortalities removed.

Water to the rearing facility was treated via foam fractionation and filtration (cartridge filter) to 1 µm prior to exposure to ozone (750 mV) and UV (wavelength 264 nm) to reduce bacterial loading (Plate 2.1b). Water temperature (pH/oxi 340i) was kept at 28.1 (± 0.42) ºC, pH at 8.1 (± 0.01) (pH/oxi 340i) and salinity (optical salinometer, Bio-marine, Inc.) at 34.5 (± 0.62) g L^{-1}. Ammonia and nitrite were below detectable levels (NH_3/NH_4 and NO_2 Kits, Aquarium Pharmaceuticals, Inc.) The light regime in the culture room was 12: 12 hrs light: dark, provided by fluorescent tubes and regulated by timers.

Following acclimation, the shrimp were paired and transferred to individual 23 L glass aquaria with lids. The experimental tanks were provided with aeration, flow-through water at a rate of 100% per half hr (sourced 400 m offshore Cape Ferguson, Townsville, and treated as described above) and an 8 cm diameter piece of bleached coral for shelter (Plate 2.1c). Water parameters and shrimp maintenance were as described in the acclimation protocol.

PLATE 2.1 Facilities at AIMS a) CEF, b) ozone system and c) broodstock tanks.

Experimental procedures

2.2.1 MP – SH pairing

The experiment run in March and April, 2007, and aimed to identify the size at which MPs were sexually mature and examine if SH size affects fecundity. Pairs made up of one MP and one SH shrimp were grouped based on three size categories of the SH:

1) Small (Sml), mean 35.0 (± 0.2) mm TL.
2) Medium (Med), mean 40.1 (± 0.2) mm TL.
3) Large (Lrg), mean 52.4 (± 1.4) mm TL.

The MPs were 34.0 (± 0.2) (range 32.4 – 34.7) mm TL and were randomly allocated across the treatments. The Sml and Med groups had seven replicates, while the Lrg group contained six replicates, due to insufficient numbers of large SHs.

Measurements of intact moults (TL, from the tip of the rostrum to the end of the telson, by digital callipers) provided information on the size of the animals (Plate 2.2a). Alternatively, if the moult was not intact, the shrimp was removed from the tank and measured to the nearest 0.1 mm. Monitoring of individuals within pairs was possible by size differences and/or differing moult stages, or in instances where moulting was likely to be synchronous, the carapace or uropods of individuals were colour-coded using permanent markers. The experiment was completed when all SHs had completed a spawning cycle.

The parameters recorded were:

a) The volume the egg mass occupied (length x width x depth), expressed as a percentage of the available egg carrying area (abdominal length x abdominal width x abdominal depth in mm^3). Zero percent represented no spawn and 100 total egg cover. Recordings were taken every 2 days (Plates 2.2b and 2.2c).

b) The number of larvae hatching at each spawn, referred to as viable fecundity. Total counts of viable larvae were made by siphoning them from the broodstock tanks each morning at 0800. To ensure that larvae were not inadvertently flushed out overnight, a 150µm filter was placed on the outlet.

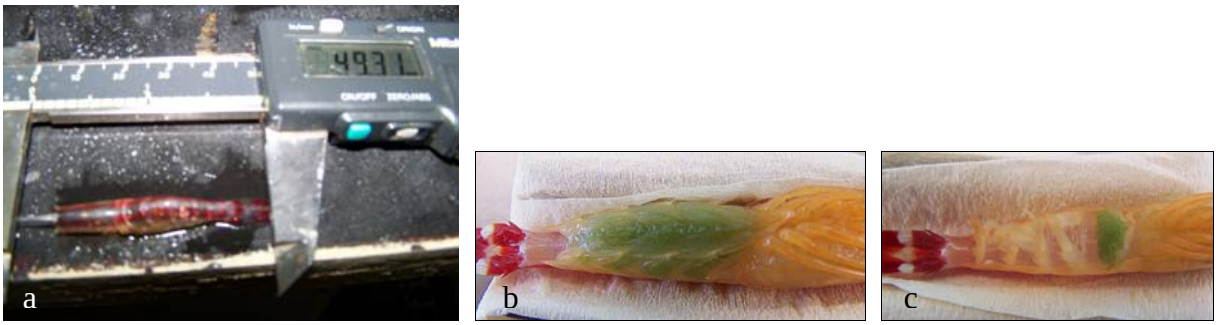

PLATE 2.2 Experimental procedures a) measurement of a moult with digital callipers, b) the equivalent of 100% egg cover on the abdomen and c) the equivalent of approximately 20% egg cover on the abdomen.

2.2.2 MP size at phase change and influence of social factors

The experiment was run in March and April, 2008, and aimed to identify the time and size at which MPs change to SHs and whether social environment plays a part in the timing of phase change. Specifically, the effect of body size and sex composition of pairs on the change to SH was tested by exposing focal MPs (phase change candidates) to four social conditions:

1) Alone (30.0 – 32.0 mm TL).
2) With a similar-sized MP (both 32.0 - 34.0 mm TL).
3) With a larger MP (focal MP 32.0 - 34.0 mm TL, larger non-focal MP 35.0 - 37.0 mm TL).
4) With an SH (focal MP 35.0 – 37.0 mm TL, SH 40.0 - 42.0 mm TL).

The size disparity of focal MPs in the 1st and 4th treatments was due to lack of more MPs of an average size 33.0 mm TL and is addressed in the statistical analysis of the results. In treatments 3 and 4 focal MPs could be identified by differing size. In treatment

2, shrimp identification was either by differing moult stage or by different colour permanent ink markers. Phase change was noted when the MP spawned for the first time. Each treatment had six replicates. The experiment was completed when all MPs (focal and non-focal) became SHs.

The parameters recorded were:

a) Time (days) taken for the focal MPs in each treatment and, in treatments 2 and 3 for the non-focal MPs, to change to SHs.
b) Final size (mm TL) at phase change, for all MPs (focal and non-focal). Size was measured preferentially from the moult, occasionally the shrimp itself (for details see 2.2.1).

Statistical analysis

The programs SAS v8.2 (SAS Institute, Inc., Cary, North Carolina) and SPSS v16.0 (SPSS Inc., Chicago) were used for statistical analysis. Percent egg cover across the 12 day incubation time was analysed using the MIXED procedure, data were arcsine-transformed. For viable fecundity counts and egg cover on the day before hatch (DBH) 1-Way ANOVA was applied, after log- and arcsine- transformation of the data respectively. Post-hoc analysis was carried out using Student-Newman-Keuls test. The final length at which shrimp changed phase, and the time taken to change, were analysed with the Least Mean Square model. All interactions were tested for significance, and the not significant ones were subsequently removed. The model corrects for differences in initial length and time to next moult.

2.3 Results

2.3.1 MP – SH pairing

The area of the abdomen covered with eggs from the day of spawning (day 0) until hatching in the three size classes of SH shrimp is illustrated in Fig. 2.1. All SH groups demonstrated a significant decrease in the initial spawn over the 12 day incubation period [$F(6, 102) = 7.14$, $P < 0.0001$]. On day 6 average egg cover in all size groups was similar, approximately 75%. In the Lrg SHs egg retention appeared to stabilize thereafter, and before hatching was approximately 70 (± 17.6) %. Egg cover in the Sml SHs decreased from 80 (± 8.7) % to 60 (± 12.3) %, whereas in the Med group reduction of the egg mass was more pronounced, reaching 57 (± 12.9) % on the day prior to hatching.

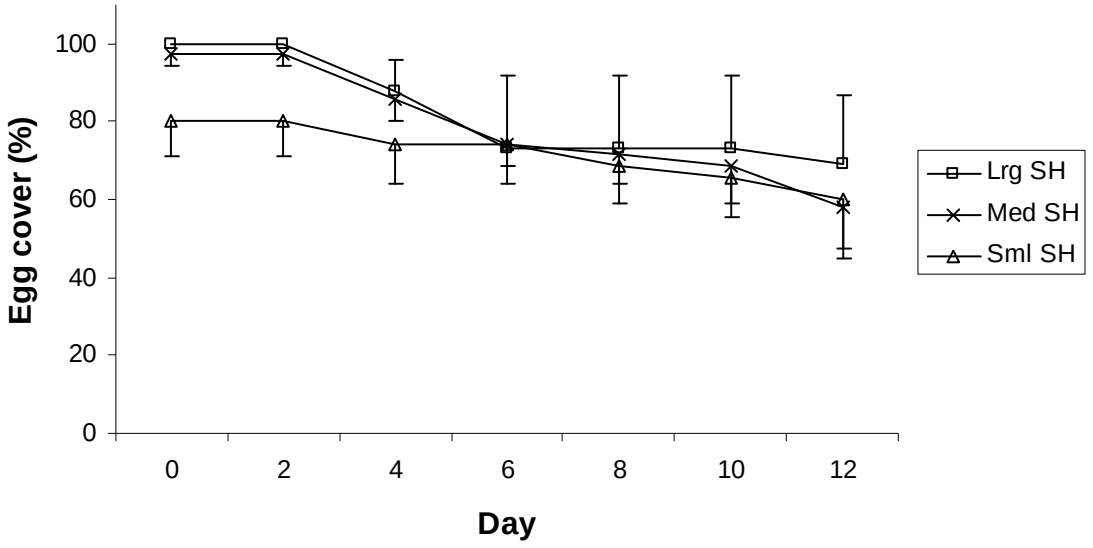

FIGURE 2.1 Progression of egg cover of the abdominal area, expressed as a percentage of total egg carrying area, during the 12 day incubation period, for each SH group (means ± SEM, n = 6 for Lrg SH, n = 7 for Med and Sml SH).

Egg cover the DBH was not significantly different among the three SH groups, ranging between 60-70% (P = 0.878) (Fig. 2.2). However, the number of larvae produced by the Lrg SHs (231 ± 102) was significantly different to the Med and Sml SH groups (70 ± 17 and 58 ± 17, respectively) (F [2, 18] = 5.15, P = 0.019) (Fig. 2.2).

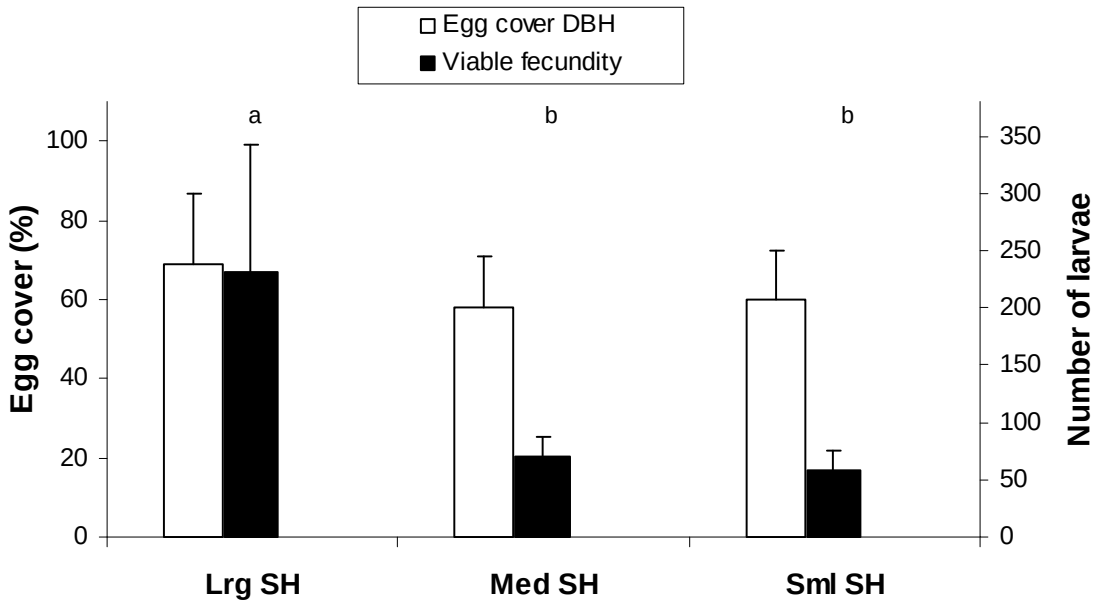

FIGURE 2.2 Percent egg cover of the abdomen the DBH and number of viable larvae produced at the end of the brooding period, for each SH size group (means ± SEM, n = 6 for Lrg SH, n = 7 for Med and Sml). Different letters indicate significant differences in viable fecundity.

2.3.2 MP size at phase change and influence of social factors

In regard to the time taken to change phase, the focal MPs paired with a larger MP or an SH changed at 24 and 20 days respectively, while those maintained alone or reared with a similar-sized MP changed in approximately 18 days (Fig. 2.3). The differences were not significant (P = 0.260).

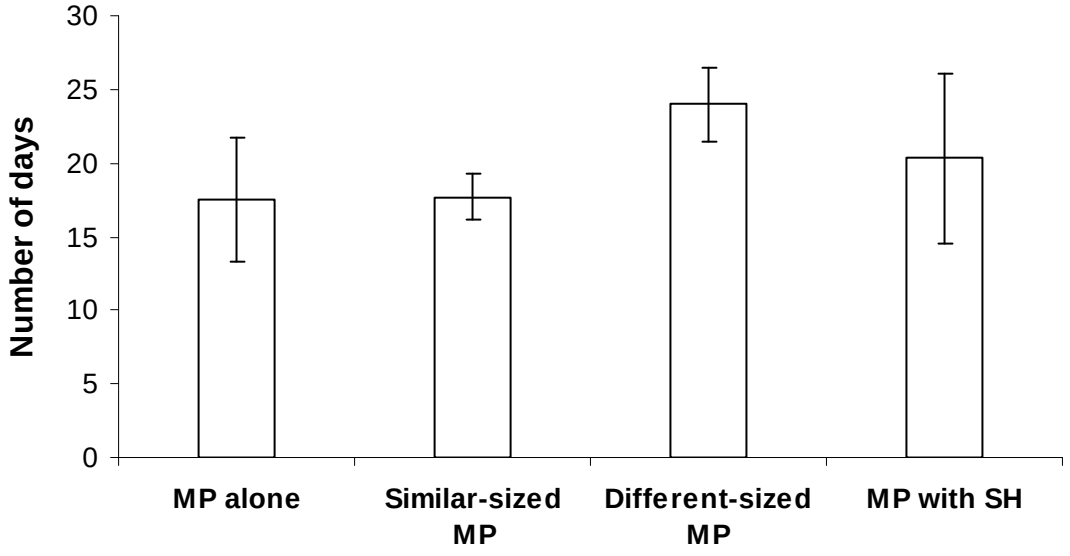

FIGURE 2.3 Time taken for focal MPs in the different treatments to change sex (means ± SEM, n = 6 reps per treatment).

Size at the time of phase change was recorded for the focal MPs in the different treatments (Fig. 2.4). Adjusting for an average initial length of 33.3 mm, the social environment had a significant effect on size at phase change ($F_{(3, 19)} = 17.132$, $P < 0.001$). The focal MPs in the different-sized treatment changed at the largest size (39.6 ± 0.5 mm TL), whereas those in the remaining paired treatments changed at approximately 36.0-36.5 mm TL. Those MPs reared alone reached a final size of 37.1 (± 0.9) mm TL before changing to SHs.

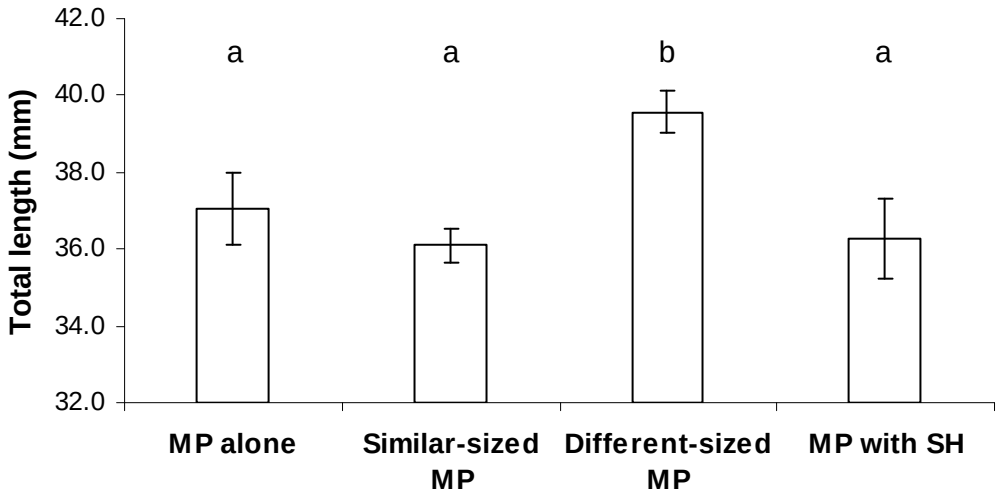

FIGURE 2.4 Final size (TL mm) at phase change of focal MPs in the different social environments (means ± SEM, n = 6 reps per treatment). Different letters indicate significant differences in final size.

The time taken for non-focal MPs to change to SHs and size at change were also monitored. There were no significant differences within the same treatment for either parameter (P = 0.339 and P = 0.118, respectively), but the non-focal MPs in the different-sized treatment changed significantly slower (23 ± 1 days) (F (1, 19) = 6.232, P = 0.022) and at a larger size (38.8 ± 0.2 mm TL) (F (1, 20) = 25.219, P < 0.001) than the non-focal MPs in the similar-sized treatment.

2.4 Discussion

The *L. amboinensis* MPs used in this study were already sexually mature at an average 34.0 mm TL, as opposed to a minimum size of 35.0 mm suggested by Fletcher *et al.*

(1995). Although seemingly a minor difference, this could have an important impact on the establishment of functional pairs; for example, in *L. wurdemanni* (a smaller temperate species found in the Western Atlantic) MPs measuring 13 mm successfully mated with an SH, but MPs of 12 mm were not successful (Zhang and Lin 2005b). Moreover, the smallest *L. amboinensis* MPs used had a mean minimum size of 32.6 (± 0.1) mm TL and successfully fertilized the eggs of their SH partners, regardless of the SH size. However, the average number of hatched larvae from all size groups was lower than that usually reported in the literature (Fletcher *et al*. 1995; Simoes *et al*. 1998). Lower larval production in the current study may be related to the observed brood loss.

Brood loss in *L. amboinensis* did not differ significantly between the SH groups, in agreement with studies that have shown brood mortality not to change with female size in crustaceans (Kuris 1991; Oh and Hartnoll 1999). In *L. seticaudata*, brood losses were similar for small, medium and large shrimp and ranged between approximately 11-14% (Calado and Narciso 2003). Such losses were attributed to egg swelling by water uptake (Amsler and George 1984; Clarke 1993; Figueiredo *et al*. 2008), resulting in an increase in egg volume and a decrease in egg number, due to limited available egg carrying space. However, in *L. amboinensis* loss was greater than would be expected if embryo volume increases were the only changes occurring.

Potential causes for early brood loss could be incomplete fertilization and egg loss at oviposition (Kuris 1991; Oh and Hartnoll 1999). Fertilization is not necessary for egg attachment and unfertilized eggs will be later removed by the female (Bauer and Holt 1998). The failure of eggs to adhere to the pleopods would reduce brood size (Kuris 1991), and this was especially noticeable in the Sml SH group, in which shrimp would

frequently have an initial spawn of 80%. This could be attributed to brooding inexperience, since the smaller hermaphrodites would only have been functioning as females for a short time (see the section on phase change).

Where crustacean brood losses are high, parasitism has been demonstrated as the cause of egg mortality (Fisher 1983a; Kuris and Wickham 1987; Kuris 1991). In carideans, losses due to parasites seem to be less common. While parasite infestations may be less likely, other causative disease agents, such as pathogenic bacterial strains (*Vibrio spp*.), were present during culture and may have contributed to 'over grooming' of the egg mass and the potential removal of healthy eggs along with the diseased. Females keep the incubated embryos healthy by grooming the egg mass with their highly specialized appendages (Fisher 1983b; Kuris and Wickham 1987; Bauer 2004). This behaviour was evident in all experimental *L. amboinensis* shrimp.

Finally, sudden changes in environmental parameters (e.g. temperature, pH) and continuous handling are known to cause stress and contribute to brood loss in berried decapod females (Kuris 1991; Smith and Ritar 2005). Even though such disturbances were kept low, it is possible that they contributed to the observed brood loss.

As is well established in crustacean studies, fecundity increases with increasing female size (Corey and Reid 1991; Kuris 1991; Mori *et al*. 1998; Calado and Narciso 2003; Figueiredo *et al*. 2008), so it would be expected that larger females would produce more eggs and be able to hold more embryos on their abdomen. Calado and Narciso (2003) found embryo volume in *L. seticaudata* to be on average the same across all SH size groups examined. Assuming this for *L. amboinensis*, and given that, proportionally, egg cover of the abdomen did not vary significantly across groups, the difference in viable

fecundity could be due to the number of eggs laid and to the available area for attachment. Accordingly, offspring output in a quantitative gradient should be higher for large SHs > medium SHs > small SHs; this was generally observed. However, the difference was only significant for the Lrg SHs, which produced approximately 3 times more larvae than the other two groups. Larval production between the Med and Sml SHs was similar and may be related to these groups being closer in average body size. Factors such as experience in brood care may also have influenced fecundity, with the Lrg SHs more capable of caring for the attached eggs. This group's prolonged experience in brooding would have led to the enhanced survival of incubated embryos.

Lysmata amboinensis is reffered to as a "low density" or "pairs" species (Bauer 2006), since in nature individuals are found in small aggregations or in pairs. It would make evolutionary sense for the MPs kept singly to become SHs early on, so that they could potentially mate with any other con-specific, if given the opportunity. On the other hand, there would be an advantage in delaying phase change because the MP could allocate more energy to growth, since sperm production is much less energetically expensive (Baeza 2006). Larger size at the time of change would result in higher reproductive success as females, since in carideans larger female body size is positively correlated to higher fecundity (Calado and Narciso 2003; Bauer 2004). The recorded 37.1 mm may be the balance point between these two influential factors. That could be considered the "default" size at phase change for *L. amboinensis*, under the current conditions of cultivation, i.e. the size at which MPs would also assume female function, regardless of social situation. However, the result should be viewed with caution, since using smaller MP individuals could potentially give different results.

In those treatments consisting of MP pairs, there would initially be no opportunity for reproduction and it would thus be advantageous for one in the pair to change. The larger MP in the different-sized pairs invariably changed phase first, confirming a size-ratio induction (Baeza and Bauer 2004). The smaller MPs in the same treatment continued to grow and finally changed at a size not significantly different to that of their partners. MPs in the similar-sized pairs took approximately the same time to change phase, and both became SHs at a similar, but smaller than the "default", size, confirming a sex-ratio induction (Baeza and Bauer 2004). This implies it was more important to start reproducing, rather than reach the "ideal" size before doing so. The fact that both MPs in the different- and similar- sized pairs changed phase at a not significantly different length to each other points to a size-assortative mating strategy. This strategy has been proposed for other "low density", gonochoristic or hermaphroditic caridean species (Correa and Thiel 2003).

Those MPs reared with an SH were immediately presented with a chance for reproduction, since they could inseminate the hermaphrodite. Therefore, they could have delayed changing sex until they reached an optimum size. However, these MPs changed at a smaller size than the "default". The reasons are not entirely clear. The smaller than expected size at phase change may be explained by competition for food with the much larger SH, further supported by the longer time it took the MP to reach that size. Another possible reason for the early change may be connected to the fact wild *L. amboinensis* pairs reportedly consist mainly of SHs (Wirtz 1997; Bauer 2000; 2006). Thus it may be the case that when MPs are paired with SHs they would change regardless of size to fulfil their ecological role.

When Baeza and Bauer (2004) examined *L. wurdemanni* for socially mediated sex phase change, they found that single MPs changed significantly faster than MPs reared with an SH, and no other significant differences were detected. In the case of *L. amboinensis*, differences in focal MPs and time to change were not significant, but the trend was similar (to the aforementioned study), in that single MPs changed earlier than those paired with an SH. Calado and Dinis (2007) reported that in *L. seticaudata* MPs reared individually did not change phase and suggested that in this species tactile interactions may be necessary to initiate sex phase change. The authors also reported that rearing density had a significant influence on the number of juvenile *L. seticaudata* changing to SHs (Calado and Dinis 2007), true also for MP *L. wurdemanni* (Lin and Zhang 2001a; Zhang and Lin 2007). In the latter study, recruitment and mortality would further trigger phase change of MPs (Zhang and Lin 2007). Although both aforementioned species are "crowd species" (Bauer 2000), the findings, along with those reported in the current study for a "pairs species", suggest that social interactions play an important role in sexual phase change in *Lysmata*.

Apart from the social conditions examined here for *L. amboinensis*, there may be numerous other factors influencing the change to SHs. Future studies should investigate if, and how, low density juvenile groups, natural recruitment and mortality, seasonal variation of abiotic parameters and natural diet may affect sex phase change in *L. amboinensis*.

Chapter 3

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The Effect of Maturation Diets on the Reproductive Output of the white-striped cleaner shrimp, *Lysmata amboinensis*

3.1 Introduction

The marine ornamental trade is a multi-million dollar industry which is growing in popularity. As a result, demand for specific species, including the high-value white-striped cleaner shrimp, *L. amboinensis*, is increasingly threatening the sustainability of the industry. Many marine species sold in the aquarium market are collected from the wild, and, to a large extent, from coral reefs (Chapman *et al*. 1997). Indirect consequences of capture of marine ornamentals include the potential to damage reef structures through destructive fishing techniques, with direct ones including reduced biodiversity and knock-on ecological imbalances caused by overexploitation of natural stocks (Lin *et al*. 2002; Bunting 2003; Chokley and Mary 2003). Captive breeding of ornamental species could provide an alternative to wild collections, alleviate pressure on coral reefs, and satisfy growing market demands as well as breeding predisposed specimens to an aquarium environment.

At present, the commercial rearing of ornamental shrimp is hindered by an extended larval phase, with metamorphosis often delayed and with low survival during larviculture (Calado *et al*. 2003a, b). This is especially true for *L. amboinensis*, with a larval duration of up to 150 days (Fletcher *et al*. 1995). Several broodstock aspects are known to affect larval quality and survival (Racotta *et al*. 2003), with nutrition being the most reviewed. Reproductive attributes affected by nutrition include fecundity, egg size and biochemical composition, and hatchability (Cahu *et al*. 1994; Wouters *et al*. 2001; Racotta *et al*. 2003). The link between the nutritional input from broodstock diets and the reproductive output and offspring quality has been demonstrated for penaeid species and the giant freshwater prawn, *Macrobrachium rosenbergii* (Bray *et al*. 1990; Bray and Lawrence 1992; Cahu *et al*. 1995; Wouters *et al*. 2002).

Overall, the evidence indicates that the nutritional status of the broodstock is reflected in egg quality and subsequent larval quality (Palacios *et al*. 1998; Racotta *et al*. 2003); hence, it is essential to provide adequate maternal nutrition to promote ovarian development and vitellogenesis, enhance fertility and improve the viability of offspring (Harrison 1997). It is likely that reproductive success in *Lysmata* species would also be improved with appropriate broodstock feeding regimes (Lin and Zhang 2001b; Lin *et al*. 2002; Lin and Shi 2002).

Although the use of formulated, commercially available feeds is desirable, due to controlled nutritional value, reduced disease transmission, more reliable supply and longer storage, the common practise in hatcheries has been to rely on fresh frozen natural foods (Harrison 1990; Bray and Lawrence 1992; Naessens *et al*. 1997). Such items include squid, mussel, polychaetes and *Artemia* biomass, with artificial diets used only as

a dietary supplement. This approach is due to generally poorer reproductive performance of artificial diets compared to fresh ones (Nascimento *et al*. 1991; Harrison 1997; Wouters *et al*. 2000), although in penaeids there has been successful partial replacement of the classic fresh food regime with dry feeds or complete replacement with moist diets (Marsden *et al*. 1997; Denece *et al*. 1999; Wouters *et al*. 2002).

There is very little published information on the nutritional requirements of ornamental shrimp broodstock and nutritional management appears to be an extrapolation of knowledge acquired with commercial food species (e.g. prawns and lobsters), combined with the experience of successful aquarists. The aim of this study was to evaluate the suitability of common fresh frozen food items, such as squid and mussel, and a commercial pellet as maturation diets for the white-striped cleaner shrimp, *L. amboinensis*.

3.2 Materials and Methods

Broodstock Maintenance

Broodstock maintenance was as described in Chapter 2, section 2.2. Following acclimation, the shrimp were paired according to size and transferred to the experimental tanks.

Experimental Procedures

The experiment was conducted between May and August 2008. The shrimp were of an average initial size 43.6 (± 0.4) mm. There were six dietary treatments (Table 3.1), each containing five replicates, each replicate consisting of a pair of shrimp. The diets were:

1) California squid, *Loligo opalescens*, and New Zealand green mussel, *Perna canaliculus* chopped into 2-3 mm pieces and fed on alternate days.
2) Frozen adult (12 days old) *Artemia* (*A. franciscana*) biomass. *Artemia* were ongrown in a mix of algae (*Chaetoceros muelleri*, *Tetraselmis suecica*, and *Isochrysis galbana* Tahitian strain), rice pollard, and spirulina. *Artemia* biomass was harvested, rinsed in ozonated seawater for 10 min, re-suspended in 20 L of seawater and treated with 400 mg L^{-1} formalin for 30 min, rinsed in freshwater for 10 min, drained and frozen at -18 °C until used.
3) Commercial Kuruma prawn diet (Ebi Star, Higashimaru, Japan). The pellets were ground and sieved to an average size of 800 µm.
4) Squid, mussel, *Artemia* fed on consecutive days.
5) Squid, mussel, pellet fed on consecutive days.
6) Squid, mussel, *Artemia*, pellet fed on consecutive days.

Data for each replicate were collected for four successive spawns and were comprised of the average spawn value per pair, rather than for individuals, to account for potential differences in feeding behaviour and intake. Prior to the data collection, *L. amboinensis* were fed for a moult cycle on the experimental diets to allow acclimation to the treatment diets and minimise the effects of the maintenance diet. Feeding quantities were the

equivalent dw of 3.5% shrimp wet wt and were based on preliminary observations of satiation, taking into account shrimp size differences within and between treatments and daily feeding response.

The parameters recorded were:

a) Shrimp growth at the end of the study, quantified as a percent increase in the initial TL (from the tip of the rostrum to the end of the telson) and calculated by the formula (final – initial size) / final size x 100. Shrimp differentiation and size measurements were conducted as described in Chapter 2, section 2.2.1.

b) Abdominal egg cover, expressed as a percentage of the available egg carrying area (egg mass volume/volume of abdomen for egg attachment *100). Data are the average of three intermoult periods. At the completion of the experiment freshly spawned eggs were stripped off the pleopods after anaesthetizing the shrimp with 40 ppm AQUI-S solution (AQUI-S, NZ Ltd.) for 5-10 min. Egg clumps were separated by soaking in 1M KOH for approximately 1 hr. Individual eggs were counted using a dissection microscope (Leica MZ16) fitted with Leica Application Suite (LAS) software (Leica Microsystems AG, Switzerland).

c) Viable fecundity, expressed as the number of larvae hatching at each of the four spawns. Collection of larvae was as described in Chapter 2, section 2.2.1.

TABLE 3.1 Summary of constituents of each broodstock diet treatment.

Diet	**Squid**	**Mussel**	***Artemia***	**Pellet**
1	✓	✓	×	×
2	×	×	✓	×
3	×	×	×	✓
4	✓	✓	✓	×
5	✓	✓	×	✓
6	✓	✓	✓	✓

Feed analysis

The food items used during the experiment (squid, mussel, *Artemia*, pellet) were analysed for their gross and FA profiles at the conclusion of the study. Gross composition of feed components was obtained using standard laboratory methods generally in accordance with AOAC International (1999). Accordingly, dry matter was determined by the loss in weight following drying at 135 ºC for 2 hrs; crude protein (N × 6.25) by the Dumas combustion method using a Leco® Nitrogen analyzer; ash as the residue following combustion at 600 ºC for 2 hrs; total carbohydrate was calculated by difference as: 100 – (protein + fat + crude fiber + ash); crude fiber by Weende extraction using the Foss® Fibercap technique; metabolizable energy (ME) was calculated according to General Appendix 2, Composition of Foods Australia (Cashel *et al*. 1989). For fat determination samples were acid hydrolysed, then extracted with petroleum spirit 40-60 °C boiling range in a Soxhlet apparatus and the extracts were dried to constant weight. A further aliquot of the extract was taken for FA analysis. The lipid FAs in the extract were

derivatized to their FA methyl esters (FAME) using 14% boron trifluoride-methanol (Van Wijngaarden 1967). FAME were analyzed on an Agilent Technologies 6890 gas chromatograph using split injection with helium carrier gas and a flame ionization detector. The column used was a DB23 fused silica capillary column, 30 m x 0.25 mm, with a 0.25 µm coating (Agilent Technologies, USA). Column oven temperature was held at 140 °C for 5 min and then elevated to 210 °C at 3 °C min^{-1} where it was held until all FAME of interest had been eluted. FAME were identified by comparing their retention times with those of authentic standards (Sigma-Aldrich Co, USA) and were quantified by comparison with the response of an internal standard, heneicosanoic acid.

Statistical analysis

Statistical analysis was conducted using SAS v8.2 (SAS Institute, Inc., Cary, North Carolina) and SPSS v16.0 (SPSS Inc., Chicago). The level of significance was $P = 0.05$. Growth data (arcsine-transformed) were analysed by 1-way ANOVA. Percent egg cover across the incubation period was analysed using the MIXED procedure, data were arcsine-transformed. Viable fecundity across each of the four spawns was analysed with a Split Plot ANOVA after log-transforms. Post-hoc analysis was carried out using Student-Newman-Keuls test.

3.3 Results

The gross composition of the food items is presented in Table 3.2. All items had similar protein content (72-76%) with the exception of *Artemia*, which had the lowest

protein (60%) but the highest fat content (22%) of the diets. Pellet had the second highest fat content whereas squid had the lowest at 6% (on a dry matter basis).

TABLE 3.2 Proximate composition and metabolizable energy (ME, Kcal/100g) of the experimental feeds squid, mussel, *Artemia* and pellet. Moisture content is a percentage of the feed wet wt. Protein, fat, ash, carbohydrate and fiber are presented as % in dw.

Dietary Components	**Moisture**	**Protein**	**Fat**	**Ash**	**Carbohydrate**	**Fiber**	**ME**
Squid	79.1	72.7	6.3	9.1	11.9	< 0.1	397
Mussel	78.5	74.0	8.1	6.5	11.4	< 0.1	416
Artemia	88.7	60.2	22.1	8.0	9.7	< 0.1	477
Pellet	14.2	76.0	14.2	12.4	< 0.1	0.9	421

The principal components of lipids are FAs. Table 3.3 shows the composition of the 14 to 22 Carbon FAs for the feeds. There are marked differences; accordingly, squid had the highest 22:6 (DHA) content and a high 20:5 (EPA) level, although the highest EPA level was recorded in the mussel. *Artemia* were deficient in DHA and also had a low EPA content, but were high in 18:2 (linoleic, LOA). Pellet had the lowest 20:4 (ARA) level, an intermediate value of DHA and high EPA.

TABLE 3.3 FA content (mg g^{-1} dw) of squid, mussel, *Artemia* and pellet.

FA	**Squid**	**Mussel**	***Artemia***	**Pellet**
14:0 (Myristic)	0.98	2.00	0.47	3.06
16:0 (Palmitic)	12.39	6.79	9.11	14.23
16:1n-7 (Palmitoleic)	0.24	2.68	1.24	3.44
18:0 (Stearic)	2.21	2.30	4.98	3.28
18:1n-9 (Oleic)	1.27	0.57	16.04	10.09
18:1n-7 (Vaccenic)	0.66	1.35	4.26	3.29
18:2n-6 (LOA)	0.16	0.77	13.30	5.30
18:3n-3 (LNA)	<0.05	0.45	1.29	0.73
20:4n-6 (ARA)	1.43	1.13	1.44	0.72
20:5n-3 (EPA)	7.60	8.83	1.95	8.36
22:6n-3 (DHA)	20.25	9.08	<0.05	10.65
SFA	16.23	12.05	15.43	21.63
MUFA	4.56	6.74	21.85	28.09
PUFA	0.44	1.56	17.37	6.50
HUFA	30.29	25.09	3.86	22.23
Σ(n-3)	28.23	19.91	3.72	22.07
Σ(n-6)	2.05	2.50	17.51	6.57

Survival of the shrimp was not affected by the experimental diets. There was no significant difference in broodstock growth in the different treatments over the duration of the feed study (P = 0.256) (Fig. 3.1). Mean overall growth ranged between 7 ± 0.8 to 11 ± 1.0 % for the diets.

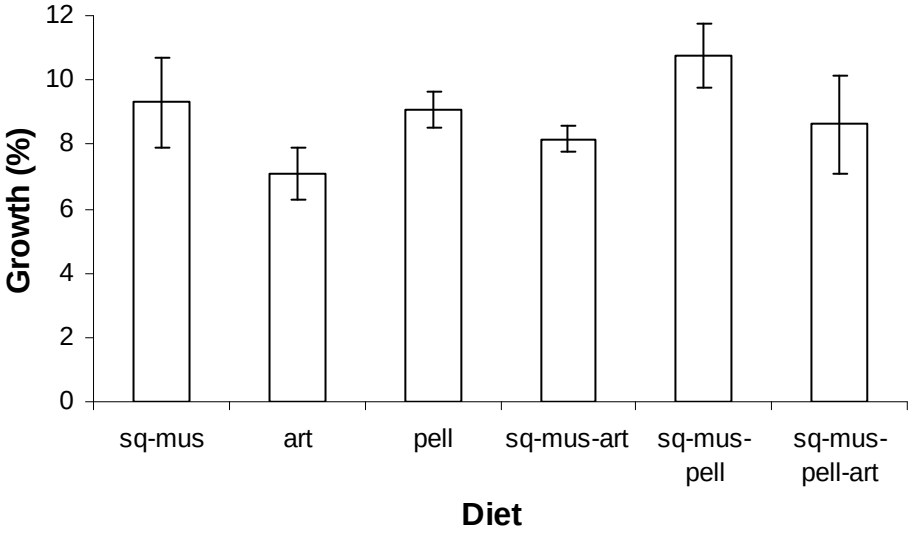

FIGURE 3.1 Shrimp growth (percent increase in TL) at the completion of the experiment (means ± SEM, n = 5 reps per treatment).

An estimate of the contribution of egg numbers to egg cover was calculated for a range of sizes used in the study. Indicatively, 100% cover of a 42.9 mm shrimp would hold 1144 eggs, of a 44.2 mm 1297, of a 46.2 mm 1532, of a 48.3 mm 1779, of a 49.6 mm 1932 eggs. Individual egg volume was constant at 0.046 ± 0.003 mm^3. Changes in egg cover from the day of spawning (day 0) until hatch are displayed in Fig. 3.2. Diet had a significant effect on egg cover [F (6, 24) = 4.76, P = 0.0025]. The *Artemia* diet had the highest initial egg cover and low egg loss during incubation, reducing from 99 (± 0.7) to 82 (± 6.1) % by day 12. Pellet, squid-mussel-pellet and squid-mussel-*Artemia* all demonstrated similar losses to the *Artemia* diet, with reductions of approximately 10-15% over the course of the 12 days. Egg cover for the squid-mussel-pellet-*Artemia* and squid-mussel diet commenced with lower amounts (81 ± 8.7 and 78 ± 4.6 %, respectively) and, while egg cover on the squid-mussel fed animals decreased significantly to 24 (± 7.3) %

prior to hatch [F (5, 20) = 32.32, *P* <0.0001], that of the squid-mussel-pellet-*Artemia* fed shrimp did not decrease at the same rate and was not significantly different to the *Artemia*, pellet, squid-mussel-pellet and squid-mussel-*Artemia* treatments.

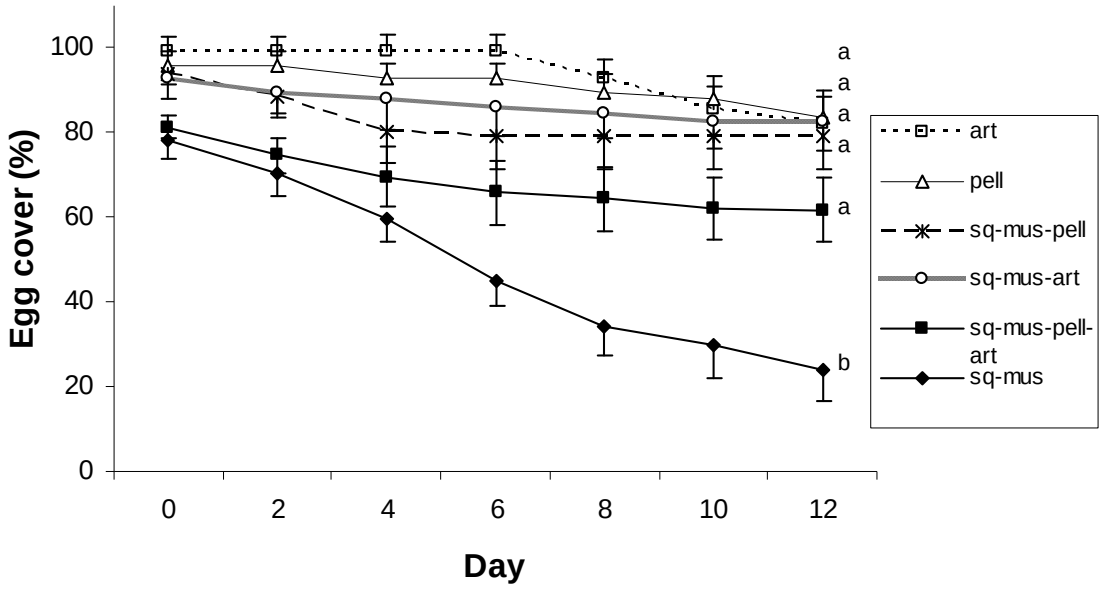

FIGURE 3.2 Egg cover, expressed as a percentage of total egg carrying area, during the 12 day incubation period for the maturation diets (means ± SEM, n = 5 reps per treatment). Different letters indicate significant differences in egg cover.

Within the same spawn cycle diet had a significant effect on viable fecundity [F (5, 19) = 10.420, *P* < 0.001] (Fig. 3.3). Shrimp fed *Artemia* had low reproductive output, with 23 (± 9) larvae in the first spawn and 12 (± 7) larvae in the second being the lowest. In spawns 3 and 4, the squid-mussel diet produced the lowest number of larvae (39 ± 19 and 22 ± 11, respectively). The observed trend for number of larvae produced to decrease for the squid-mussel diet from 184 (± 76) to 22 (± 11) and increase for the pellet from

394 (± 63) to 682 (± 131) over time (i.e. by spawn 4) was not statistically significant (*P* = 0.128) (Fig. 3.3).

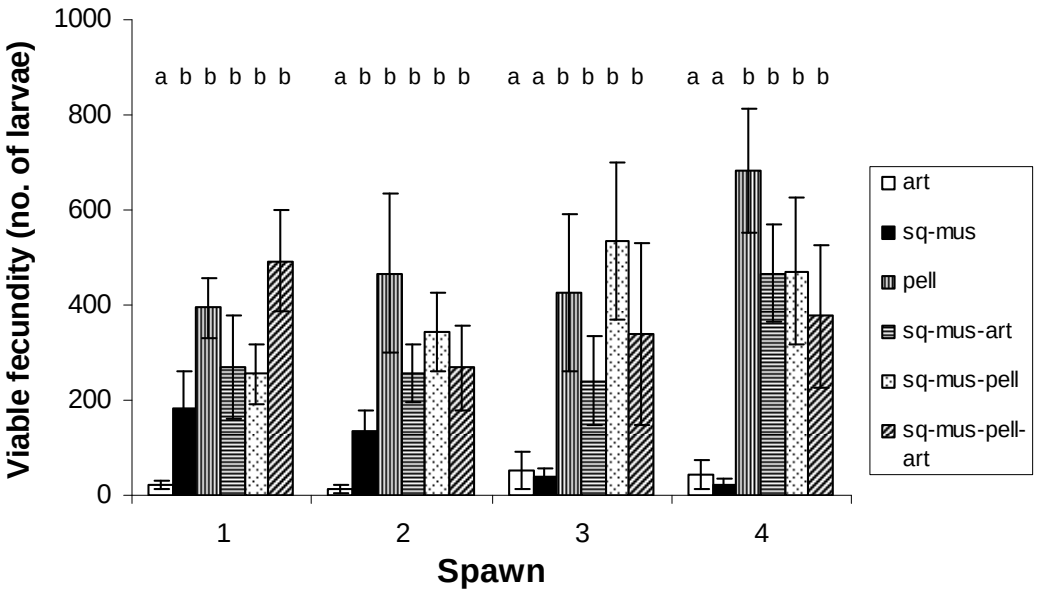

FIGURE 3.3 Number of larvae produced, referred to as viable fecundity, for each diet treatment at each spawn cycle (means ± SEM, n = 5 reps per treatment). Different letters indicate significant differences in viable fecundity.

3.4 Discussion

Culture vessels and the basic environmental parameters of water quality, temperature, salinity, photoperiod and light intensity were constant throughout the study and, therefore, unlikely to have preferentially affected reproductive performance. Likewise, handling stress, a known contributor to egg loss (Smith and Ritar 2005), was kept low and randomly distributed.

Broodstock survival was not adversely affected by any of the diets and suggests all diets are suitable for maintenance of adults. Similarly, the growth rate of *L. amboinensis* broodstock did not differ across treatments. The slightly lower but, not significantly different, growth rate recorded for shrimp fed *Artemia* biomass could be related to lower protein content of this diet (Glencross *et al*. 2001; Smith *et al*. 2005). In contrast, diet lead to marked differences in the reproductive output of *L. amboinensis*. Provided that the diets satisfied essential maintenance requirements of the sexually active SHs, there were differences in dietary energy directed to gonadal maturation (Calado and Dinis 2007).

The dietary items selected had a significant effect on a number of reproductive parameters in *L. amboinensis* broodstock, including egg deposition and retention. Broodstock that were fed pellet or frozen *Artemia* biomass produced broods of greater size, measured as abdominal egg cover, and displayed lower egg mass loss over the incubation period, compared to shrimp fed squid-mussel or a combination of all four items. That may be related to the lipid content of the experimental diets, as lipids have been shown to provide suitable energy for continuous spawning (Middleditch *et al*. 1980; Xu *et al*. 1994; Naessens *et al*. 1997). The *Artemia* had the highest total lipid content and resulted in the best spawning performance, followed by the pellet, the mixed diets and lastly by the squid-mussel, which contained the lowest total lipids and exhibited lower egg fecundity and poor egg retention.

Various essential FAs can impact on spawning quality; in *M. rosenbergii* increased dietary levels of LOA (n-6 family) increased fecundity, measured as egg number per female weight (Cavalli *et al*. 1999). Beneficial effects of high LOA on reproduction and offspring survival have also been reported for penaeids (Luis and Ponte 1993; Palacios *et*

al. 2001). A high concentration of C18 PUFAs (including LOA) in the *Artemia* diet of *L. amboinensis* could be associated with high egg production; however, viable fecundity was low and may suggest a requirement for a different mix of FAs. The poor embryo hatchability of the *Artemia* diet could be attributed to the observed DHA deficiency, a FA component often associated with hatching success in penaeids and carideans (Xu *et al*. 1994; Cavalli *et al*. 1999).

The potential role of LOA in the reproduction of *L. amboinensis* may be further supported by the fact that squid and mussel were both low in LOA and shrimp fed the diet exhibited poor spawning. Viable fecundity for squid-mussel was variable and generally low, despite both items having high n-3 HUFA levels and may be partly explained by the lower egg cover prior to hatch compromising larval production. The very high DHA content of squid could also be affecting hatchability negatively as inhibitory effects of elevated FA levels have been previously reported for penaeids (Bray *et al*. 1990). The Kuruma pellet had moderate to high LOA and DHA levels and yielded good spawning and hatching performance. Further studies are needed to confirm the discussed hypotheses on the role of these FAs.

Variability in larval production across the reproductive cycles for the applied mixed diets may be related to differential dietary intake of the individual components, due to animal preferences (Williams *et al*. 2005; Johnston *et al*. 2007). Overall, the various combinations of squid, mussel, pellet and *Artemia* tested performed well compared to squid-mussel or *Artemia* alone. Other studies have also demonstrated the inadequate nutrition provided by single or double item fresh diets, and that a combination of natural and dry feeds as maturation outperforms fresh feeds hatchery practices (Nascimento *et al*.

1991; Naessens *et al*. 1997; Perez-Velazquez *et al*. 2002; Wouters *et al*. 2002). Indeed, when pellet was included in the diet of *L. amboinensis* in this study, larval production increased significantly. It should be noted that the improved performance of the pelleted diet may be related not only to a high PUFA and HUFA content but also to an adequate supply of vitamins, minerals and carotenoids (Perez-Velazquez *et al*. 2003; Williams 2007).

In penaeid prawn culture it is generally recommended that pellets constitute up to 50% of the broodstock diet to maintain high reproductive performance (Primavera *et al*. 1979; Harrison 1990; Bray and Lawrence 1992; Wouters *et al*. 2001; 2002). However, a sole dry feed regime yielded satisfactory results in terms of egg cover and retention, hatchability and resulting viable fecundity over the duration of this study. As such, it would be more cost effective to feed *L. amboinensis* broodstock on Kuruma pellet only instead of a combination of fresh frozen and dry feeds, without compromising reproductive potential. Kuruma diet is an expensive crustacean aquaculture diet (US$ 3400–6500 tonne^{-1}, Barclay *et al*. 2006), but due to the quantitatively low feed requirements of the ornamental species considered, it would still be an economically viable option. To the author's knowledge, this is the only reported case in crustacean culture where a commercial dry feed has performed better as maturation diet than common fresh frozen feeds or fresh frozen feeds combined with dry feeds. Future studies should investigate the manipulation of FA dietary components to ascertain the link between specific nutritional elements and their role in reproduction in *L. amboinensis.*

Chapter 4

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Evaluation of lipid enriched *Artemia* on the reproductive performance of the white-striped cleaner shrimp, *Lysmata amboinensis*

4.1 Introduction

Artemia species have been used in fish and crustacean aquaculture since the 1930s, solely or as a component of larval dietary regimes (Seale 1933; Léger *et al*. 1985; Millamena *et al*. 1988). While *Artemia* are not the natural diet for most cultured species, the use of this prey item has become widespread due to their ready availability, long-term storage as cysts, simplicity of production and acceptable biochemical composition (Dhert *et al*. 1993; Coutteau and Sorgeloos 1997). Supposedly, live *Artemia* are of higher nutritive value (Conklin 1995), but nevetheless, harvested *Artemia* can be frozen, freeze-dried or acid-preserved to obtain biomass for later use (Dhert *et al*. 1997). As *Artemia* proved to be a satisfactory replacement for bloodworms, an expensive ingredient in broodstock fresh feeds, inclusion of *Artemia* biomass in crustacean maturation diets became increasingly common practise (Rhodes *et al*. 1992; Naessens *et al*. 1997). Broodstock maturation diets are an important component of captive breeding programs in

prawn and shrimp hatcheries around the world. Much research has focused on addressing the nutritional requirements of commercial species, in an effort to reduce feeding costs by utilising cheaper, readily available, effectual ingredients, minimizing feed waste and optimising reproductive performance.

It has been demonstrated that the nutritional condition of spawners can impact on several reproductive aspects of marine crustaceans, such as fertility and spawning frequency in females and sperm quality in males (Marsden *et al*. 1997; Perez-Velazquez *et al*. 2002; Wouters *et al*. 2002). Racotta *et al*. (2003) noted the critical role that broodstock diet had on gonadal maturation, vitellogenesis, spawning, egg biochemical composition, embryo hatchability, viable fecundity, larval quality and early larval survival in shrimp. For *Litopenaeus vannamei*, inclusion of frozen enriched *Artemia* biomass in a fresh items diet yielded better ovarian maturation, spawning, hatching and larval survival (Wouters *et al*. 1999). Lin and Shi (2002) found that a mixed diet of frozen adult *Artemia* biomass and hard clam achieved high relative fecundity in *Stenopus scutellatus*, the golden banded coral shrimp.

The lipid profile of administered diets has been a focal point in studies investigating maturation in crustaceans. Lipids serve many roles; they are a major form of energy storage, structural components of biological membranes, carriers for fat-soluble vitamins and sterols, and precursors of prostaglandins (reproductive hormones) (Harrison 1997; Sales and Janssens 2003). EFAs, including LOA, linolenic (LNA), ARA, EPA and DHA, must be provided in the diet, as many crustaceans have limited or no ability to synthesize them *de novo* (Middleditch *et al*. 1980; Xu *et al*. 1994; Glencross and Smith 2001). Feeding *M. rosenbergii* broodstock with high LOA and n-3 HUFAs improved fecundity

and overall offspring quality (Cavalli *et al*. 1999). In a study by Cahu *et al*. (1995), low dietary HUFA concentration resulted in poor embryonic development.

Given *Artemia spp*. are relatively poor in EPA and DHA, both FAs linked to successful reproduction and healthy development of marine species, enriching *Artemia* with lipid emulsions is standard procedure in many hatcheries (Sorgeloos *et al*. 2001). There are numerous enrichment techniques applied to boost the nutritive value of *Artemia*, some targeting *Artemia* nauplii for feeding to larvae and others *Artemia* adults for feeding to PL or broodstock (Coutteau and Sorgeloos 1997; Nelson *et al*. 2002a). The various enrichment techniques account for differences in ingestion and metabolism of enrichment media by the different *Artemia* life stages (Dhont and Lavens 1996).

The aims of this study were to further investigate the suitability of frozen adult *Artemia* biomass as a maturation diet for *L. amboinensis*, examine if lipid enrichment of *Artemia* improves the reproductive output of the cleaner shrimp and elucidate the role of EFAs in the reproductive performance of *L. amboinensis*.

4.2 Materials and Methods

Broodstock maintenance

Lysmata amboinensis acquisition, acclimation and experimental set up were as described in Chapter 2, section 2.2. The values of the water parameters only differed slightly, specifically water temperature was 27.9 (± 0.88) ºC (pH/oxi 340i), pH 8.1 (± 0.06) (pH/oxi 340i) and salinity 34.7 (± 0.20) g L^{-1} (optical salinometer, Bio-marine,

Inc.). The major difference was the maintenance diet, which was frozen, un-enriched adult *Artemia* biomass, instead of the squid-mussel diet. The experiment was conducted during September and October, 2008.

Artemia production

Decapsulated *A. franciscana* cysts (INVE Aquaculture Inc., USA) were hatched in a 50 L fiberglass cone at 28.1 (± 0.23) ºC. At 24 hrs, the *Artemia* nauplii were removed from the tank and stocked into a 680 L PVC conical tank, at a density of 5 ml^{-1}. They were cultured on a mix of algae (*C. muelleri*, *T. suecica* and *I. galbana* Tahitian strain), rice pollard and spirulina. On day 11, adult *Artemia* were harvested, rinsed in freshwater for 10 min, wet weight (wet wt) taken and equally divided between 2 x 200 L fibreglass cones filled with static, ozonated, aerated seawater. *Artemia* in one vessel received a 16 hr Algamac 3000 enrichment (Aquafauna Biomarine Ltd, USA), with 100% water exchange and application of fresh enrichment at 8 hrs. Algamac 3000 was blended in ozonated seawater for 30 s prior to distributing at a rate of 0.2 g L^{-1} for each 8 hr enrichment. *Artemia* in the second vessel were fed rice pollard and spirulina at a rate of 0.1 g L^{-1} and 0.02 g L^{-1}, respectively. Both *Artemia* batches were antibiotic-treated (oxytetracyclin, erythromycin, streptomycin and ciprofloxacin mix) for the last 3 hrs prior to harvesting. At harvest, the two *Artemia* batches were mixed in different proportions to produce a graduated lipid concentration across four diets. Prepared diets were then stored at -18ºC.

Experimental procedures

There were four dietary treatments, each containing five replicates, each replicate consisting of a *L. amboinensis* pair. They ranged in size from 43.2-50.5 mm TL and were allocated to the treatments randomly. The diets were:

1) Un-enriched *Artemia* (*Artemia* fed rice pollard and spirulina) ("unenr").
2) Enriched *Artemia* (fed Algamac 3000) ("enr").
3) 1/3 enriched *Artemia* (produced by mixing 2/3 un-enriched and 1/3 enriched *Artemia*) ("1/3 enr").
4) 2/3 enriched *Artemia* (produced by mixing 1/3 un-enriched and 2/3 enriched *Artemia*) ("2/3 enr").

The *Artemia* of the different nutritional profiles were mixed homogenously prior to freezing. The diets were fed to the shrimp once daily. Feeding quantities were the equivalent *Artemia* dw of 3.5% broodstock wet wt.

Data for each replicate were collected for three successive spawns over 2 months (September-October, 2008), and were comprised of the average of the pair. Prior to the data collection, the experimental diets were fed to the broodstock for a moult cycle, to allow acclimation to the treatment diet and to negate any carry-over effects of the maintenance diet.

The parameters recorded were:

a) Percent growth by the end of the study. Growth was calculated as in Chapter 3, section 3.2. Shrimp differentiation and size measurements were as in Chapter 2, section 2.2.1.

b) Percent egg cover of the abdomen for three intermoult periods, calculation formula as in Chapter 3, section 3.2.

c) Viable fecundity, i.e. the number of larvae hatching, at each of three spawns. Larvae were collected as in Chapter 2, section 2.2.1.

Also, at the conclusion of the experiment samples of the four diets were analysed to obtain lipid content and FA profile. Lipids were extracted from the samples with chloroform/methanol by the method of Folch *et al*. (1957). Total lipid was determined gravimetrically on an aliquot of the extract by drying for 4 hrs at 80°C in a pre-weighed glass vial. A further aliquot of the extract was taken for FA analysis. For more details on FA analysis please see the protocol at Chapter 3, section 3.2.

Statistical analysis

SAS v8.2 (SAS Institute Inc., Cary, North Carolina) and SPSS v16.0 (SPSS Inc., Chicago) were used for the statistical analysis. The level of significance was set at 0.05. Growth data, egg cover the day before hatching (data arcsine-transformed) and overall viable fecundity (data log-transformed) were analysed by 1-way ANOVA. To analyse percent egg cover across incubation time the MIXED procedure was applied, after arcsine-transformation of the data. Viable fecundity data across the three spawns were analysed with a Split Plot ANOVA, after log-transforming the data. Post-hoc analysis was carried out using the Student-Newman-Keuls test.

4.3 Results

The lipid content of the diets was similar and ranged between 20-22% dw. The 14 to 22 Carbon FA composition of the four *Artemia* diets is displayed in Table 4.1. The MUFA 18:1n-9 (oleic) was the most abundant FA in the *Artemia*, decreasing from 40.3% in the "unenr" to 29.5% in the "enr". In all *Artemia* treatments, the PUFA 18:2 (LOA) occupied a high percentage of total FAs. The highest content was found in the "unenr" at 36.6% and the lowest in the "enr" at 25.6%. The level of the PUFA 18:3 (LNA) was similar in the *Artemia* treatments, ranging between 1.4 to 1.8 %. Also, the HUFA 20:4 (ARA) was low, increasing sequentially in "unenr" < "1/3 enr" < "2/3 enr" < "enr" *Artemia* and attaining a final value of 1.2%. The 20:5 (EPA) content followed a similar pattern, commencing at 0.9% in "unenr" and reaching 3.0% in "enr" *Artemia*. No 22:6 (DHA) was detected in "unenr" *Artemia*, but it had sequentially greater concentrations in "1/3 enr" < "2/3 enr" < "enr" *Artemia*, with 15.8 mg g^{-1} dw in the "enr" treatment corresponding to 10.7% of its total FAs. Total n-3 content was highest in the "enr" treatment (15.8%), whereas the "unenr" *Artemia* were dominated by n-6 FAs (39.8%). The n-3/n-6 ratio was generally low, due to the high LOA content, with the "enr" treatment displaying a 1.0: 2.1 ratio.

TABLE 4.1 Major FAs of the un-enriched (“unenr”), 1/3 enriched (“1/3 enr”), 2/3 enriched (“2/3 enr”) and enriched (“enr”) *Artemia* diets.

FA	**Qualitative data (% in total FA)**				**Quantitative data (mg g^{-1} dw)**			
	“unenr”	**“1/3 enr”**	**“2/3 enr”**	**“enr”**	**“unenr”**	**“1/3 enr”**	**“2/3 enr”**	**“enr”**
14:0 (Myristic)	0.3	0.7	1.1	1.6	0.5	1.1	1.7	2.4
16:0 (Palmitic)	9.0	9.8	10.9	12.3	13.7	15.6	16.3	18.3
16:1n-7 (Palmitoleic)	1.0	0.9	0.9	0.9	1.5	1.4	1.4	1.3
18:0 (Stearic)	3.7	3.6	3.5	3.6	5.7	5.7	5.3	5.3
18:1n-9 (Oleic)	40.3	37.2	33.3	29.5	61.7	58.9	49.8	43.8
18:1n-7 (Vaccenic)	2.1	2.1	2.0	1.9	3.3	3.3	3.0	2.8
18:2n-6 (LOA)	36.6	33.5	29.7	25.6	55.9	53.1	44.4	38.0
18:3n-6	2.0	1.9	1.8	1.6	3.1	3.0	2.7	2.4
18:3n-3 (LNA)	1.8	1.7	1.5	1.4	2.7	2.6	2.3	2.0
20:2	0.7	0.7	0.7	0.6	1.1	1.1	1.0	0.9
20:4n-6 (ARA)	0.2	0.5	0.9	1.2	0.4	0.8	1.3	1.7
20:5n-3 (EPA)	0.9	1.6	2.4	3.0	1.5	2.6	3.6	4.5
22:5n-6	-	1.3	2.8	4.4	-	2.1	4.2	6.5
22:6n-3 (DHA)	-	3.3	6.9	10.7	-	5.2	10.3	15.8
EPA/ARA	-	-	-	-	3.8	3.3	2.8	2.6
DHA/EPA	-	-	-	-	-	2.0	2.9	3.5
Σ(n-3)	3.0	6.9	11.4	15.7	4.7	11.0	17.0	23.3
Σ(n-6)	39.5	37.9	35.9	33.4	60.5	60.1	53.6	49.6
n-3/n-6	-	-	-	-	0.1	0.2	0.3	0.5
Total FA	-	-	-	-	152.9	158.5	149.4	148.2

Lysmata amboinensis survival was not affected by the experimental diets. There were no significant differences in growth across treatments for the 2 month study (P = 0.487). Mean overall growth ranged between 6.2 to 8.8 %.

The decrease in abdominal egg cover over the 12 day incubation period was not significant for any group (P = 0.665) (Fig. 4.1). Initial egg cover for the *Artemia* diets was estimated to be approximately 100% and reduced to between 80-85% by day 12.

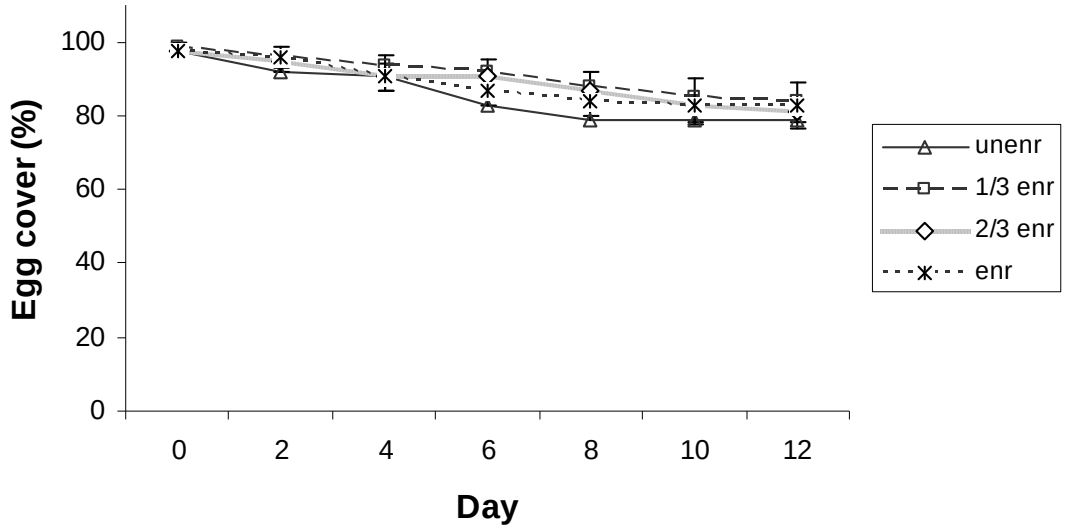

FIGURE 4.1 Egg cover, expressed as a percentage of total egg carrying area, during the 12 day incubation period for the four *Artemia* diets (means ± SEM, n = 5 reps per treatment).

The egg cover on the abdomen the day before hatch (DBH) and the average number of larvae that hatched at the end of the incubation period provided a series of contrasting results (Fig. 4.2). While egg cover the DBH was not significantly different among shrimp

fed the *Artemia* diets, with values between 80-85% (P = 0.793), viable fecundity differed significantly [F (3, 16) = 59.31, P < 0.001], with increased larval production noted from "unenr" to "1/3 enr", "2/3 enr" and "enr" *Artemia*. The minimum number of larvae was 49 (± 11) and the maximum was 529 (± 77), these were recorded for the "unenr" and "enr" diets, respectively.

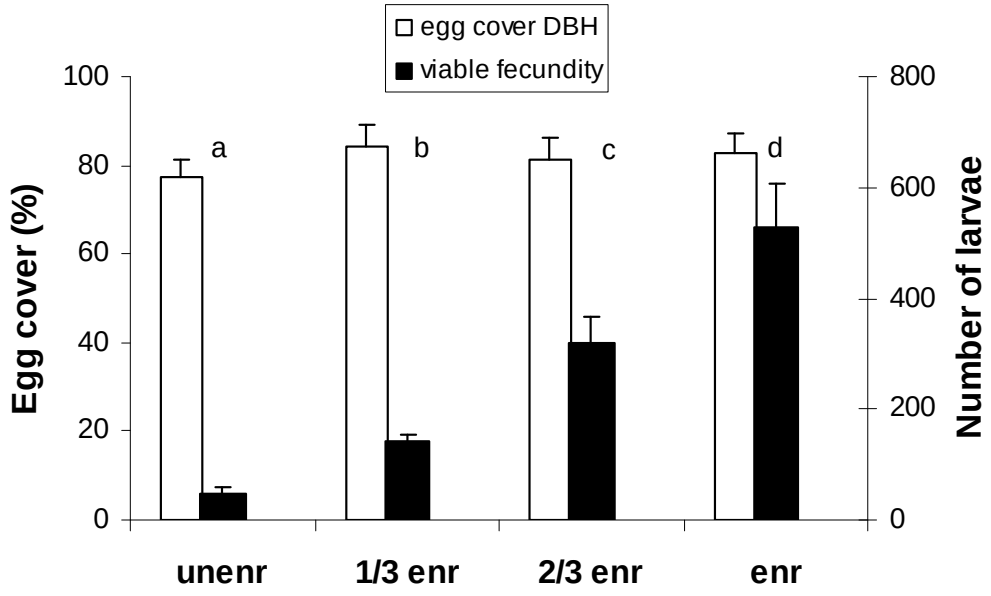

FIGURE 4.2 Percent egg cover of the abdomen the DBH, and number of viable larvae produced at the end of the brooding period for each diet treatment (means ± SEM, n = 5 reps pet treatment). Different letters indicate significant differences in viable fecundity.

For any single diet, differences in viable fecundity from one reproductive cycle to the next were not significant (P = 0.440), but within the same spawn cycle diet had a significant effect on viable fecundity [F (3, 16) = 53.19, P < 0.001] (Fig. 4.3). In spawn 1, those shrimp fed "enr" *Artemia* produced the highest number of larvae at 618 ± 144.48.

Larval production of the "1/3 enr" *Artemia* was half of the yield of the 2/3 enrichment and almost 3-fold of that of the "unenr" *Artemia*. Broodstock fed "unenr" *Artemia* had low reproductive output at all spawns, with the lowest being 25 (± 11) larvae in spawn 3. At the same spawn (i.e. spawn 3), viable fecundity of the "2/3 enr" *Artemia* reached its highest at 373 (± 114) larvae, whereas larval production for the "enr" *Artemia* dropped to 490 (± 121).

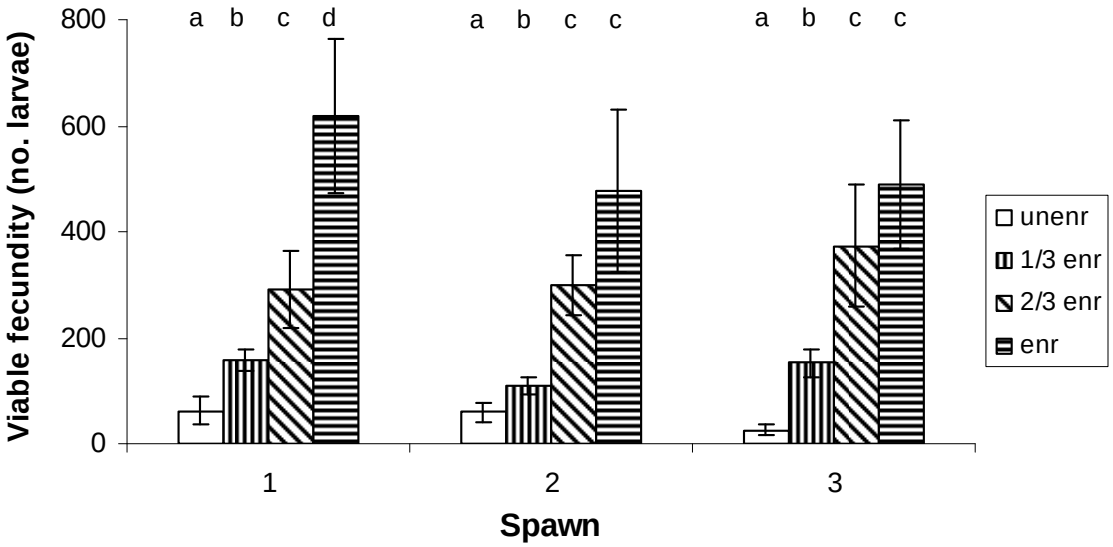

FIGURE 4.3 Number of larvae produced, referred to as viable fecundity, at each of the 3 spawn cycles for each diet (means ± SEM, n = 5 reps per treatment). Different letters indicate significant differences in viable fecundity.

4.4 Discussion

Good survival of the broodstock is indicative that *Artemia* satisfied the basic nutritional requirements for adult shrimp maintenance. Growth in the 2 month study was

also satisfactory, ranging between 7-11%. There were, however, marked differences in reproductive output between the different dietary treatment groups.

It must be noted that *L. amboinensis* preference for "unenr" or "enr" *Artemia* in the mixed diets was considered unlikely and would not have affected the outcome. When introducing the *Artemia* to the tanks, the feeding response was immediate and non-discriminatory, with the majority of the feed being consumed within 5-10 min. There was no difference across the treatments in the amount of uneaten *Artemia* (< 5% of the daily feed), with the exception of a cessation in feed intake across any treatment in the day prior to a moult or at hatch.

Lysmata amboinensis are successive breeders, producing broods all year round, an energetically demanding process occurring, on average, every 2 weeks. While the SHs incubate the developing embryos externally, their ovaries are filling with yolky oocytes, in preparation for the next reproductive cycle. Once the embryos hatch, the SHs will moult, mate and then spawn a new brood for attachment to the pleopods on the abdomen. Several studies in penaeids have established that lipids are an optimal energy substrate for oogenesis, vitellogenesis and embryogenesis (Middleditch *et al*. 1980; Teshima *et al*. 1988a, b; Xu *et al*. 1994; Harrison 1997; Naessens *et al*. 1997).

The *Artemia* diets fed to the *L. amboinensis* broodstock had a high total lipid level at > 20%, which promoted ovarian maturation and continuous spawning. Initial egg cover was high at 97-99% for all shrimp fed the *Artemia* diets and was reduced to a minimum of 80% during the 12 days of incubation, indicating good egg deposition and retention. During incubation, an increase in egg volume results in a decrease in egg number, due to the limited egg carrying space available (Calado and Narciso 2003; Figueiredo *et al*.

2008). Additionally, maternal grooming activities, stimulated by environmental stress factors or disease, have been related to egg mass losses (Kuris 1991; Oh and Hartnoll 1999). Therefore, it is not unusual in crustacean culture for a portion of the spawned brood to be lost during embryo incubation.

Despite a similar total lipid content, there were differences in the concentration of the main PUFAs and HUFAs in the *Artemia* diets. The most abundant PUFA in all dietary treatments was LOA, while the most abundant HUFA was DHA, with the exception of the "unenr" treatment where it was absent. It is likely that both FAs were sequestered from dietary sources. When algal diets containing excessive LOA were fed to penaeid larvae, this FA accumulated in the tissues of the animals (D'Souza and Loneragan 1999). Rice pollard, a primary component of the feeding regime for *Artemia*, is known to be rich in LOA (Karunajeewa and Tham 1987). LOA is a precursor of ARA, however, the low ARA levels present in the *Artemia*, in particular the "unenr" treatment that contained the greatest concentration of LOA, imply the ARA probably originated from the microalgae *C. muelleri* and *T. suecica* that were fed to the *Artemia* (D'Souza and Loneragan 1999).

The low EPA concentration in the *Artemia* may be indicative of low dietary availability and/or active catabolism to complete a range of physiological processes (Sargent *et al*. 1993). Conversion of LNA to EPA was considered unlikely as both FAs were not abundant in the *Artemia*. Enrichment of *Artemia* with DHA is inherently difficult because of their ability to retroconvert DHA to EPA (Evjemo *et al*. 1997). However, DHA uptake from the enrichment product (Algamac 3000) was adequate and retroconversion was probably minimal, as evidenced by the low EPA level noted in the "enr" *Artemia*.

The ability of crustaceans to elongate and desaturate the short-chain PUFAs into the long-chain HUFAs is limited (Kanazawa *et al*. 1979a, b; Mourente 1996; González-Félix *et al*. 2003); hence, in culture, it is recommended to provide PUFAs and HUFAs by dietary means (Xu *et al*. 1994; Harrison 1997; Glencross *et al*. 2002). Predominantly ARA, but also EPA, is involved in the synthesis of prostaglandins and other eicosanoids, which regulate crustacean moulting and reproduction (Brett and Muller-Navarra 1997). For *Penaeus chinensis*, ARA was of greater nutritional value than its precursor LOA (Xu *et al*. 1993), and a correlation was found between EPA and egg fecundity, and DHA and hatching rate (Xu *et al*. 1994).

Embryo hatchability in the current study improved significantly when DHA boosted *Artemia* were fed to *L. amboinensis* and, based on the findings, elevated DHA concentrations of >7 and up to 11% (in total FAs) in maturation diets intended for *L. amboinensis* are recommended. During the study, it was noted that shrimp egg production and attachment proceeded despite the low concentration of ARA and EPA in the diets, and in shrimp fed "unenr" *Artemia* of a DHA absence as well, and perhaps suggests a minimal role for these n-6 and n-3 HUFAs during this phase of reproduction. Marsden *et al*. (1997) also found that EPA did not contribute to reproductive output in *P. monodon*. It may be that the EFA requirements of *L. amboinensis* were partly met by the C18 PUFAs, similar to reports for other crustaceans (Luis and Ponte 1993; Cavalli *et al*. 1999; Palacios *et al*. 2001).

It has been argued that for crustaceans, the dietary DHA/EPA ratio is more critical to lipid nutrition and FA metabolism rather than the absolute concentration of either FA (Rees *et al*. 1994; Glencross *et al*. 2002). In a study by Arendt *et al*. (2005) on the

calanoid copepod *Temora longicornis*, hatching success varied from 80 to 100 % when the DHA/EPA ratio was 2.5 and 4.8, respectively. It was proposed by the authors that egg production may be more related to food quantity, whereas hatching may be more dependent on food quality, expressed by the DHA/EPA ratio. In the current study, the amount of food remained constant across all treatments; this may provide an explanation for the similarity in egg cover. In the *Artemia* enrichments, the DHA/EPA ratio increased from 2.1 in the “1/3 enr” to 3.5 in the “enr”, with a trend of increased embryo hatchability and viable fecundity with increasing dietary DHA/EPA ratio.

Chapter 5

The effect of prey density, size and quality on survival, growth, development and fatty acid profile of *Lysmata amboinensis* early stage larvae

5.1 Introduction

The larval cycle of *L. amboinensis* is amongst the longest reported for any decapod, reaching up to 150 days from hatch to settlement (Fletcher *et al*. 1995; Wunsch 1996), partly attributed to mark-time moulting (Gore 1985), especially of older larvae. Accordingly, the larvae moult and grow but no obvious morphological differences are noted and they do not progress to the next zoeal stage. During the prolonged larval phase mortality is high. Furthermore, settlement is not synchronous and, within the same batch, can range from less than 3 months to over 5 months (pers. obs.). Completion of the larval cycle in captivity consistently and in high enough numbers to allow production of new shrimp broodstock has not yet been achieved. Currently, only a small percentage of larval batches survive to the juvenile stage.

Improving the nutrition of broodstock and larvae could potentially advance captive mass production of *L. amboinensis*, by improving larval quality and increasing larval survival and growth rates (Lin *et al*. 2002; Rhyne and Lin 2004; Calado *et al*. 2008b). Broodstock feeding regime has been dealt with in previous chapters, and this study will

concentrate on suitability of various larval diets. Feed quality and quantity are key factors in regulating the duration of larval development (Harms and Seeger 1989).

Lysmata amboinensis larvae are planktonic carnivores (Bauer 2004), feeding in the wild on a diversity of zooplankton, this way satisfying their nutritional requirements. Collecting and feeding natural plankton is neither practical, nor hygienically safe, in hatchery operations. Instead, the general practice has been to feed shrimp larvae on cultivated rotifers (*Brachionus spp*.) and/or brine shrimp (*Artemia spp*.) (Lubzens *et al*. 1989; Samocha *et al*. 1989; Sorgeloos *et al*. 1998; Kumlu 1999; Dhert *et al*. 2001). This approach has been based more on convenience in production and acceptability by predators and less on nutritive value *per se*. Besides, the nutritional composition of live preys can be manipulated through enrichment with essential amino acids, EFAs, vitamins, carotenoids and other elements (Sorgeloos *et al*. 1991; Treece 2000; Dhert *et al*. 2001). *Artemia* has been the most common enriched live food used in crustacean larviculture (Sorgeloos *et al*. 2001).

While application of rotifers has been limited to first feed, due to their small size, different life stages of *Artemia* can be used throughout the culture of many marine animals, from polychaetes to fish (Sorgeloos 1980; Naegel 1999; Faulk and Holt 2005). Newly hatched and on-grown *Artemia*, as well as *Artemia* and rotifers, differ in shape, size, swimming behavior, biochemical composition and energetic content. For instance, adult *Artemia* are 20 times larger than freshly hatched nauplii, and have an increased protein and a reduced fat content (Sorgeloos 1980). Feeding *Artemia* nauplii resulted in lower survival of the lobster *Homarus americanus* larvae, than feeding adult *Artemia* (Fiore and Tlusty 2005). For Z1 and Z2 of the crab *Chasmagnathus granulata*, a diet of

small rotifers allowed for better survival than the larger *Artemia* nauplii (Ostrensky *et al*. 1997). Hence, to provide cultured aquatic organisms with the right prey type, especially at the onset of exogenous feeding, is of major concern to aquaculturists.

Correct prey density is also a contributing factor to a successful feeding regime in hatcheries (Samocha *et al*. 1989; Minagawa and Murano 1993). Overfeeding generates waste and build-up of toxic metabolites, thus deteriorating water quality and affecting larval survival. Underfeeding may impair larval growth and development and, in the case of crustaceans, may result in cannibalism (Barros and Valenti 2003). Providing optimal prey concentrations to the larvae reduces production costs and increases profitability of aquaculture operations.

It is generally accepted that shrimp larvae have a limited ability to produce *de novo* sufficient amounts of PUFAs, making it necessary to provide these in the diet (Shenker *et al*. 1993; Deering *et al*. 1997; Ritar *et al*. 2003). The research focus has been on the HUFAs ARA, EPA and DHA. Accordingly, EPA and ARA help maintain cell membrane structure and are precursors of eicosanoids (which include molting hormones), and DHA promotes the development of neural and visual systems in larvae (Brett and Muller-Navarra 1997; Sargent *et al*. 1997). The importance of these FAs differs between species; for example, EPA was the FA most strongly related with *Daphnia* growth (Muller-Navarra 1995). DHA enhanced development to the megalopa of the crab *Eurypanopeus depressus* (Levine and Sulkin 1984), and was preferentially incorporated over EPA into the tissues of *P. monodon* PL (Rees *et al*. 1994). High ARA levels promoted better survival in some penaeid larvae (D'Souza and Loneragan 1999). Because *Artemia* are poor in HUFAs, they are routinely enriched with selected microalgae, or micro-

encapsulated products, or emulsified preparations (Sorgeloos *et al*. 1998). The enrichment of rotifers appears to be less important than the enrichment of *Artemia.* This is, perhaps, because of the shorter time they are used as a larval feed and because of an inherently higher DHA content (Narciso and Morais 2001; Faulk and Holt 2005).

Mostly *Artemia*, nauplii or metanauplii, but also rotifers, have been used for rearing early *Lysmata* larvae with varying results (Zhang *et al*. 1998a, b; Rhyne and Lin 2004; Calado *et al*. 2005a; Palmtag and Holt 2007; Calado *et al*. 2008a; Cunha *et al*. 2008).No study to date has concomitantly compared the suitability of rotifers and *Artemia* for *L. amboinensis*, in terms of larval survival, growth and development. This was the aim of the first experiment conducted within this study. Calado *et al*. (2008a) reported that newly hatched *L. amboinensis* fed for 24 hr consumed more *Artemia* nauplii than metanauplii, but did not relate this to survival or other culture parameters. Thus, the second experiment examined if the early *L. amboinensis* larval stages can survive and grow equally well on *Artemia* nauplii and metanauplii, given the changes in size, behavior and profile of the prey. In the third experiment, 1-day old *Artemia* metanauplii were used as a vessel to deliver different HUFAs to the larvae, in order to assess their role and importance in the cleaner shrimp early development.

5.2 Materials and Methods

The experiments were conducted consecutively from April to end of June, 2009, at AIMS.

Broodstock maintenance

Forty adult *L. amboinensis* were kept in pairs in 23 L glass aquaria. Environmental parameters, aquarium set up and water treatment were as previously described (Chapter 2, section 2.2). Temperature was 27.5 (± 0.07) ºC and salinity 35.0 (± 0.04) g L^{-1}. The shrimp were fed chopped squid, mussel and Kuruma pellet on alternating days, once daily, to satiation.

Larval experimental procedures

5.2.1a Rotifers vs newly hatched Artemia from Z1 to Z2

For the first experiment of the series, 750 actively swimming, photo-tactic newly hatched larvae were collected from a single broodstock tank early morning and separated equally into 15 x 2 L cylindrical containers (height = 15 cm, diameter = 14 cm), i.e. 50 larvae in each. The jugs were filled with ozonated seawater up to the 1 L mark, with two glass pipettes at opposite sides providing gentle aeration and were kept in a water bath at 28 (± 0.06) °C. The treatments were:

1. Rotifers, at a density 18 ml^{-1} (R18).
2. Rotifers, 54 ml^{-1} (R54).
3. Newly hatched *Artemia*, 1 ml^{-1} (AN1).
4. Newly hatched *Artemia*, 3 ml^{-1} (AN3).
5. Starved larvae (control) (Z1St).

The rotifer densities to be used were calculated based on the *Artemia* biomass, to account for prey size differences. Newly hatched *Artemia* have an individual dw of 3.26 x 10^{-3} mg, whereas rotifers of 1.81 x 10^{-4} mg (Genodepa *et al*. 2004). The experimental design was randomized, with three replicates per diet. During the trials the larvae were transferred every morning into clean containers, water volume adjusted to maintain the same stocking density to the initial, and fed the diets once daily, early afternoon. The experiment ended when larvae changed to Z2 (i.e. at 3 days). Z2 can be easily recognized by the presence of stalked eyes, as opposed to sessile eyes at Z1.

5.2.1b Rotifers vs newly hatched Artemia from Z2 to Z3

A thousand Z1 larvae (from a single broodstock tank) were split in 2 x 50 L U-shaped upwellers, filled to the 10 L mark, and fed rotifers at 54 ml^{-1} until Z2. The system was flow-through, to avoid waste accumulation and prevent bacterial buildup. A flow rate of 0.2 L min^{-1} was set overnight, to maintain the rotifers in suspension. At 0800 hrs the outlet screen size was changed (from 75 to 150 µm) and the flow rate increased to 0.5 L min^{-1} to allow the old food to be flushed from the system. At 1600 hrs the outlet screen was changed again (150 to 75 µm) and fresh rotifers were introduced to the tanks.

When the majority of larvae had changed to Z2, they were collected and counted. The most active ones were transferred to the containers described in 5.2.1a. Experimental set up, stocking density and treatments were the same as for 5.2.1a, with the only difference being the trial was conducted from Z2 to Z3. Stage Z3 can be recognized by the presence of uropods at the telson. The treatments were:

1. Rotifers, at a density 18 ml^{-1} (RR18).
2. Rotifers, 54 ml^{-1} (RR54).
3. Newly hatched *Artemia*, 1 ml^{-1} (RAN1).
4. Newly hatched *Artemia*, 3 ml^{-1} (RAN3).
5. Starved larvae (Z2St).

5.2.1c Rotifers vs newly hatched Artemia from Z3 to Z4

Two thousand Z1 larvae (from two broodstock tanks) were collected, mixed and separated equally between four upwellers, i.e. 500 larvae in each (Plate 5.1). The larvae were fed rotifers (54 ml^{-1}) until Z2. When the majority of the larvae had moulted, they were counted, staged and returned to the tanks, ensuring the same Z2 stocking density in all. Then, two randomly chosen upwellers were changed to *Artemia* feeding, at 3 ml^{-1}. Thus, at Z2, two upwellers were receiving rotifers at 54 ml^{-1} and the other two *Artemia* at 3 ml^{-1}. The system was flow-through (as previously, 5.2.1b), but the screens used for the *Artemia* tanks were bigger, specifically 150 µm to keep the *Artemia* in, 210 µm to flush them out. When the majority of the larvae became Z3, they were collected, counted and transferred to the 2 L containers in the water bath. The set up was the same as for the previous experiments, though the treatments were different. Accordingly, the larvae from the *Artemia* tanks were separated in nine jugs; three jugs continued to be fed on *Artemia* (3 ml^{-1}), three changed to rotifers (54 ml^{-1}) and three were starved until Z4. The same arrangement applied for the larvae from the rotifer tanks (i.e. three jugs kept feeding on

rotifers (54 ml^{-1}), three changed to *Artemia* (3 ml^{-1}) and three were starved). In Figure 5.1 a diagrammatic representation of this experiment can be seen.

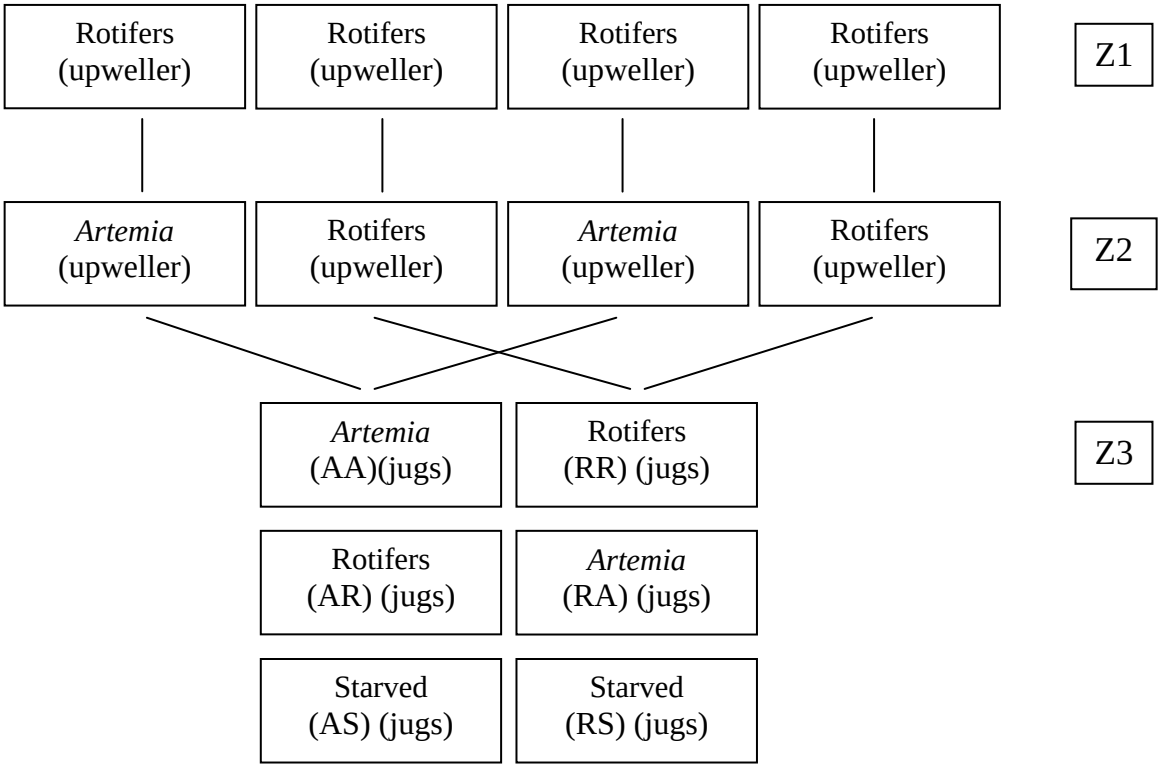

FIGURE 5.1 *Lysmata amboinensis* larval culture from hatch to Z4 (experiment 5.2.1c).

Plate 5.1 U-shaped upwellers and cylindrical container/jug used in the rearing and feeding trials of early *L. amboinensis* larval stages.

5.2.2 Artemia nauplii vs Artemia metanauplii from Z1 to Z4

Next, 750 larvae were collected from a single broodstock tank, stocked in 18 x 2 L containers and two different life stages of *Artemia* were tested. Arrangement of the jugs, larval stocking density, replication and feeding schedule were as described in 5.2.1a. The experiment was conducted continuously until Z4 (i.e. 8 days). The treatments were:

1. Newly hatched *Artemia*, 3 ml^{-1} (NH3).
2. *Artemia* metanauplii enriched with *Chlorella* paste, 3 ml^{-1} (A-c3).
3. *Artemia* metanauplii enriched with *Chaetoceros muelleri*, 1 ml^{-1} (A-ch1).
4. *Artemia* metanauplii enriched with *C. muelleri*, 3 ml^{-1} (A-ch3).
5. Starved larvae (St).

5.2.3 Artemia metanauplii enriched with different HUFAS

For the last experiment, 600 larvae (collected from a single broodstock tank) were held in 12 x 2 L jugs, to examine the effect of different lipid enrichments using *Artemia* as the vessel. The experimental design was as in 5.2.1a. On day 0 the larvae were fed immediately on newly hatched *Artemia* (3 ml^{-1}) (to avoid starvation exposure) and enriched metanauplii were given to the larvae from day 1 onwards. The experiment was conducted continuously until Z4 (i.e. lasted 8 days). The treatments were:

1. 1-day old *Artemia* enriched with rice pollard (RP).
2. 1-day old *Artemia* enriched with Algamac ARA (AM-A).
3. 1-day old *Artemia* enriched with Algamac 3000 (AM-3).
4. Starved larvae (S).

Experimental parameters

The experimental parameters recorded were:

1. Larval survival on a daily basis to the end of the experiment, expressed as a percentage (exp. 5.2.1a, b, and c, 5.2.2, 5.2.3). Initial stocking density was always 50 larvae L^{-1}. Larvae in each replicate were counted individually to determine the number remaining.
2. Larval development every second day, calculated as percent contribution of each zoeal stage to the total number of remaining larvae (exp. 5.2.2 and 5.2.3). At Z4, larvae were examined under a dissection microscope with an attached camera

lucida (Leica $MZ7_5$), also used in the case there were any doubts regarding Z3. Extra care was taken not to induce any physical damage to the larvae, as they were transferred back to the containers after examination. The developmental rate was also calculated, using the formula (no. of larvae at the zoeal stage x no. of days to the zoeal stage) / no. of larvae surviving.

3. Larval growth (TL mm), measured from the tip of the rostrum to the telson (exp. 5.2.2 and 5.2.3). Five larvae per replicate, of the same stage, were measured every 3 days. Photos of the larvae were taken with a dissection microscope, with an attached camera lucida (Leica $MZ7_5$). The larvae were then transferred back to the containers, and the images were subsequently analyzed.

Only for experiment 5.2.3 the larval FA profile was also generated. For this, larvae were mass cultured under the four diet regimes, and at 8 days all surviving larvae in each treatment were harvested, rinsed in 0.5 M ammonium bicarbonate and stored at -80 ºC until FA analysis. The enriched *Artemia* diets were also analyzed.

Rotifer production

Rotifers, *Brachionus rotundiformis*, were obtained from a continuous laboratory culture at JCU. They were kept in 2 x 20 L containers, static system with feeble aeration, at an average density of 1200 ml^{-1}, at 26.5 (± 0.20) ºC. They were raised on a paste of the green alga *Chlorella* (Aquasonic Ltd, Australia), fed twice daily, at 0.8 ml L^{-1} in the morning and 0.3 ml L^{-1} in the afternoon. The required rotifer numbers for the feeding

trials were harvested every day, alternating between vessels. There was a 10% water exchange daily and every 3 days the rotifers were transferred to clean vessels.

Artemia production

Decapsulated *A. franciscana* were hatched daily in a 50 L fiberglass cone at 28 (± 0.42) ºC. At 12 hr, the newly hatched *Artemia* nauplii were removed from the tank. Depending on the experiment, they were either stored in a refrigerator (4 ºC) to slow down their metabolism and maintain the nutritional profile until they were fed to the larvae (exp. 5.2.1a, b, c and 5.2.2), or they were kept in 10 L aerated buckets, at a density of 10 ml^{-1}, for enrichment. The *Artemia* enrichments were:

1. *Chlorella* paste, at 13.0 x 10^9 cells ml^{-1} (experiment 5.2.2).
2. *C. muelleri*, at 1.2 x 10^6 cells ml^{-1}, live culture at the AIMS facilities (experiments 5.2.2).
3. Rice pollard, added at 0.05 g L^{-1} (experiment 5.2.3).
4. Algamac ARA (Aquafauna Biomarine Ltd, USA), added at 0.2 g L^{-1} (experiment 5.2.3).
5. Algamac 3000 (Aquafauna Biomarine Ltd, USA), added at 0.2 g L^{-1} (experiment 5.2.3).

All enrichments were administered in one dosage for 8 hr in experiment 5.2.2 and for 12 hr (overnight) in experiment 5.2.3. The rice pollard and the Algamac products were blended in ozonated seawater for 30 s and then poured into the *Artemia* containers through a 100 µm screen. In the *Chaetoceros* case the container was filled with the alga and the *Artemia* were then added. At the end of the enrichment period, the 1-day old

Artemia were harvested, rinsed briefly in freshwater to remove remnants of the feed products and fed to the shrimp larvae.

Feed analysis

For lipid analysis see Chapter 4, section 4.2, for FA analysis the protocol at Chapter 3, section 3.2.

Statistical analysis

The statistical package was SPSS v19.0 (SPSS Inc., Chicago), significance was accepted at $P \leq 0.05$. Survival was analyzed by 2-way ANOVA in 5.2.1a, b and in 5.2.2 (prey type and density the independent variables), also in 5.2.1c (previous diet and current diet). For survival and development in 5.2.3, 1-way ANOVA (prey type) was used. Because arcsine transformation of size data in 5.2.3 did not satisfy homogeneity, Kruskal-Wallis test was used. A 2-way ANOVA was applied for growth and development in 5.2.2 (prey type and density). Post-hoc analyses were carried out with Student-Newman-Keuls test.

5.3 Results

5.3.1 Rotifers vs newly hatched Artemia from Z1 to Z4

Larval survival from Z1 to Z2 was approximately 10% higher when larvae were fed on rotifers at either 18 or 54 ml^{-1}, as opposed to *Artemia* at 1 or 3 ml^{-1} (for prey type P = 0.003, for prey density P = 0.80). Of the starved larvae, 43% survived to Z2 (Fig. 5.2a).

From Z2 to Z3, the RR54 diet yielded 70% larval survival, RAN3 79%, RR18 and RAN1 73%, the differences were not significant for either prey type or density (P = 0.23 and P = 0.50, respectively). The survival percentage for the Z2St was 35 (Fig. 5.2b). It should be noted that starved larvae were fed rotifers at 54 ml^{-1} during Z1.

Finally, larvae starved during Z3, but fed rotifers (54 ml^{-1}) during Z2, showed poor survival (27%) compared to those fed *Artemia* (3 ml^{-1}) prior to starvation (50%) (P < 0.001). Diet AA performed better (89% larval survival) than RR (73%), with the alternating diets AR and RA at 82% mean survival (Fig. 5.2c); differences were significant (P = 0.02).

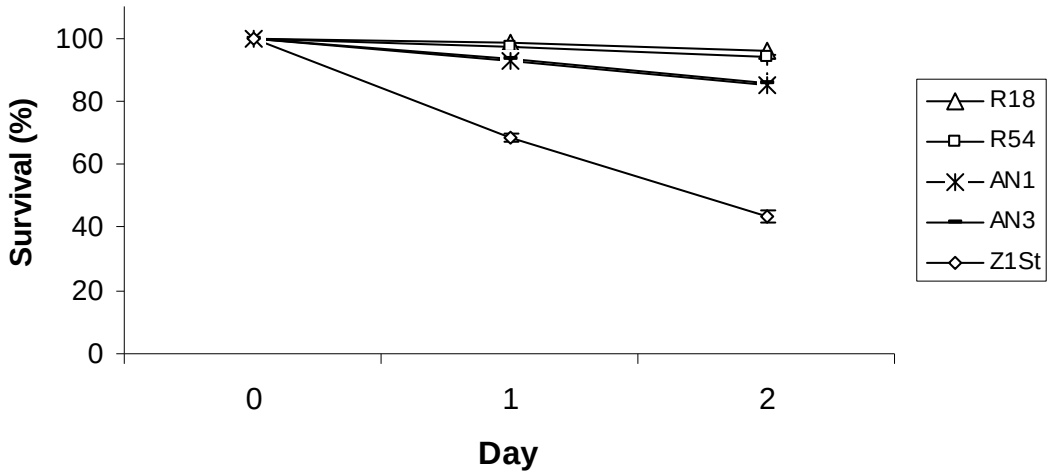

FIGURE 5.2a Survival of Z1 larvae, either starved (Z1St), or fed rotifers at 18 ml^{-1} (R18) or 54 ml^{-1} (R54) or newly hatched *Artemia* at 1 ml^{-1} (AN1) or 3 ml^{-1} (AN3) (means ± SEM, n = 3 reps per treatment).

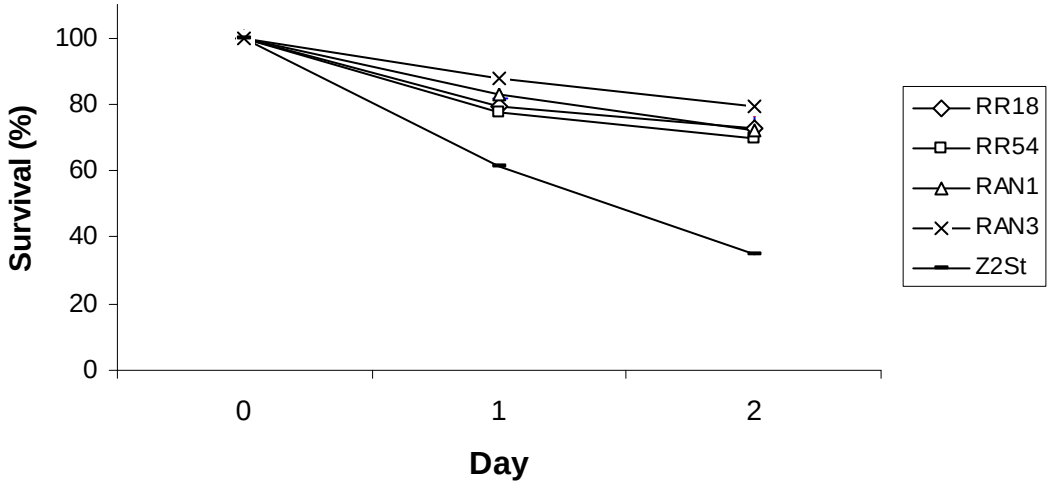

FIGURE 5.2b Survival of Z2 larvae, either starved (Z2St), or fed rotifers at 18 ml^{-1} (RR18) or 54 ml^{-1} (RR54) or newly hatched *Artemia* at 1 ml^{-1} (RAN1) or 3 ml^{-1} (RAN3) (means ± SEM, n = 3 reps per treatment).

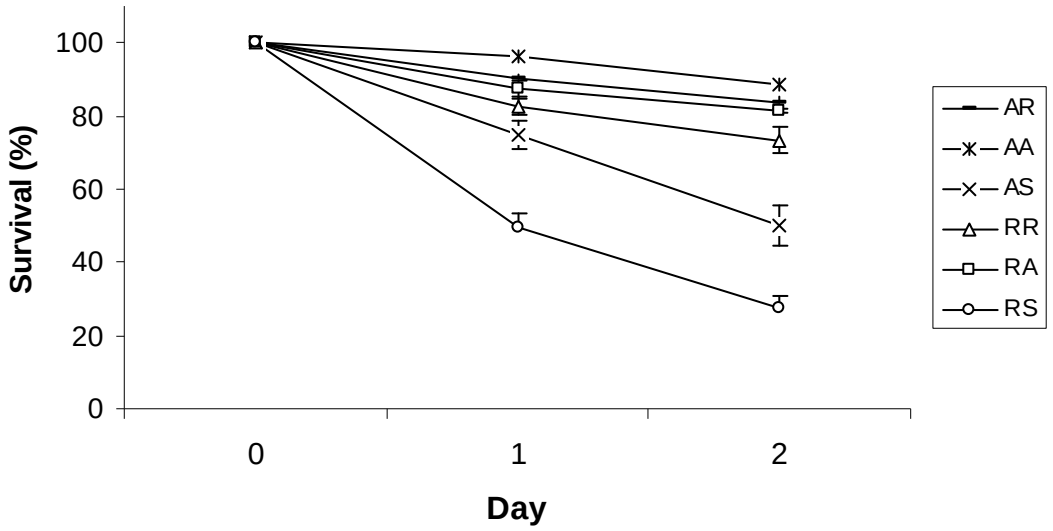

FIGURE 5.2c Survival of Z3 larvae, either starved (previous diet *Artemia* (AS) or rotifers (RS)), or fed rotifers (previous diet *Artemia* (AR) or rotifers (RR)) or newly hatched *Artemia* (previous diet *Artemia* (AA) or rotifers (RA)) (means ± SEM, n = 3 reps per treatment).

5.3.2 Artemia nauplii vs Artemia metanauplii from Z1 to Z4

Survival was similarly high for all diets, between 86-89%, neither prey type nor density affected survival (P = 0.99 and P = 0.49, respectively) (Fig. 5.3). The number of surviving starved larvae reduced gradually, with 5% still alive on the last day of the experiment.

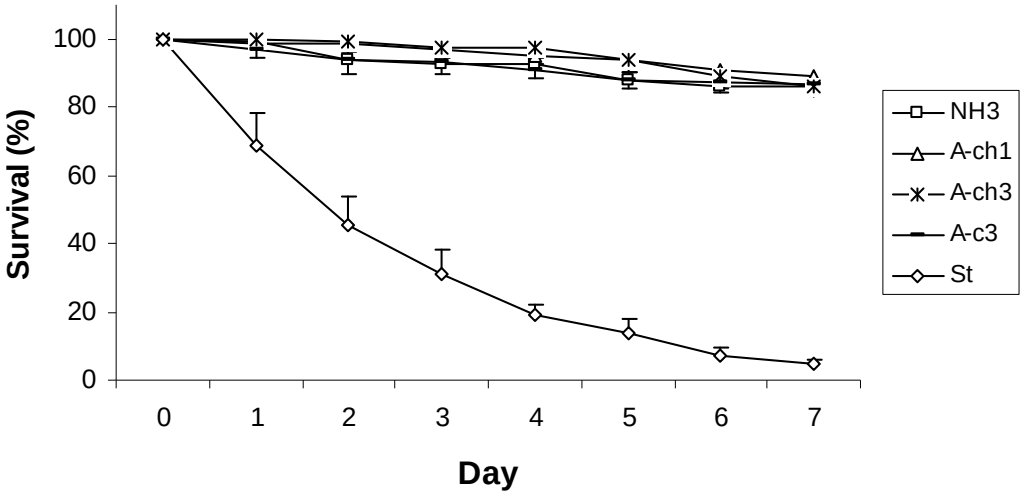

FIGURE 5.3 Survival of *L. amboinensis* larvae fed newly hatched *Artemia* at 3 ml^{-1} (NH3), or *Chaetoceros* enriched *Artemia* metanauplii at 1 ml^{-1} (A-ch1) or 3 ml^{-1} (A-ch3), or *Chlorella* enriched *Artemia* metanauplii at 3 ml^{-1} (A-c3), and of starved larvae (St) (means ± SEM, n = 3 reps per treatment).

Lysmata amboinensis larvae hatch as Z1, therefore all larvae stocked at day 0 were Z1 (Fig. 5.4). Approximately half of the larvae in NH3 and A-ch3 were Z2 on day 2, and so were 23% of the starved larvae. A-ch3 showed the highest percentage of Z3 on day 4 (40%), followed by A-ch1 at 33%. Diet A-c3 had the lowest Z4 proportion on day 6 at 4% (P = 0.03), whereas 10% of the larvae were still Z2. Prey density had no effect on development (P = 0.48). The developmental rate to Z4 was highest for NH3 and A-ch3 at 0.8 and 0.7, respectively, and lowest for A-c3 at 0.3.

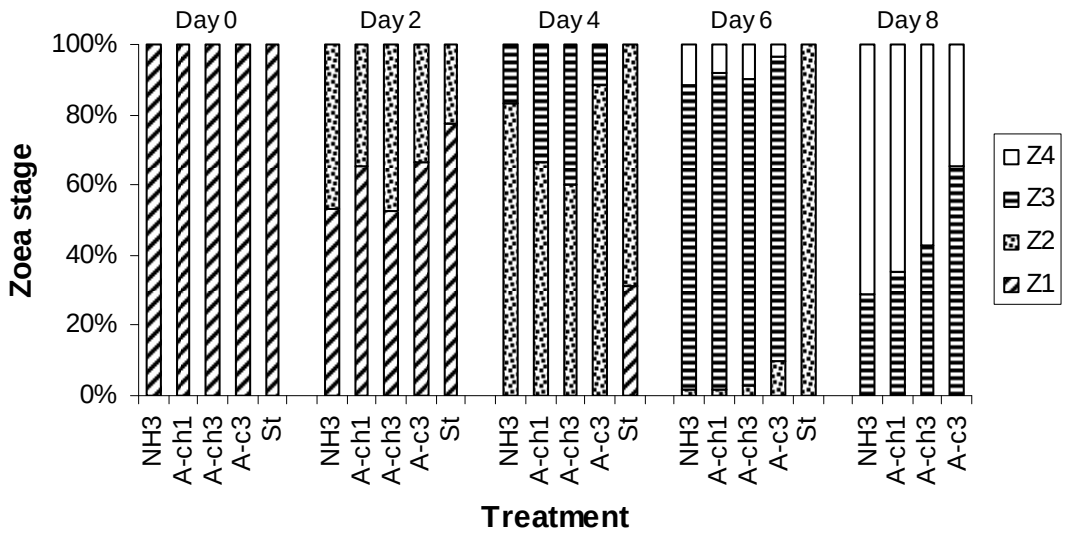

FIGURE 5.4 Per cent larvae at each zoeal stage in the *Artemia* nauplii (NH3), or *Chaetoceros* enriched *Artemia* metanauplii at 1 ml^{-1} (A-ch1) or 3 ml^{-1} (A-ch3), or *Chlorella* enriched *Artemia* metanauplii at 3 ml^{-1} (A-c3) treatments, and of starved larvae (St), at five sampling points (means ± SEM, n = 3 reps per treatment).

Newly hatched larvae were 2.77 (± 0.01) mm TL, the same size recorded for Z2 starved larvae (Table 5.1). Across all diets, larval size at the same developmental stage did not differ significantly (P = 0.06 for prey type, P = 0.27 for prey density).

Table 5.1 Size (TL mm) of each larval stage (Z1-Z4) for starved larvae (St), larvae fed newly hatched *Artemia* (NH3), or *Chaetoceros* enriched *Artemia* metanauplii at 1 ml^{-1} (A-ch1) or 3 ml^{-1} (A-ch3), or *Chlorella* enriched *Artemia* metanauplii at 3 ml^{-1} (A-c3) (means ± SEM, n = 15 reps per treatment).

	Z1	**Z2**	**Z3**	**Z4**
NH3		2.99 (± 0.01)	3.30 (± 0.01)	3.59 (± 0.03)
A-ch1		3.01 (± 0.01)	3.32 (± 0.03)	3.56 (± 0.03)
A-ch3	2.77 (± 0.01)	3.04 (± 0.02)	3.33 (± 0.03)	3.61 (± 0.02)
A-c3		3.00 (± 0.02)	3.26 (± 0.03)	3.51 (± 0.03)
St		2.77 (± 0.01)	-	-

5.3.3 Artemia metanauplii enriched with different HUFAS as larval feeds

Larval survival at the end of the 8 day experimental period was high across all treatments, with the lowest being recorded for the RP diet at 85%; differences were not significant (P = 0.33) (Fig. 5.5). Approximately half of the starved larvae were still alive on day 3, none by day 7.

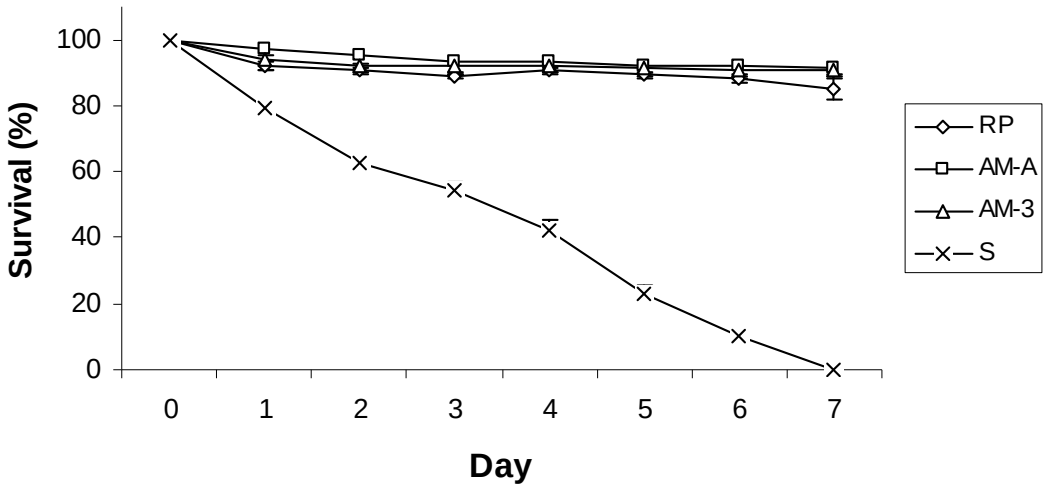

FIGURE 5.5 Survival of *L. amboinensis* larvae fed *Artemia* metanauplii enriched with rice pollard (RP), or Algamac ARA (AM-A),or Algamac 3000 (AM-3), and of starved larvae (S) (means ± SEM, n = 3 reps per treatment).

On day 2, the dominant stage in the diet treatments was Z2 (98-100%), whereas in the starved larvae treatment only 12% had changed (Fig. 5.6). By day 4, 80% of the AM-3 larvae were Z3 and by day 6, 28% were Z4, which was over 3 times the percentage of the RP diet. At the end of the trials RP and AM-A had similar proportion of Z4, while the highest was recorded for AM-3 at 96% (P = 0.04). The developmental rate to Z4 was 1.9 for AM-3, and 0.5 and 1.0 for RP and AM-A diets, respectively.

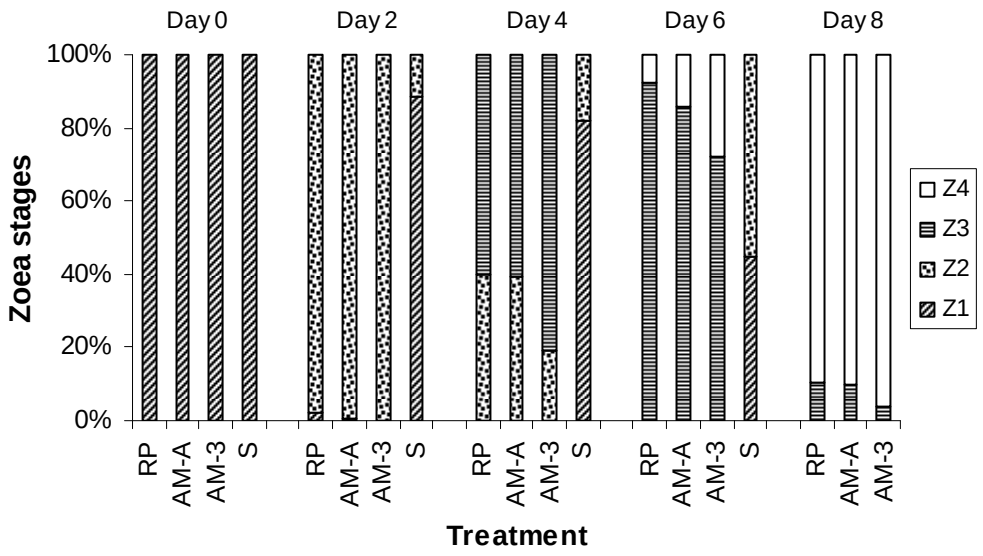

FIGURE 5.6 Per cent larvae at each zoeal stage in the *Artemia* enriched with rice pollard (RP), or Algamac ARA (AM-A), or Algamac 3000 (AM-3), and in the starved (S) treatments, at five sampling points (means ± SEM, n = 3 reps per treatment).

At Z1, the average size of the batch was 2.71 (± 0.01) mm TL (Table 5.2). The starved larvae that changed to Z2 were a mean of 2.75 (± 0.02) mm TL, compared to 2.99 mm for the diet treatments. There was no significant difference among the fed larvae for the same developmental stage (P = 0.10).

Table 5.2 Size (TL mm) of each larval stage (Z1-Z4) for starved larvae (S) and larvae fed *Artemia* enriched with rice pollard (RP), or Algamac ARA (AM-A), or Algamac 3000 (AM-3) (means ± SEM, n = 15 reps per treatment).

	Z1	**Z2**	**Z3**	**Z4**
RP		3.00 (± 0.02)	3.31 (± 0.03)	3.51 (± 0.05)
AM-A		2.94 (± 0.02)	3.26 (± 0.03)	3.55 (± 0.02)
AM-3	2.71 (± 0.01)	3.04 (± 0.02)	3.34 (± 0.02)	3.62 (± 0.03)
S		2.75 (± 0.02)	-	-

The lipid and FA composition of the shrimp larvae and their diets are presented in Table 5.3. Overall, *Artemia* had a higher lipid and FA content than the larvae. Specifically, AM-A diet had the highest lipid content and AM-3 diet the lowest, whereas AM-3 larvae contained the highest lipids. The *Artemia* diets had similar levels of 16:0 (palmitic), 18:0 (stearic), 18:1n-7 (vaccenic) and 18:3n-3 (LNA). RP diet had the highest 18:1n-9 (oleic) concentration at 42.7 mg g^{-1} dw, AM-A diet had the highest 20:4n-6 (ARA) at 37.5 mg g^{-1} dw and AM-3 diet had the highest 22:6n-3 (DHA) at 7.2 mg g^{-1} dw. The 18:2n-6 (LOA) was also abundant in the *Artemia*, with RP and AM-A diets showing twice the amount of AM-3. Lower LOA and oleic concentrations contributed to lower PUFA and MUFA content, respectively, of AM-3 as opposed to the other diets. All the aforementioned FAs were also prominent in the larval samples, with AM-3 larvae showing the highest levels for all, except for LOA and ARA, both of which were higher in the AM-A fed larvae. The n-3/n-6 ratio was high at 2.2 in the AM-3 larvae but only 0.6 at the AM-A larvae. PUFAs and HUFAs were highest in AM-3 and AM-A larvae and lowest in RP larvae.

Table 5.3 Lipid content (% in dw) and major FAs (mg g^{-1} dw) of larvae fed *Artemia* enriched with rice pollard (RP), or Algamac ARA (AM-A), or Algamac 3000 (AM-3), and of the *Artemia* diets.

	***Artemia* diets**			***L. amboinensis* larvae**		
	RP	**AM-A**	**AM-3**	**RP**	**AM-A**	**AM-3**
Lipid content	29	32	20	14	17	19
FA						
14:0 (Myristic)	0.8	0.9	1.2	0.5	0.8	1.5
16:0 (Palmitic)	14.6	13.7	14.3	8.5	9.2	13.9
16:1n-7 (Palmitoleic)	2.0	2.3	1.9	0.9	1.2	1.4
18:0 (Stearic)	7.1	7.7	6.4	5.4	6.3	7.3
18:1n-9 (Oleic)	42.7	34.0	23.6	19.1	19.3	20.6
18:1n-7 (Vaccenic)	8.0	7.9	7.2	5.7	6.0	7.2
18:2n-6 (LOA)	23.8	23.6	10.5	8.8	11.0	7.9
18:3n-6	0.6	3.9	0.6	0.2	1.0	0.4
18:3n-3 (LNA)	29.5	28.6	27.7	9.9	11.0	14.9
18:4n-3	4.8	4.6	4.6	0.6	0.6	0.9
20:1n-9	1.1	1.0	0.9	0.6	0.7	0.8
20:3n-3	1.1	1.1	1.1	1.0	1.2	1.4
20:4n-6 (ARA)	2.3	37.5	1.8	2.3	17.9	3.4
20:5n-3 (EPA)	2.9	2.7	3.9	4.7	2.9	6.6
22:5n-6	-	-	2.9	-	-	3.8
22:6n-3 (DHA)	1.7	5.7	7.2	2.6	3.9	11.2
EPA/ARA	1.3	0.1	2.2	2.0	0.2	1.9
DHA/EPA	0.6	2.1	1.8	0.6	1.3	1.7
n-3/n-6	1.5	0.6	2.8	1.6	0.6	2.2
SFA	23.1	23.0	22.4	15.8	17.9	24.6
MUFA	53.8	45.2	33.6	26.4	27.3	29.9
PUFA	55.1	58.0	40.2	20.6	25.0	25.1
HUFA	11.7	50.3	20.3	10.2	25.3	26.4
Total FA	143.8	176.5	116.5	73.2	95.7	106.4

Discussion

It is noteworthy that early stage *L. amboinensis* larvae displayed great starvation tolerance. In the two occasions larvae were continuously being deprived of food (from hatch to the conclusion of the experiment), they were able to withstand > 8 days of starvation. Comparing starvation resistance of four *Lysmata* species, *L. seticaudata*, *L. ankeri*, *L. debelius* and *L. amboinensis*, Calado *et al*. (2008a) found no larvae survived beyond 5 days, under the same light conditions and a lower water temperature of 25 ºC. During starvation at higher temperatures, the depletion rate of endogenous reserves is more rapid, which translates to greater larval mortality (Ritar *et al*. 2004). In an earlier study by Calado *et al*. (2007), *L. amboinensis* larvae did not even tolerate 24 hr starvation, whereas *Lysmata boggessi* and *L. seticaudata* displayed 100 % survival. The longer survival of starved *L. amboinensis* larvae exhibited in the current study may imply better larval quality and could be related to maternal provision of essential nutritional elements via the broodstock diet. Broodstock maturation diets are an important component of shrimp hatchery management, since the nutritional status of spawners affects gonadal maturation, egg fecundity, hatchability and early larval survival (Wouters *et al*. 2002; Racotta *et al*. 2003).

More importantly, between 12-23% of the Z1 starved larvae across treatments changed to Z2, whereas in the aforementioned papers by Calado *et al*. (2007; 2008a) no *L. amboinensis* progressed to Z2 in the absence of food. Such change was previously recorded for *L. seticaudata*, *L. boggessi* and *L. ankeri* and was regarded as FPL. In FPL, larvae posses enough yolk reserves to moult to Z2 under nutritional stress, while at the

same time retaining their ability to capture, ingest and digest prey if it became available (Anger 2001). Our study demonstrates that *L. amboinensis* are also facultative primary lecithotrophs. A similar suggestion was made by Cunha *et al*. (2008) based on oxygen consumption rate and metabolite content of the 24 hr post hatch larvae, but was not substantiated. Lecithotrophy is a strategy common to carideans, and may be a response to the variable environmental conditions planktotrophic larvae are exposed to (Rosa *et al*. 2007).

Secondary facultative lecithotrophy (SFL), where larvae use up the energy reserves accumulated during previous zoeal stages, has been reported for *L. amboinensis* larvae (Calado *et al*. 2007). The finding was also confirmed in this study, with starved Z2 larvae originating from fed Z1 being able to moult to Z3 and, equally, starved Z3 larvae from fed Z2 moulting to Z4.

It must be noted that the increase over time in the proportion of Z2 larvae in the starved treatments is “artificial”, as this was not caused by more larvae progressing to Z2, but from poorer survival of the Z1s. Cannibalism has been observed in many crustacean larvicultures (Wickens and Lee 2002; Fiore and Tlusty 2005; Penha-Lopes *et al*. 2005) but, although it cannot be ruled out completely, it is unlikely to be the main reason for the persistence of Z2 *L. amboinensis* larvae, as most of the Z1s were found dead at the bottom of the vessels, with few occasions of missing larvae during daily counts. Calado *et al*. (2007) stated that newly hatched *Lysmata* larvae were not considered cannibals and indeed remained under continued starvation.

Prey density did not affect the survival of early stage *L. amboinensis* larvae, since similar results were obtained when feeding rotifers at either 18 or 54 ml^{-1}, *Artemia* nauplii

at 1 or 3 ml^{-1}, *Artemia* metanauplii at 1 or 3 ml^{-1}. The finding was rather surprising, given *Lysmata* larvae are not active hunters but, rather, depend on "chance encounter", with a predatory response triggered only when the prey approaches the thoracic region near the exopods of the maxillipeds (Calado *et al*. 2008a). That means the higher the number of preys in the vessel, the greater the chance the larvae will "find" them, and in that respect, 54000 rotifers or 3000 *Artemia* in 1L of seawater provides a better feeding opportunity than 18000 rotifers or 1000 *Artemia*.

However, findings of food quantity independent larval survival are not uncommon in the crustacean literature. Feeding rotifers to Z2 *Penaeus semisulcatus* at 10, 20 or 30 ml^{-1} yielded no significant difference in survival to PL, survival was also independent of *Artemia* nauplii density (between 3-15 nauplii ml^{-1}) at the same study (Samocha *et al*. 1989). The treatment of 1 *Artemia* nauplii ml^{-1} did not affect survival of the zoeal stages of the ornamental crab *Mithraculus forceps*, compared to higher food concentrations of 4, 7 or 12 nauplii ml^{-1}, differences only became significant at the metamorphosis to megalopa (Penha-Lopes *et al*. 2005).

Cunha *et al*. (2008) also reported that survival of *L. amboinensis* to Z2 was not dependent on prey density or the relation of larval and prey density, the prey being enriched rotifers, *Brachionus plicatilis*, and the densities 20, 35 and 50 rotifers ml^{-1}. The authors finally proposed using 35 rotifers ml^{-1} in Z1 *L. amboinensis* cultures (Cunha *et al*. 2008). The observations of Calado *et al*. (2008a) that Z1 *L. amboinensis* would consume on average 32 *Artemia* nauplii or 28 metanauplii per larva per day, at a prey density of 3 ml^{-1}, may designate the maximum ingestion values possible. In those trials, larvae were kept separately and had 60 *Artemia* available daily, but consumed half. Zhang *et al*.

(1998a) reported the ingestion rate of *L. wurdemanni*, when the *Artemia* density was maintained at 5 nauplii ml^{-1} and the temperature at 28 ºC, was higher for larvae kept individually (as in the paper by Calado *et al*. 2008a), instead of in cultures with other larvae, probably due to lack of interactions with con-specifics.

The above findings can help us understand why at 1 or 3 *Artemia* nauplii or metanauplii ml^{-1} *L. amboinensis* larval survival remained unaffected. Making available to each larva 20 nauplii/metanauplii (as in the 1 ml^{-1} density) may have been below their capacity, but was apparently sufficient for *L. amboinensis* to survive and grow. And this relation did not change until the end of the study (at Z4). The larvae of another caridean, *M. rosenbergii*, displayed a constant ingestion rate of *Artemia* nauplii from Z1 to Z4 (Barros and Valenti 2003).

Prey (*Artemia* newly hatched or metanauplii) size at first larval feeding was not a major concern for the survival and larval duration of *L. seticaudata* (Calado *et al*. 2005a). The live preys offered to *L. amboinensis* larvae during trials in the present study, represent three size ranges, with rotifers being at the lower end (203 ± 15.71 µm) and *Artemia* metanauplii at the higher (893 ± 42.28 µm). *Artemia* nauplii were inbetween, measuring 493 (± 11.22) µm. Combining the survival results from the current experiments shows that from the 1st larval stage *L. amboinensis* were able to capture all tested preys. Slightly better survival was achieved with rotifers at Z1, followed by *Artemia* (nauplii or metanauplii) from Z2 onwards. Ruscoe *et al*. (2004) also found the inclusion of rotifers in the feeding regime of *Scylla serrata* larvae valuable, but confined only to Z1. In the larviculture of *L. wurdemanni* and *L. seticaudata*, a feeding regime of

rotifers (at 15 ml^{-1}) during Z1 and enriched *Artemia* metanauplii thereafter yielded 60 and 70 % survival to PL, respectively (Calado *et al*. 2003c).

In the present study, *Artemia* constituted the preferred diet for Z2, not because of diet performance *per se*, since the contribution of either diet to larval survival was similar, but because there was indication of beneficial effects that extended to the next larval stage. This became most clear when the survival percentage of larvae fed on *Artemia* nauplii during Z2 and subsequently starved (during Z3), was double that of larvae previously fed on rotifers. Also, feeding rotifers to the larvae during Z3 supported the lowest survival, especially if the same prey was provided during Z2. Perhaps rotifers with their small size and slow swimming velocity (Dhert *et al*. 2001) are more suitable for newly hatched *L. amboinensis*, but as the larvae get older they require larger preys, like *Artemia* nauplii or metanauplii, to satisfy their energetic requirements. This has been also suggested for *L. seticaudata* (Figueiredo and Narciso 2006). *Lysmata amboinensis* larvae could still feed on rotifers at Z3, if that was the only food offered, however, they performed better on *Artemia*. Calado *et al*. (2005a) similarly referred to the plasticity exhibited by *L. seticaudata*, because even under an inadequate feeding regime no significant increase in mortality was recorded.

Using un-enriched or enriched rotifers and *Artemia* has generated erratic success in the culture of commercial decapod species. Increased growth and survival rates were recorded for larvae and PL belonging to the families Palaemonidae and Penaeidae when fed HUFA enriched *Artemia* (Narciso and Morais 2001; Immanuel *et al*. 2004). *Lysmata spp.* (gulf coast variety) fed on 2-day old enriched *Artemia* metanauplii developed with high survival to Z5 (>99%) (Rhyne and Lin 2004), whereas survival of the fire shrimp *L.*

debelius did not benefit from the use of DHA enriched rotifers and *Artemia* during the first larval stages (Palmtag and Holt 2007). Feeding either *Artemia* nauplii or C*hlorella* enriched rotifers to *L. wurdemanni* larvae did not affect survival, but development to PL was approximately 3 days faster with *Artemia* (Zhang *et al*. 1998b). Figueiredo and Narciso (2006) found the enrichment of *Artemia* metanauplii to have no effect on larval duration of *L. seticaudata*.

Enriching *Artemia* with diatoms (*Chaetoceros*) or chlorophytes (*Chlorella*) or commercial Algamac products made no difference to the survival of early stages of *L. amboinensis*. On the other hand, the rate of development appeared to be affected by the quality (related to the nutritional profile) of the prey. Typically, *Chlorella* contains high levels of C_{16} and C_{18} FAs, whereas *C. muelleri* is rich in EPA and poor in C_{18} and C_{22} PUFAs (Lora-Vilchis and Voltolina 2003; Ritar *et al*. 2004). *Chlorella*, however, does not enhance *Artemia* quality, according to a study comparing *C. muelleri* and *Chlorella capsulata* as diets for *A. franciscana* (Lora-Vilchis and Voltolina 2003). The reason given by the authors was the lower absorption efficiency of *Chlorella*, owing to the thick cell walls of this alga. This may explain why feeding *L. amboinensis* larvae *Chlorella* fed *Artemia* resulted in slower development than a diet of *Chaetoceros* fed *Artemia*. Even though freshly hatched *Artemia* are generally deficient in HUFAs, they are considered a high value feed if presented to the larvae promptly after hatching (Tamaru *et al*. 1993). Feeding larvae with newly hatched *Artemia* indeed gave good results in regards to zoeal development, confirming their aptness for early stages of *L. amboinensis*.

Among rice pollard and the two Algamac products, Algamac-3000 was the most successful for larval development, displaying the highest proportion of Z4s at the

conclusion of the experiment. Algamac 3000, made of spray-dried algal cells of *Schizochytrium spp*., is rich in C_{22} FAs, particularly DHA. Indeed the *Artemia* fed Algamac-3000 attained a high DHA content and, subsequently, the larvae fed those *Artemia* also showed increased DHA levels. In fact, incorporation of DHA in the larval tissues of AM-3 *L. amboinensis* was greater than measured levels in their enriched diet, pointing to selective retention of this EFA during the culture period. The same could be argued for EPA, which was higher in the larvae than available dietary amounts in any of the *Artemia* treatments. Because of the superior DHA and EPA inclusion, n-3 HUFAs became the dominant FA group in the AM-3 larvae.

On the contrary, AM-A fed larvae were rich in n-6 FAs as seen by the low n-3/n-6 ratio. To that (i.e. high n-6 FA content) contributed the greatly elevated ARA concentration in the *Artemia* diet. Algamac-ARA provides a phospholipid source of ARA, originating from spray-dried *Mortierella alpine* fungi. In the last few decades, PUFA and HUFA from the n-3 series have been considered more important than those of the n-6 series for marine animals (Sargent *et al*. 1997; Narciso and Morais 2001; Calado *et al*. 2005a). This is also supported by the current study, with an increased number of larvae developing to the next stage when receiving a diet rich in n-3 HUFAs, especially DHA.

Rice pollard is produced from a mixture of rice brans and rice polishings and contains large quantities of oleic and LOA (Balnave 1982). These elements were also reflected in the corresponding *Artemia* profile. Because of the high oleic concentration, MUFAs were the most abundant FA group in the *Artemia* fed rice pollard. Nevertheless, this was not the case for the RP fed larvae, which showed a similar oleic and MUFA content to the

rest larval samples. Accumulation of MUFAs in an organism's tissues is considered an indicator of EFA deficiency (Suprayudi *et al*. 2004); in *L. amboinensis*, MUFA and PUFA larval content was lower than the equivalent dietary amounts, which indicates these FA groups are preferentially catabolized to satisfy metabolic functions of the larvae (Cavalli *et al*. 2001; Rosa *et al*. 2007).

Allegedly, *Artemia* may either contain high LNA and low EPA or moderate levels of both; normally, *A. franciscana* display the former in their profile (Bengtson *et al*. 1991). This was also observed in this study. Levels of LNA were similarly high, and EPA was similarly low, in all the *Artemia* diets. Perhaps the abundance of LNA, as well as LOA, explain the satisfactory performance of the Algamac and/or rice pollard enriched *Artemia*, which improved further with the addition of dietary DHA (in the case of AM-3). Perhaps the EFA requirements of *L. amboinensis* larvae can be partly fulfilled by dietary C_{18} PUFAs, as previously reported for penaeids (Glencross and Smith 2001; Palacios *et al*. 2001). Despite similar levels of SFAs in the diets, AM-3 larvae had accrued more saturates than AM-A or RP larvae, which contributed to a higher total FA content. Higher FAs means greater energy capital available to the larvae to invest in development.

The developmental rate calculated here was a function of time to first appearance of Z4 larvae and the equivalent Z4 number in the samples. There were no differences in timing, thus the rate was determined by larval numbers. In an increasing order, the rate was A-c3 < RP < A-ch3 < NH3 < AM-A < AM-3. Thus, the highest developmental rate was achieved with *Artemia* enriched with Algamac-3000, almost double that of Algamac-ARA enriched *Artemia* and triple that of *Chaetoceros* fed *Artemia*. The key constituent seems to be DHA (n-3 HUFA). It must be noted that the FA profile of the diet did not

only affect developmental rate, but larval stage distribution as well.. For example, in the cultures fed *Chlorella* or *Chaetoceros* there were three developmental stages (Z2, Z3 and Z4) on day 6, whereas larvae were either Z3 or Z4 in the Algamac treatments, confirming that well nourished larval batches exhibit, normally, a more homogeneous stage assemblage (Wickens 1972; Anger 2001).

Factors influencing decapod larval growth include season, temperature, salinity, light intensity and photoperiod, larval size at hatch and larval quality, diet quality and quantity (Fiore and Tlusty 2005). The experiments in the present study were conducted within a short time-period from each other and under constant environmental parameters. The similar broodstock size and uniform maturation diet may explain the similar size of larvae at Z1 (allowing for genetic variability) and, likely, a similar nutritional profile determining larval quality. Larval food quantity and quality were the two parameters varying in the trials. However, neither of these appeared to affect growth of *L. amboinensis* larvae, since, at the same zoeal stage, larvae were of a similar size, regardless of batch of origin and, more importantly, prey type and density. The Z2 starved larvae were similar in size to the Z1 larvae, suggesting growth and development may be conflicting features in *L. amboinensis*, with the latter a priority.

For *L. seticaudata*, feeding larvae with un-enriched *Artemia* metanauplii resulted in PL with a shorter carapace length, compared to those larvae fed *Artemia* nauplii or Algamac 2000 enriched metanauplii (Figueiredo and Narciso 2006). Smaller larval size would be linked to reduced growth between moults and points to an inadequate diet (Anger 2001). On that assumption, all diets tested in the study of *L. amboinensis* were

suitable. However, only the first four larval stages were examined and results may differ with older larval stages and development to PL and juveniles (Racotta *et al*. 2004).

Chapter 6

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A comparison of the fatty acid profiles of adult tissues and, newly hatched, fed and starved *Lysmata amboinensis* larvae

6.1 Introduction

The high-value white-striped cleaner shrimp, *L. amboinensis*, has recently been the focus of intense studies looking into developing suitable mass rearing methods. Studies have addressed larval culture conditions, including stocking density, suitability of live preys, optimum food concentration, feeding behavior and starvation tolerance (Calado *et al*. 2007; Calado *et al*. 2008a, b; Cunha *et al*. 2008). Growing evidence suggests that satisfying specific dietary requirements of early stages may ensure that larval survival and development are not prematurely compromised and could potentially reduce late larval mortality and settlement delay (Simoes *et al*. 2002; Calado 2008).

In the wild, the nutritional requirements of this and other planktonic larval forms are thought to be satisfied by the diversity of natural prey (Jones *et al*. 1997). However, in practice, studying the natural diet of planktonic larvae is difficult, due to dispersion by currents, their transient nature and small physical size. Thus, alternative techniques must be applied to ascertain suitable feeding regimes in aquaculture. In crustacean research,

knowledge of the biochemistry of adults and resulting larvae is a key to understanding metabolic processes and providing cultured animals with essential nutrients for broodstock maturation and larval development (Spaargaren and Haefner 1994; Cavalli *et al*. 2001; Palacios *et al*. 2001; Smith *et al*. 2004; Smith *et al*. 2008).

Given the superiority of wild over cultured larvae in trials testing survival and stress resistance, many studies have suggested using the biochemical composition of eggs or embryos from wild broodstock as a reference for formulating larval diets (Rainuzzo *et al*. 1997; Narciso and Morais 2001; Rosa *et al*. 2003). It would be reasonable to assume that such a diet (i.e. with a composition equivalent to the yolk reserves) would indeed approximate the nutritive needs of the cultured larvae, once exogenous feeding commences. Furthermore, changes in the profile of successive larval stages, reared under constant hatchery conditions, may be indicative of energy substrates used during development. This information would assist in devising stage-specific larval feeds through inclusion of corresponding nutritional elements (Roustaian *et al*. 1999).

Lipid deposition during maturation is vital to successful reproduction; lipids act as energy reserves (triacylglycerols), basic constituents of cellular membranes (phospholipids) and hormone substrates (sterols) (Harrison 1990; Anger 2001). In crustaceans, the primary storage and processing organ for lipids is the hepatopancreas (Harrison 1997). During maturation, nutrients, including lipids, are transported to the ovaries from the hepatopancreas to support oocyte development and egg yolk deposition (Harrison 1997; Vazquez Boucard *et al*. 2004). A dramatic increase in the concentration of ovarian lipids is reported for crustaceans during vitellogenesis, often comprising up to

40% of the total ovarian dry mass by the completion of maturation (Harrison 1990; Lee and Walker 1995; Ravid *et al*. 1999).

The HUFAs EPA and DHA have been identified for their essential role in female maturation, embryo development and larval survival (Bray and Lawrence 1992; Alava *et al*. 1993; Cahu *et al*. 1995). The quality, quantity and rate of preservation of FAs are strong indicators of the lipid requirements of crustaceans (D'Souza and Loneragan 1999; Smith *et al*. 2003; Rosa *et al*. 2007). Information on EFA requirements can be obtained by analyzing tissues of wild caught animals, or animals subjected to different feeding regimes or nutritional stress (Reigh and Stickney 1989; Tidwell *et al*.1998; Ritar *et al*.2003).

The study was conducted to indicate the qualitative scope of FAs present in *L. amboinensis*. To achieve this, the FA profile of tail muscle, hepatopancreas and ovaries of SHs, and that of newly hatched larvae, were examined. A comparison between the latter and broodstock tissues would allow an assessment of maternal contribution to larval nutrient reserves. Changes to the FA profile of Z1 larvae during feeding or starvation may suggest dietary contribution to larval nutrient reserves and denote the relative importance of certain FAs through preferential conservation, respectively. These results may aid in ultimately defining the lipid requirements in an artificial diet for the species.

6.2 Materials and Methods

Broodstock maintenance

The experiment was conducted during July and August, 2009, at AIMS. A total of twelve pairs of *L. amboinensis* were used. Environmental parameters, set up of broodstock tanks and water pre-treatment were as described in Chapter 2, section 2.2. Water temperature and salinity were stable throughout the experimental period at 27.3 (± 0.10) ºC and 35 (± 0.02) g L^{-1}, respectively. Broodstock were fed Kuruma pellet 3 days per week, and squid or mussel on alternating days during the remainder of the week. Food quantity was the equivalent dw of 3.5% shrimp wet wt. At the completion of the larval component of the experiment, three intermoult broodstock were removed from the tank, at approximately 3-5 days prior to brood hatching. These shrimp were cooled down in a -20 ºC freezer for 30 min, and the TL and wet wt were recorded prior to dissection. Tail muscle, hepatopancreas and ovaries were removed, weighed individually, and stored at -80 ºC until FA analysis.

Larviculture

Actively swimming larvae were removed from the broodstock tanks immediately post-hatch by siphoning into a screened vessel. A total of nine batches were collected during this study, each batch consisted of 1000 (±159) larvae. Three batches were immediately processed (rinsed with 0.5M Ammonium Bicarbonate, wet wt recorded) and stored (-80

ºC) for FA analysis. Three batches were fed rotifers until Z2, and the remaining three batches were starved until Z2. For the “fed” and “starved” treatments, only one batch of larvae was reared at a time. For each treatment, the larvae were equally divided in 4 x 35 L U-shaped upwellers (i.e. 250 larvae in each) to reduce con-specifics competition and cannibalism, respectively. Environmental parameters and water treatment were the same as noted for the broodstock holding tanks. For the “fed” treatments, a water flow rate of 0.2 L min^{-1} and a 75 µm outlet screen were used overnight to keep the rotifers in suspension. During the day the screen was changed to 150 µm and the flow rate adjusted to 0.5 L min^{-1} to flush rotifers from the system before introducing fresh feed. Prey density was 54 rotifers ml^{-1}. In the case of the starved larvae, the larger mesh size (150 µm) and higher flow rate (0.5 L min^{-1}) were used at all times. When 1-2% of the fed or starved larvae changed to Z2 (distinguished by stalked eyes) the batch was harvested and stored, as per the procedure for newly hatched larvae.

Rotifer culture

Rotifer (*B. rotundiformis*) production was carried out as described previously, Chapter 5, section 5.2. At the end of the experiment a rotifer sample was also analyzed for FAs as a dietary reference.

Feed analysis

For lipid analysis see Chapter 4, section 4.2, for FA analysis the protocol at Chapter 3, section 3.2.

Statistical analysis

The package SPSS v17.0 (SPSS Inc., Chicago) was used for statistical analysis. The level of significance was set at $P \leq 0.05$. Lipid and FA-group abundance for the larvae was compared by one-way between-groups ANOVA, with Tukey-Kramer HSD or Student-Newman-Keuls post-hoc test. Whereas the adult data were analyzed via one-way repeated measures ANOVA, and a Paired t-test (after Bonferroni correction) post-hoc test. The qualitative profile of adult tissues and larvae was compared by non-metric multidimensional scaling (MDS). Based on the MDS results, the quantitative FA profile of tail muscle and of newly hatched, starved and fed larvae were compared by MANOVA, followed by one-way between-groups ANOVA (post-hoc). MANOVA was also applied to the broodstock tissues quantitative data, but with Paired t-test post-hoc. Pearson correlation was applied to describe the relationship between the qualitative profile of fed larvae and their diet, rotifers. To meet assumptions of normality and homogeneity, the data were arcsine or log transformed as necessary.

6.3 Results

Broodstock used for the production of larvae meausred 53.4 (± 2.0) mm TL. This was not significantly different from broodstock analyzed for FA content at 55.5 (± 1.3) mm (P = 0.14). Survival of starved and fed larvae prior to collection for FA analysis was > 90%.

In the adults, lipids accounted for over 40% of the hepatopancreas dw and nearly 30% of the ovary dw (Fig. 6.1). The tail muscle had the lowest, amongst broodstock tissues, lipid content at 6.4% (F (1.00, 2.00) = 250.9, P = 0.004). Of the larval samples, the starved larvae contained the lowest total lipids at 10.5%, whereas the newly hatched larvae had the highest lipid content at 17.2% of dw (Fig. 6.1). Lipid content of the fed larvae was similar to that in their diet. There were no significant differences (P = 0.22) among the larval lipid contents.

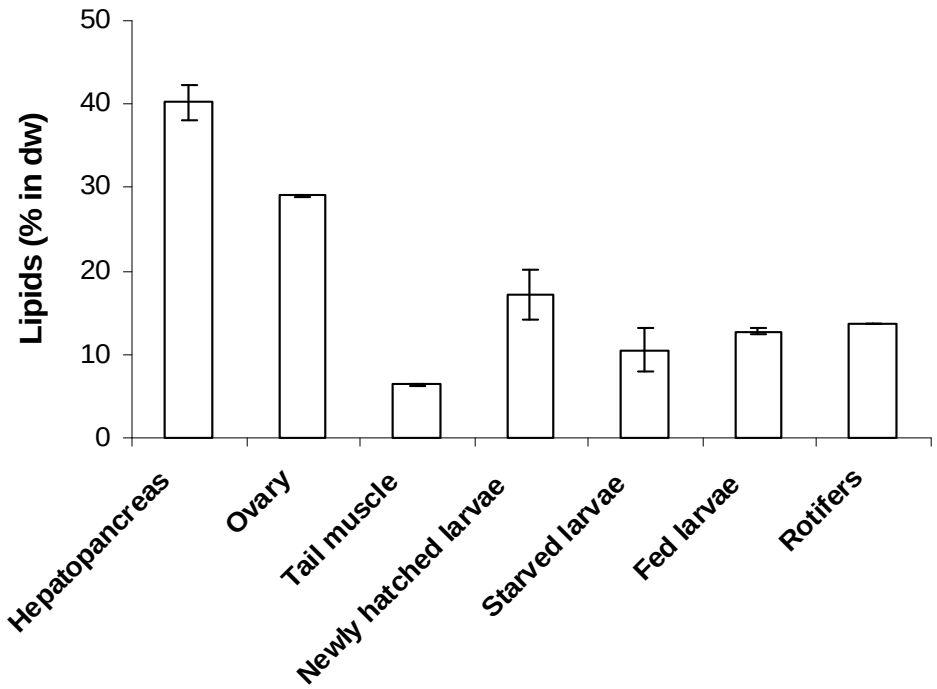

FIGURE 6.1 Percent lipid content of broodstock tail muscle, hepatopancreas and ovary, and of newly hatched, starved and fed larvae, and their food source rotifers (means ± SEM, n = 3 reps per treatment, single rotifer sample).

Qualitatively, the SFAs and HUFAs were the most abundant groups in broodstock tissues and larvae (Table 6.1 and Fig. 6.2). HUFAs were dominant in the tail at 37% ($F_{(3, 8)} = 765.1$, $P < 0.001$), SFAs were the dominant FAs in the hepatopancreas at 35% ($F_{(3, 8)} = 308.2$, $P < 0.001$), whereas the ovaries had equal percentages of HUFAs and SFAs. The starved larvae had the highest HUFA content amongst larvae at 40% ($F_{(2, 6)} = 6.19$, $P = 0.04$), whereas the highest PUFA was recorded for the fed larvae at 13.5% ($F_{(2, 6)} = 17.0$, $P = 0.003$), as well as their diet, rotifers (Table 6.1).

TABLE 6.1 Qualitative FA composition (% in total FA) of broodstock hepatopancreas, ovary and tail muscle, and newly hatched, starved and fed larvae (means ± SEM, n = 3 reps per treatment), and their rotifer diet. TR indicates < 0.05%.

FA	Broodstock			Larvae			Feed
	Hepatopancreas	**Ovary**	**Tail**	**Newly hatched**	**Starved**	**Fed**	**Rotifers**
14:0 (Myristic)	3.9 ± 0.1	2.2 ± 0.0	1.8 ± 0.1	1.9 ± 0.5	1.3 ± 0.0	1.2 ± 0.0	0.8
15:0	0.8 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.0	0.5
16:0 (Palmitic)	20.5 ± 0.4	20.3 ± 0.1	18.8 ± 0.4	22.1 ± 0.9	19.8 ± 0.7	17.2 ± 0.3	17.8
16:1n-7 (Palmitoleic)	4.4 ± 0.1	4.3 ± 0.1	2.9 ± 0.1	2.2 ± 0.1	1.2 ± 0.3	1.5 ± 0.2	0.8
17:0	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	0.4
18:0 (Stearic)	7.8 ± 0.1	9.5 ± 0.2	10.8 ± 0.1	11.3 ± 0.3	12.9 ± 0.4	10.5 ± 0.1	4.0
18:1n-9 (Oleic)	10.0 ± 0.4	14.5 ± 2.5	16.3 ± 0.4	14.0 ± 0.4	13.0 ± 0.4	10.5 ± 0.7	4.9
18:1n-7 (Vaccenic)	8.6 ± 0.1	5.2 ± 0.1	4.3 ± 0.1	5.5 ± 0.2	5.1 ± 0.4	4.8 ± 0.3	1.0
18:2n-6 (LOA)	3.3 ± 0.1	3.5 ± 0.4	3.3 ± 0.1	3.1 ± 0.2	3.7 ± 0.8	9.7 ± 0.9	24.6
18:3n-6	0.3 ± 0.0	0.2	0.2	0.1	TR	0.2 ± 0.0	TR
18:3n-3 (LNA)	0.7 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.4	1.5 ± 0.2	5.8
18:4n-3	0.8 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	TR	TR	TR	0.1
20:0	0.6 ± 0.0	0.3	0.2 ± 0.0	TR	TR	TR	TR
20:1n-11	1.3 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.4	TR	0.3 ± 0.0	1.0
20:1n-9	3.0 ± 0.0	1.5 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.4	0.9 ± 0.1	2.5
20:1n-7	0.9 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2	TR	TR	0.1
20:2n-6	1.0 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.7	0.7 ± 0.2	1.6 ± 0.1	3.4
20:3n-3	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2	TR	0.5 ± 0.1	1.4
20:4n-6 (ARA)	2.1 ± 0.3	2.0 ± 0.4	2.8 ± 0.3	3.4 ± 0.5	3.8 ± 0.1	3.0 ± 0.2	0.9
20:5n-3 (EPA)	10.9 ± 0.4	12.0 ± 0.8	17.6 ± 0.4	16.2 ± 0.3	20.1 ± 0.2	15.5 ± 0.3	6.9
22:0	0.3 ± 0.0	0.1	0.2 ± 0.0	0.2	TR	0.2 ± 0.0	0.2
22:1n-9	0.5 ± 0.0	0.3 ± 0.0	TR	0.1	TR	0.2 ± 0.0	1.1
22:4n-6	0.2 ± 0.0	TR	TR	TR	TR	0.2	0.2
22:5n-6	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2	TR	0.2 ± 0.0	0.3
22:5n-3	1.2 ± 0.1	0.8 ± 0.0	0.5 ± 0.1	1.3 ± 0.7	0.7 ± 0.0	2.2 ± 0.0	5.4
22:6n-3 (DHA)	14.9 ± 0.4	18.5 ± 1.5	16.0 ± 0.4	15.5 ± 0.1	15.8 ± 0.3	15.6 ± 0.1	15.3
24:0	TR	TR	0.3 ± 0.0	TR	TR	TR	TR
24:1n-9	0.5 ± 0.0	0.2	0.2 ± 0.0	TR	TR	0.3 ± 0.0	0.6
SFA	35.2 ± 0.9	34.0 ± 0.3	33.6 ± 0.6	37.5 ± 1.3	36.2 ± 1.4	31.4 ± 0.1	23.7
MUFA	29.2 ± 1.0	26.8 ± 2.7	24.7 ± 0.5	22.6 ± 0.6	19.4 ± 0.6	18.5 ± 1.4	12.0
PUFA	5.4 ± 0.2	5.3 ± 0.5	4.6 ± 0.1	3.8 ± 0.4	4.3 ± 1.2	13.5 ± 1.3	35.2
HUFA	30.2 ± 1.0	34.1 ± 2.9	37.3 ± 0.9	36.0 ± 1.3	40.1 ± 0.7	36.6 ± 0.3	29.1

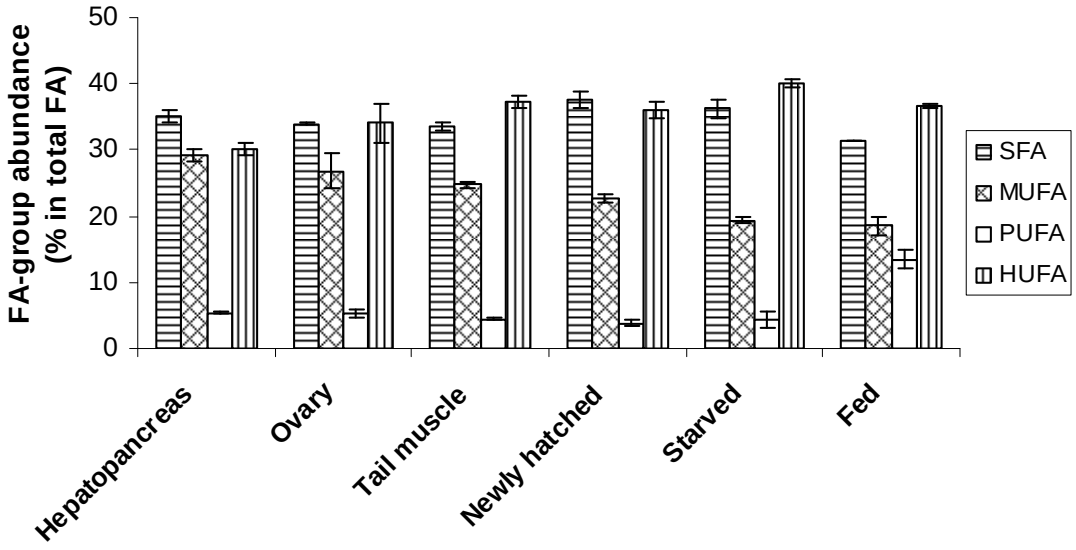

FIGURE 6.2 Percent FA-group abundance (in total FAs) in broodstock tail muscle, hepatopancreas and ovary, and in newly hatched, starved and fed larvae (Sum of means ± SEM, n = 3 reps per treatment).

The major FAs noted in the broodstock also dominated the larval profiles with 16:0 (palmitic), 20:5n-3 (EPA) and 22:6n-3 (DHA) accounting for approximately 50% of the FAs present (Table 6.1). Specifically, in the tail muscle, hepatopancreas and ovaries of the broodstock, palmitic (approx. 19-20% of total FAs) and 18:0 (stearic, 8-11% of total FAs) were the most abundant SFAs. The equivalent amounts in the larvae were 17-22% for palmitic and 10.5-13% for stearic. The dominant MUFAs were 18:1n-9 (oleic), highest at 16% in the tail muscle, and 18:1n-7 (vaccenic), highest at 9% in the hepatopancreas, while the dominant PUFA was 18:2n-6 (LOA), highest in the fed larvae and the dominant FA in rotifers (25%). The HUFA profile was dominated by EPA and

DHA. These two FAs constituted approximately one third of the total FAs in all samples. The qualitative FA profile of newly hatched and starved larvae was similar to the profile of the tail muscle (Fig. 6.3, stress 0.02). For example, DHA was 15.5% in the newly hatched larvae, 15.8 % in the starved larvae, and 16.0 % in the tail muscle (Table 6.1).

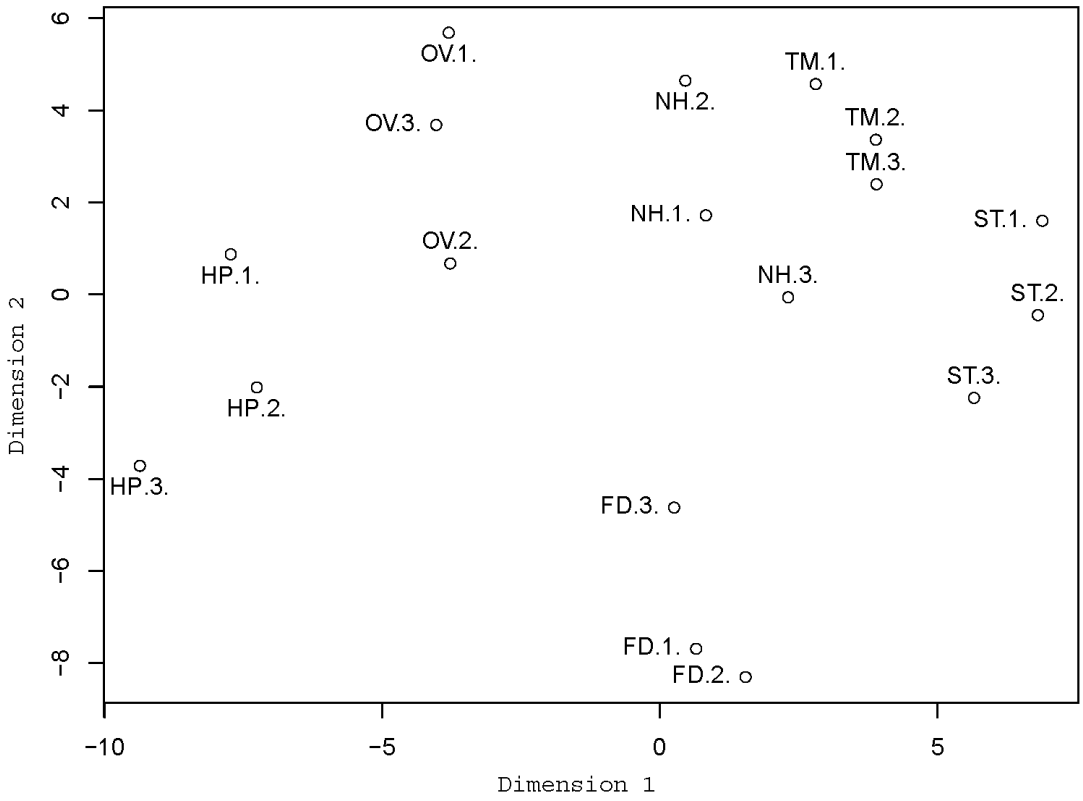

FIGURE 6.3 MDS graph of the qualitative FA profile of broodstock tail muscle, hepatopancreas and ovary, and of newly hatched, starved and fed larvae. Abbreviations: FD, fed larvae; HP, hepatopancreas; NH, newly hatched larvae; OV, ovary; ST, starved larvae; TM, tail muscle.

DHA accounted for 16% of total FAs in the tail muscle (Table 6.1), which corresponded to 4.2 mg g^{-1} dw (Table 6.2). That was significantly less (P = 0.009) than the amount of DHA in the hepatopancreas or ovary, with 33.8 and 30.5 mg g^{-1} dw, respectively (Table 6.2). Due to the low lipid content of the tail muscle, the pattern was repeated across its entire FA profile, with a total FA content of 25.9 (± 0.8) mg g^{-1} dw (P = 0.004), as opposed to 225.7 (± 34.8) mg g^{-1} dw in the hepatopancreas and 164.1 (± 2.8) mg g^{-1} dw in the ovary. The newly hatched larvae had a higher concentration of palmitic (P = 0.001), 16:1n-7 (palmitoleic) (P < 0.001), stearic (P = 0.002), oleic (P = 0.001) and 20:4n-6 (ARA) (P = 0.002), compared to fed and starved larvae. They also had higher vaccenic (P = 0.002), EPA (P = 0.012) and DHA (P = 0.002), when compared to the tail muscle. Both LOA (P < 0.001) and LNA (P = 0.041) concentrations in the fed larvae were 3 times greater than in newly hatched and 5 times greater than in starved larvae. The starved larvae contained significantly less total FAs (20.5 ± 2.1 mg g^{-1} dw) (P = 0.001) compared to newly hatched and fed larvae (39.7 ± 2.7 and 34.1 ± 2.4 mg g^{-1} dw, respectively) (Table 6.2).

TABLE 6.2 Major quantitative FAs (mg g^{-1} dw) in adult hepatopancreas, ovary and tail muscle, and of newly hatched, starved and fed larvae (means ± SEM, n = 3 reps per treatment) and the larval diet, rotifers.

FA	Broodstock			Larvae			Feed
	Hepatopancreas	Ovary	Tail	Newly hatched	Starved	Fed	Rotifers
14:0 (Myristic)	8.8 ± 1.3	3.6 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	0.3 ± 0.0	0.4 ± 0.0	0.5
16:0 (Palmitic)	46.1 ± 6.6	33.3 ± 0.7	4.9 ± 0.2	8.8 ± 0.7	4.1 ± 0.5	5.9 ± 0.5	10.0
16:1n-7 (Palmitoleic)	9.8 ± 1.4	7.0 ± 0.3	0.7 ± 0.1	0.9 ± 0.0	0.2 ± 0.1	0.5 ± 0.0	0.4
18:0 (Stearic)	17.5 ± 2.6	15.6 ± 0.5	2.8 ± 0.1	4.5 ± 0.2	2.7 ± 0.3	3.6 ± 0.2	2.2
18:1n-9 (Oleic)	22.9 ± 6.1	23.7 ± 3.7	4.2 ± 0.2	5.6 ± 0.5	2.7 ± 0.3	3.5 ± 0.1	2.8
18:1n-7 (Vaccenic)	19.4 ± 3.2	8.6 ± 0.1	1.1 ± 0.0	2.2 ± 0.2	1.1 ± 0.2	1.6 ± 0.0	0.5
18:2n-6 (LOA)	7.4 ± 1.3	5.7 ± 0.5	0.9 ± 0.0	1.2 ± 0.1	0.7 ± 0.1	3.4 ± 0.5	13.8
18:3n-3 (LNA)	1.6 ± 0.2	1.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1	0.5 ± 0.1	3.2
20:4n-6 (ARA)	4.6 ± 0.5	3.3 ± 0.6	0.7 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	1.0 ± 0.0	0.5
20:5n-3 (EPA)	24.4 ± 3.3	19.7 ± 1.7	4.6 ± 0.2	6.4 ± 0.4	4.1 ± 0.4	5.3 ± 0.5	3.9
22:6n-3 (DHA)	33.8 ± 5.5	30.5 ± 3.1	4.2 ± 0.2	6.1 ± 0.5	3.2 ± 0.3	5.3 ± 0.4	8.6
EPA/ARA	5.2 ± 0.2	6.1 ± 0.7	6.4 ± 0.5	4.9 ± 0.5	5.2 ± 0.1	5.1 ± 0.4	7.8
DHA/EPA	1.4 ± 0.1	1.5 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	2.2
n-3/n-6	4.2 ± 0.2	5.1 ± 0.5	5.3 ± 0.2	4.8 ± 0.1	4.7 ± 0.4	2.4 ± 0.1	1.2
Total FA	225.7 ± 34.8	164.1 ± 2.8	25.9 ± 0.8	39.7 ± 2.7	20.5 ± 2.1	34.1 ± 2.4	56.2

The n-3/n-6 ratio was similar for tail muscle, hepatopancreas, ovary, newly hatched and starved larvae (between 4.0 to 5.0) (Table 6.2). The fed larvae differed, with a low of 2.4 ($P < 0.001$), and a dietary rotifer ratio of 1.2. The EPA/ARA ratio was high in adults (> 5.2) and larvae (> 4.9). The DHA/EPA ratio was approximately 1.5 for hepatopancreas and ovaries, but significantly lower in the tail muscle (1.0, $P < 0.001$) and similar to the larval samples (0.8-1).

The major FA constituents of the rotifers were LOA at 25% of total FAs, palmitic at 18%, DHA at 15%, EPA at 7% and LNA at 6%. The fed larvae had a higher content of stearic, oleic, vaccenic and EPA than the rotifers, and a lower LOA and LNA content.

However, there was a significantly positive relationship between the qualitative FA profile of the larvae and their diet (r = 0.71, $P < 0.001$) (Fig. 6.4).

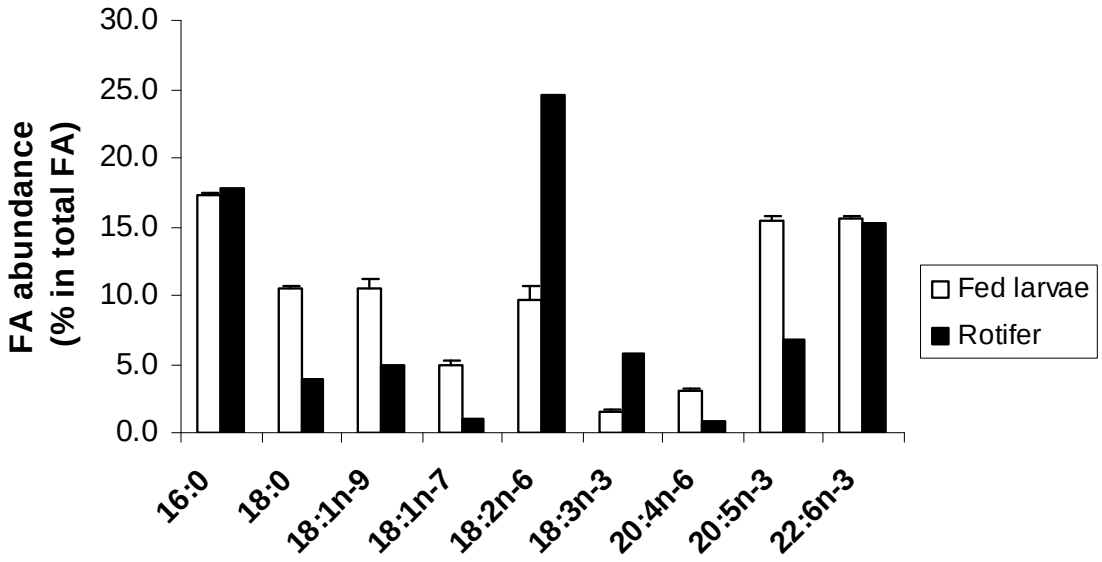

FIGURE 6.4 Major FA abundance (% in total FAs) in fed larvae and their rotifer diet (means ± SEM, n = 3 reps for larvae, single rotifer sample).

6.4 Discussion

The role of the hepatopancreas as a lipid modifying centre and a storage organ has been noted for other crustaceans, for example *P. monodon*, *Penaeus kerathurus* and *M. rosenbergii* (Millamena and Pascual 1990; Mourente and Rodriguez 1991; Cavalli *et al*. 2001). In *L. amboinensis*, the high hepatopancreatic lipid content was dominated by SFAs (the building blocks for lipid classes) (Guary *et al*. 1974; Mourente 1996), suggesting the hepatopancreas as a processing and reservoir organ. Lipids also comprised a high

percentage of the cleaner shrimp's ovarian dw, in agreement with reports on *Penaeus aztecus* and *Jasus edwardsii* (Castille and Lawrence 1989; Smith *et al*. 2004). In the ovaries, both SFAs and HUFAs were of equal prominence; SFAs are required for vitellogenesis (Mourente 1996) and HUFA presence has been positively associated with fecundity, hatchability and offspring quality in penaeids (Xu *et al*. 1994; Wouters *et al*. 2001; Racotta *et al*. 2003). The biology of *Lysmata* SHs involves continuous reproduction with a short maturation period of approximately 2 weeks, and individual broodstock acting as both male (fertilization of other individuals) and female (ovarian development and brooding) in the same reproductive cycle (Fiedler 1998). This validates the need for continual high FA content in both the hepatopancreas and ovaries, which in this study was satisfied largely by the constant provision of a high quality broodstock diet. Muscle lipid content was low, in accord with the level recorded in other carideans (e.g. *M. rosenbergii* in Cavalli *et al*. 2001), as the tail is a functional, rather than storage organ, with HUFAs dominant.

The lower lipid content of the starved larvae suggests that lipids are utilized as the major metabolic source of energy (Rosa *et al*. 2003; Smith *et al*. 2003) to support survival and lecithotrophic development to Z2 after 3 days starvation. Newly hatched larvae in this study were high in total lipids, comprising > 17% of dw. This is similar to the amount present in other caridean shrimp, such as *Palaemon serratus* at 21.5% of dw (Narciso and Morais 2001), and unlike that in temperate larval spiny lobsters, where lipid only comprises 7-12% of dw (Ritar *et al*. 2003). Large energy reserves (i.e. higher lipid and FA content) is a typical reproductive trait of carideans (Anger *et al*. 2004), and may

be a response to the variable environmental conditions planktotrophic larvae are exposed to (Rosa *et al*. 2007).

The FA profile of the tail muscle mirrored that of newly hatched larvae and, therefore, may be a good indicator of the FAs required during this stage of development in *L. amboinensis*. The prominent FA components in newly hatched *L. amboinensis* were palmitic, stearic, oleic, vaccenic, EPA, DHA; this suite of FAs is also common in other carideans, *P. serratus* (Narciso and Morais 2001) and *M. rosenbergii* (Roustaian *et al*. 1999), penaeids, *Penaeus japonicus*, *P. semisulcatus* and *P. monodon* (D'Souza and Loneragan 1999), the Norway lobster *Nephrops norvegicus* (Rosa *et al*. 2003), the spiny lobster *J. edwardsii* (Smith *et al*. 2003) and portunid crabs (Rosa *et al*. 2007).

By examining the profile of the starved larvae, the FA importance for cell structural maintenance and metabolic functioning can be assessed. Preferential retention of certain FAs during nutritional stress is an indicator of what is essential for the development of an organism (Tidwell *et al*. 1998; Ritar *et al*. 2003). Palmitic, stearic, oleic, EPA and DHA were most prominent in the starved animals, indicating these were catabolized slower and to a lesser extent than other FAs during starvation. It has been argued, however, that accumulation of short-chain saturates like palmitic and stearic may be due to the ease of their synthesis and storage, rather than an absolute requirement for them (Olsen 1998; Smith *et al*. 2003). HUFAs were the most abundant group in the starved larvae, with EPA and DHA being proportionally high, confirming a high nutritional value in crustaceans (Rees *et al*. 1994; Brett and Muller-Navarra 1997). EPA serves as a precursor to eicosanoids, crucial for a wide range of physiological processes in invertebrates (Brett

and Muller-Navarra 1997). DHA is found in high quantities in brain and retina in fish, suggesting a specific role in neural membranes (Sargent 1995).

The same pattern of high EPA and DHA was observed in the fed larvae. Rotifers appear to accumulate DHA effectively, compared to other larval preys, such as *Artemia* (Narciso and Morais 2001), but less so for EPA. Despite a lower EPA concentration in the food source, the fed larvae still maintained the same DHA/EPA ratio displayed in newly hatched larvae and tail muscle. This is likely to have been through preferential sequestering of EPA from the diet, which still contained significant quantitative amounts of EPA, and, perhaps, some retroconversion of DHA to EPA (Evjemo *et al*. 1997). It is unlikely that any significant amounts of EPA would have originated through desaturation and elongation of LNA, demonstrated to be poor in crustaceans (Jones *et al*. 1979; D'Abramo and Sheen 1993). This may need to be investigated with a radioactive tracer study, in the light of reports of the greater bioconversion ability of *L. seticaudata* PL (Calado *et al*. 2005a). The ARA content in the fed larvae was greater than in the rotifers, suggesting selective sequestering and preferential storage of ARA by *L. amboinensis* larvae. Overall, the low ARA and high EPA and DHA concentrations in the larval samples, and the high n-3/n-6 ratio in the starved larvae, suggest an increased requirement for n-3 HUFA and a limited requirement for n-6 HUFA in *L. amboinensis*, in agreement with findings for other crustaceans (Xu *et al*. 1994; Wickens *et al*. 1995).

It has been argued that for crustaceans, the DHA/EPA ratio is more critical for lipid and FA metabolism, than the absolute concentration of either FA (Rees *et al*. 1994; Glencross *et al*. 2002). The DHA/EPA ratio in the fed larvae was maintained at 1.0, despite the variable amounts found in their dietary source, and similar to the newly

hatched larvae. When Calado *et al*. (2008b) assessed the FA profile of newly hatched *L. amboinensis* larvae from wild broodstock, these had a high DHA/EPA ratio (3.7), compared to the newly hatched larvae of the present study, although the general distribution of individual FAs was similar. The high DHA/EPA ratio of the wild larvae did not, however, support moulting to Z2 following starvation (Calado *et al*. 2008b). Whereas, survival and lecithotrophic development of the starved larvae during this study suggest a balanced DHA/EPA ratio, which has also been noted in newly hatched *L. debelius* (Palmtag and Holt 2007), may be advantageous.

PUFAs appeared to contribute minimally to the lipid profile of newly hatched and starved larvae, and only in the fed larvae a significant increase was noted, potentially linked to dietary intake of LOA. A similar observation was made for *J. edwardsii* phyllosoma (Smith *et al*. 2003). Abundance of LOA and LNA was far greater in the rotifers than the amounts recorded in the fed larvae. It is likely that when these FAs are available, they are continuously catabolized to be incorporated in lipid classes (Shenker *et al*. 1993; Mourente 1996). A greater contribution of PUFA to the overall lipid catabolism has been previously reported for embryos of *Palaemon spp*. and *L. seticaudata* (Rosa *et al*. 2007).

MUFAs also appeared to be preferentially catabolized, with high dietary content not demonstrated by increased larval content. Oleic and vaccenic were the only two MUFAs higher in the fed larvae than their diet. Higher values could be the result of bioconversion of stearic to oleic/vaccenic acids (Roustaian *et al*. 1999). It is widely accepted that MUFAs serve energetic purposes (Cavalli *et al*. 2001; Rosa *et al*. 2003), further supported in *L. amboinensis* by the low MUFA fraction in the starved larvae.

Overall, the results suggest that FAs may be involved in broodstock maturation and larval development in *L. amboinensis*. It would thus be of benefit to aquaculturists to make available a wide array of FAs to the shrimp, especially n-3 HUFAs, through the diet. Future investigations should examine the roles of individual EFAs more systematically and throughout the life cycle of the cleaner shrimp.

Chapter 7

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Functional morphology of mouthparts and digestive system during larval development of the cleaner shrimp, *Lysmata amboinensis*

7.1 Introduction

Feeding mechanisms and behavior of crustaceans have been of interest since the 1900s (Williams, 1907; Borradaile, 1917; Yonge, 1924; Orton, 1927). The works of Manton (1964, 1977) described in detail the jaw mechanisms of major classes of arthropods, including crustaceans, and the investigations of Fryer (1963, 1977) helped clarify the operation of feeding appendages. The crustacean feeding structures include the paired maxillipeds, maxillae, maxillules, paragnaths and mandibles, as well as the labrum (Hunt *et al*., 1992). Masticated food enters the alimentary system, which consists of the foregut, the midgut, the midgut gland or hepatopancreas and the hindgut, for further processing and digestion (Anger, 2001).

The link between functional morphology and diet has been well documented, with many feeding studies connecting the anatomy of the mouthparts and digestive system with behavioral observations (Roberts, 1968; Schaefer 1970; Greenwood, 1972; Caine

1975; Barker and Gibson, 1978; Schembri, 1982; Alexander and Hindley, 1985; Paffenhofer and Lewis, 1989; Lavalli and Factor, 1992; Mcmillan *et al*., 1997; Crain, 1999; Nelson *et al*., 2002b; Drumm, 2004; Richter and Kornicker, 2006). The feeding strategy for a particular species can be assessed through the study of mouthparts and gut. For example, increased robustness of the mandibles and complexity of the gastric mill can be correlated to a carnivorous diet in crustaceans (Kunze and Anderson, 1979). The paragnaths are more complex in microphagous species than in carnivorous macrophages (Hunt *et al*., 1992).

Information generated through studies of the functional morphology of mouthparts and gut could assist in identifying appropriate prey characteristics, or, in developing effective formulated diets that better meet the nutritional requirements of each life stage of important aquaculture organisms. This approach has been used with considerable success for various crustaceans, for example slipper lobsters, spiny lobsters, blue crabs and prawns (Alexander and Hindley, 1985; Johnston and Alexander, 1999; McConaugha, 2002; Cox *et al*., 2008).

The larval cycle of *L. amboinensis* is defined by 14 zoeal stages, prior to metamorphosis to the PL (see Appendix A for details on larval staging). Z1 has sessile eyes and three pairs of thoracopodal appendages. In Z2, the eyes are stalked, in subsequent instars appendages are added in an anterior to posterior sequence, with Z14 identifiable by the *appendix interna* on pleopods 2-5. Presently, larviculture of *L. amboinensis* is hindered by an extended larval phase, which can reach up to 150 days and is punctuated by periods of high mortality (Fletcher *et al*., 1995).

Little is known about the natural diet of *L. amboinensis* larvae, their feeding behavior and digestive capabilities. This contributes to an inadequate feeding regime under culture conditions and impedes hatchery production. From direct laboratory observations it was noted that older larvae would consume a greater variety of feeds, including commercial prawn pellets. Whether these behavioral changes correspond to morphological changes of the mouthparts and digestive system is currently unknown. External and internal feeding mechanisms have not yet been described in this species.

The aim of this study was (i) to describe the mouthpart appendages and digestive system of the larvae to understand the mechanisms involved in feeding and (ii) to highlight morphological changes in these structures during larval development, which may indicate changes in dietary preferences and enable the provision of suitable food items.

7.2 Materials and Methods

The study was conducted from September 1st to November 30th, 2009. The histology and scanning electron microscopy (SEM) were performed at JCU.

Larviculture

The larvae were reared at AIMS, from captive broodstock. Actively swimming newly hatched larvae from two SHs, were transferred to a 50 L U-shaped upweller filled with seawater. Water parameters were maintained at 27 (± 0.41) °C (pH/oxi 340i) and 35 (±

0.25) g L^{-1} salinity (optical salinometer, Bio-marine, Inc.). Ten randomly selected larvae were examined under a Leica $MZ7_5$ microscope every 4 days to determine the developmental rate of the batch. When the desired zoeal stages were reached, batches of twenty larvae were removed from the culture vessel, morphologically examined to confirm larval stage and each batch placed in fixative specific for electron microscopy or histology. The only exception was Z14, for which limited larval availability reduced the number to ten. During the culture period larvae were fed with progressively larger *A. franciscana* (at a concentration of 3 *Artemia* ml^{-1}), the diet was further supplemented at the late larval stages with finely chopped mussel gonad (*Mytilus edulis*) and squid flesh (*L. opalescens*) (1 ml of the blend L^{-1} of seawater).

Scanning Electron Microscopy

Larvae destined for SEM were fixed in 3% glutaraldehyde (25% EM grade aqueous solution buffered with filtered seawater) and dissected (Leica MZ16 dissection microscope) to remove the maxillipeds and/or maxillae and/or maxillules. Larvae were dehydrated through a series of graded ethanols to 100% ethanol, sonicated for 10 sec to remove debris, critical point dried in a Pelco CPD2 and sputter-coated with gold in a JEOL JUC-5000 magnetron. Observations were made on a JEOL JSM-5410LV scanning electron microscope operated at 10 Kv. Mouthparts of ten newly hatched shrimp larvae (Z1) and of two last stage larvae (Z14) were examined. To aid in setae identification, the removed maxillae and maxillules were preserved in 70% ethanol and further examined under a Zeiss Axioscope with an attached Axiocam for image capture. The software used

was Axiovision *v4.5*. The terminology of Crain (1999) was followed for the description of the mouthpart setae, with Factor (1978) used as an additional source for setal types not included in Crain (1999).

Histology

The larvae were fixed for 24 hr in Davidson's fixative (ethyl alcohol, 37-39% formalin, glacial acetic acid, DI water), embedded in paraffin and serially sectioned (transversely or longitudinally) at 3-5 µm, using a Leitz manual rotary microtome. Cut sections were stained either with hematoxylin-eosin (H & E) or Alcian blue and Periodic Acid Schiff (P.A.S.) reagent. The digestive tract of five shrimp larvae at each of Z1, Z5 and Z9, and of two larvae at Z14, were examined and photographed under an Olympus DP12 Microscope Digital Camera System. The terminology of Bell and Lightner (1988) was used for the description of the alimentary canal, with the exception of the proventriculus for which Johnston and Ritar (2001) was followed.

7.3 Results

Mouthparts

The mouthparts are listed in sequence, starting with the most distal to the mouth opening and followed by those involved in the oral cavity. Where major structural

differences were recorded between Z1 and Z14, images of the corresponding structures of both larval stages are displayed.

The maxillae of Z1 and Z14 were comprised of the scaphognathite, the endopodite, the bilobed (referred to as distal and proximal) basal endite, and the coxal endite (bilobed in Z1 - distal and proximal coxal endites; unilobed in Z14) (Plate 7.1). In Z1, the scaphognathite had five long plumose setae, the endopodite had nine plumodenticulate setae, the basal endite had four short plumose setae on the distal lobe and four on the proximal lobe, while the coxal endite had four and eight short plumose setae on the distal and proximal lobes, respectively (Plate 7.1). In Z14 larvae, the scaphognathite was larger and completely fringed with in excess of 100 long plumose setae. There were eight plumodenticulate setae on the Z14 endopodite. The distal and proximal basal endites of Z14 each possessed more than twenty short plumose setae and the coxal endite more than thirty short plumose setae.

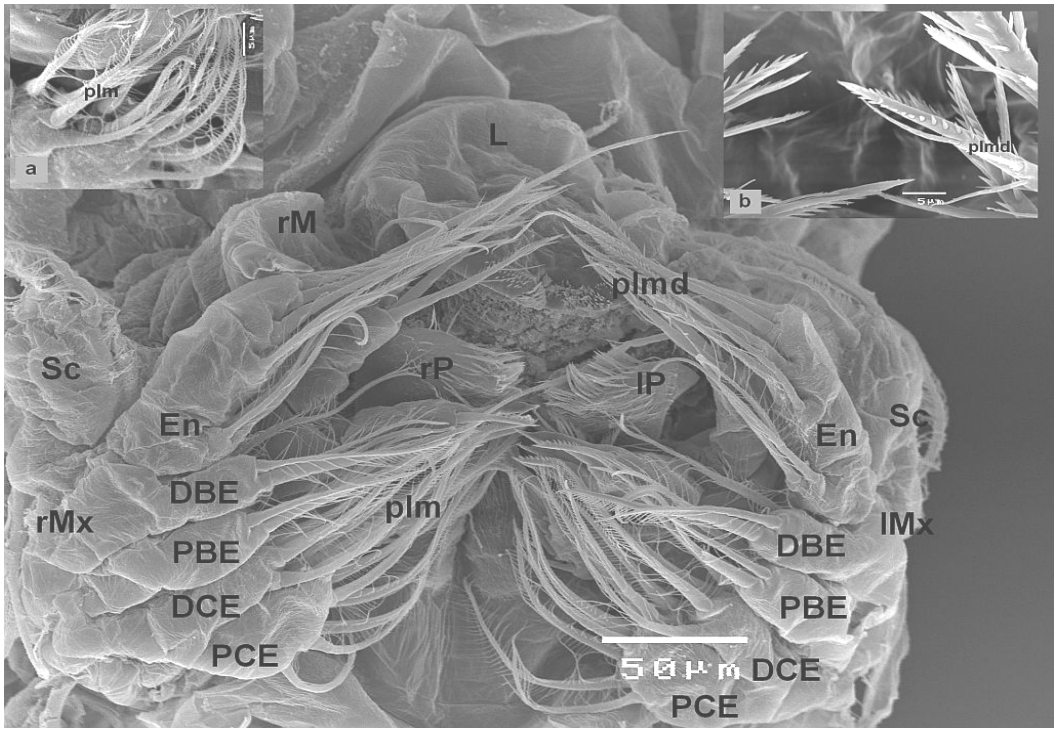


PLATE 7.1 SEM of the maxillae of Z1 *L. amboinensis*. Insets: a) plumose setae, b) plumodenticulate setae. Abbreviations: DBE, distal basal endite; DCE, distal coxal endite; En, endopodite; L, labrum; lMx, left maxillae; lP, left paragnath; plmd, plumodenticulate setae; plm, plumose setae; PBE, proximal basal endite; PCE, proximal coxal endite; rM, right mandible; rMx, right maxillae; rP, right paragnath; Sc, scaphognathite.

The maxillules, in both Z1 and Z14 larvae, were comprised of the endopodite and the basal and coxal endites (Plate 7.2). The endopodite was configured to curve around the mandibles; in Z1 larvae, it had three terminal and two sub-terminal plumodenticulate setae, in Z14, these had developed into three and four respectively. The basal endite extended over most of the paragnath surface; in Z1, it possessed two large cuspidate setae (with setules) flanked by three serrate setae, by Z14 the number of cuspidate and serrate

setae had increased to approximately thirty. The coxal endite curved in an anterior direction towards the paragnath base, with seven pappose setae in Z1 larvae increasing to approximately thirty in Z14. In both larval stages there were microtricha (Wunsch, 1996) on the margins of the coxal endite (Plate 7.2).

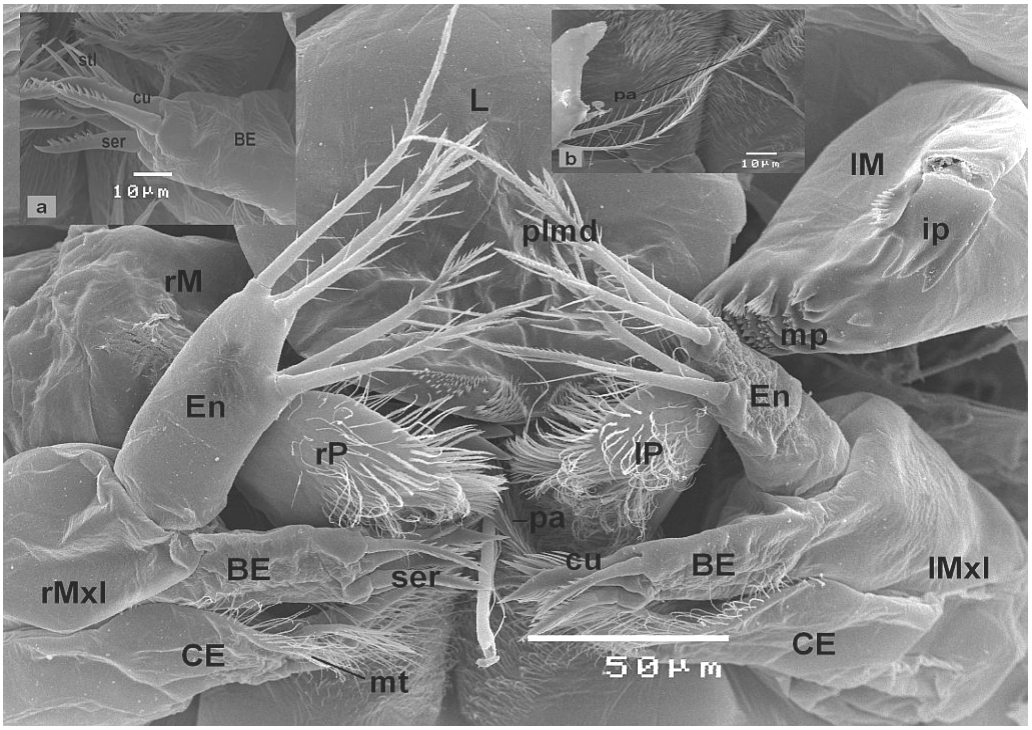
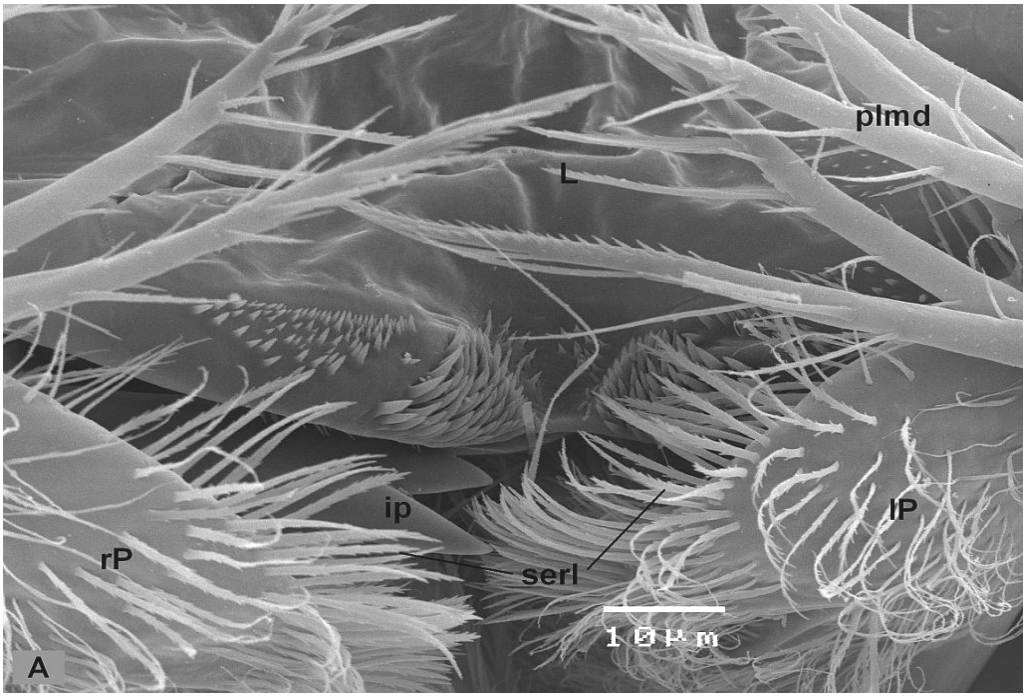


PLATE 7.2 SEM of the maxillules of Z1. Insets: a) serrate and cuspidate setae on the basal endite, b) pappose setae on the coxal endite. Abbreviations: BE, basal endite; CE, coxal endite; cu, cuspidate setae; En, endopodite; ip, incisor process; L, labrum; lM, left mandible; lMxl, left maxillules; lP, left paragnath; mt, microtricha; mp, molar process; pa, pappose setae; plmd, plumodenticulate setae; rM, right mandible; rMxl, right maxillules; rP, right paragnath; ser, serrate setae; stl, setules.

The paragnaths were flattened exoskeletal extensions (Alexander, 1988), located postero-ventrally to the mandibles. In Z1 larvae, the paragnathal lobes were located closer together forming a semi-enclosed chamber (Plate 7.3A), whereas in Z14, they were less curved and further apart, and exposed the incisor process of the mandibles (Plate 7.3B). There were no pores on the paragnaths of Z1 and the serrulate setae were long and thin (Plate 7.3A), compared to the shorter and thicker serrulate setae of Z14 larvae and numerous “ring” pores (term due to the raised lip) present on the surface of their paragnaths (Plate 7.3B).



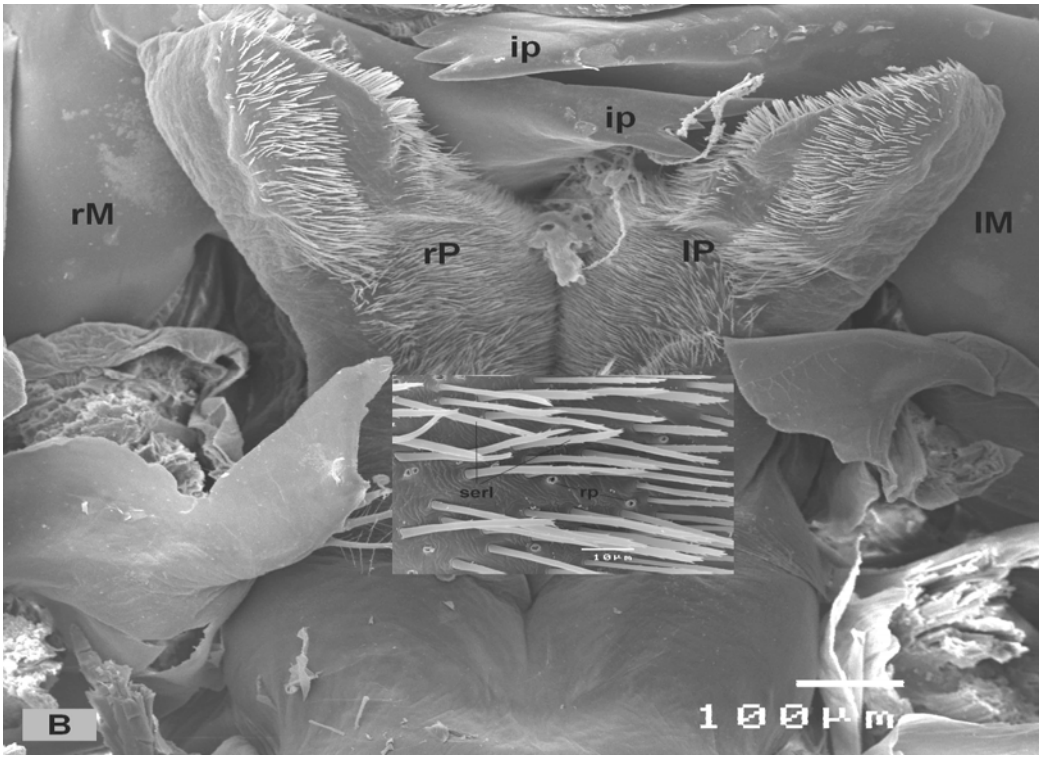
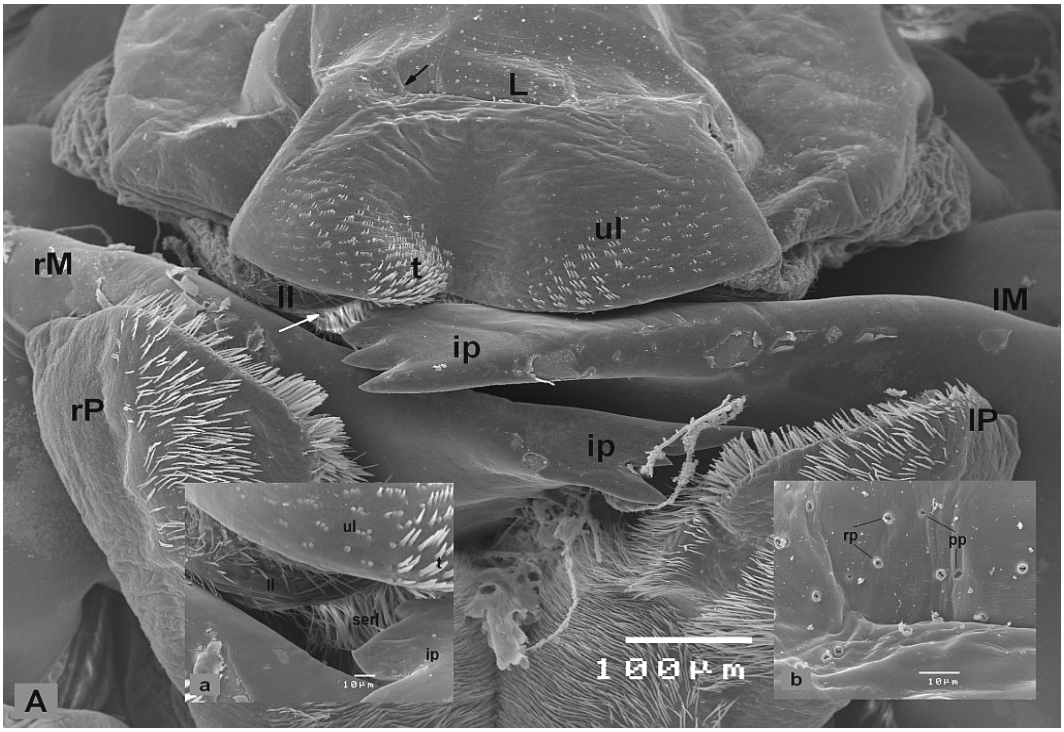


PLATE 7.3 SEM of the paired paragnaths of A. Z1, forming a semi-enclosed chamber. B. Z14, exposing the mandibles. Inset: the pores and setae on the aboral surface of the paragnaths. The arrow corresponds to the inset. Abbreviations: ip, incisor process; L, labrum; lM, left mandible; lP, left paragnath; plmd, plumodenticulate setae; rM, right mandible; rP, right paragnath; rp, ring pore; serl, serrulate setae; t, teeth; ul, upper lip.

The labrum of Z1 and Z14 was a large, muscular, sub-quadrate lobe, located antero-dorsally to the mandibles (Plate 7.4A; also Plate 7.2). In both larval stages, the dentate spines/teeth of the upper lip were organized in rows and directed upwardly (Plate 7.4B), whereas the lower lip of the labrum had numerous long serrulate setae on its ventral face (Plates 7.4A and 7.4C). “Ring” and “plain” (i.e. no raised lip) pores were scattered on the labrum of Z14 (Plate 7.4A) as opposed to only one central “ring” pore on Z1 (Plate 7.4B).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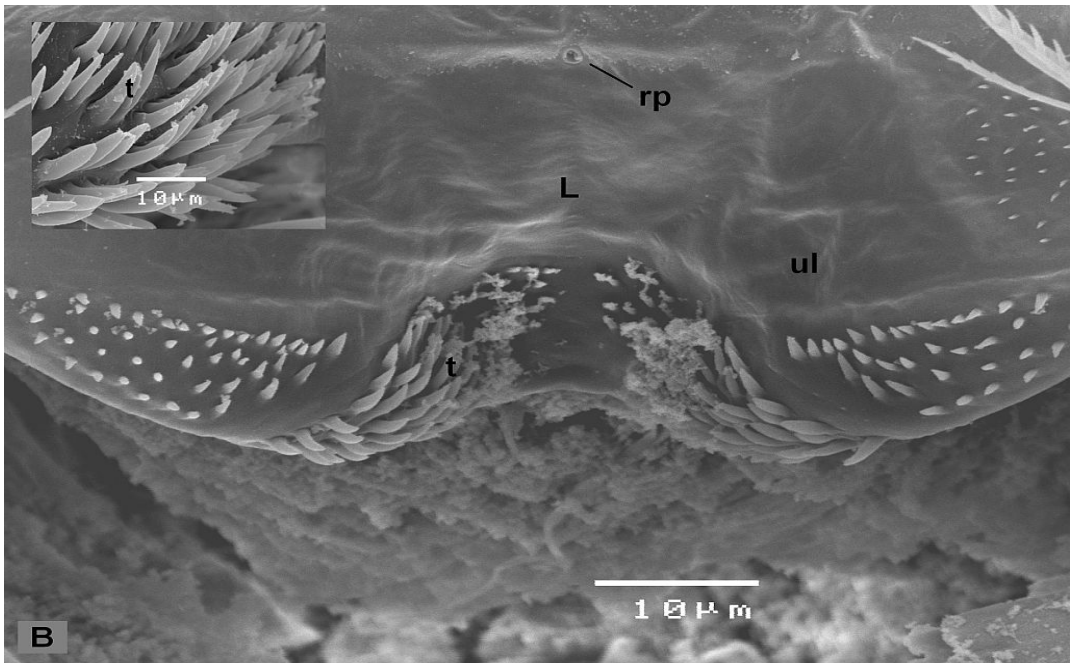
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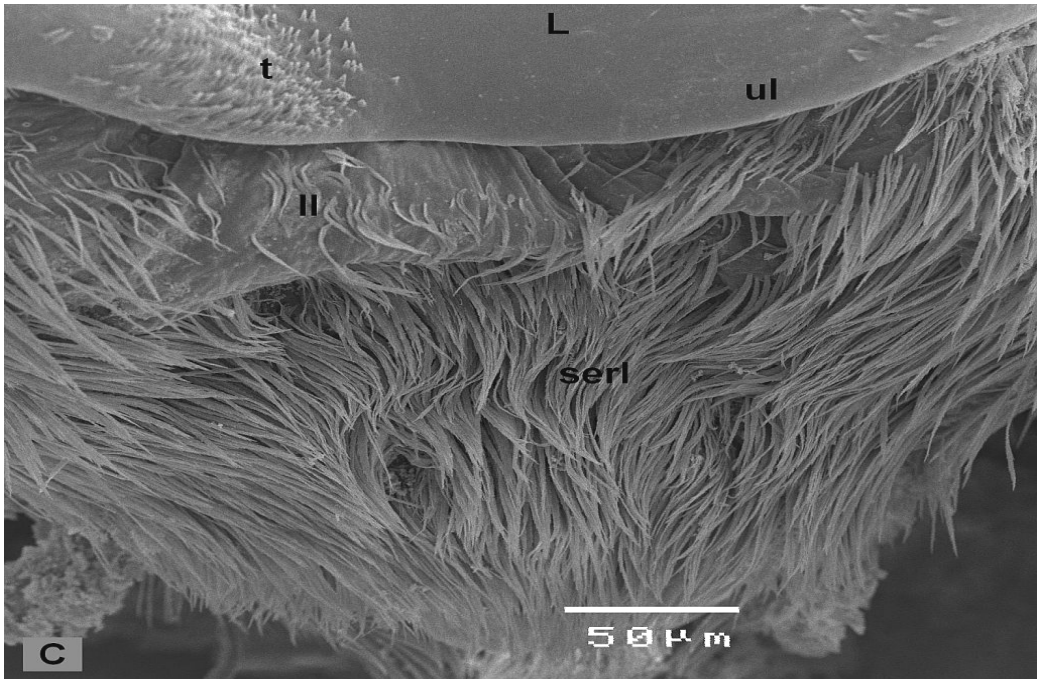
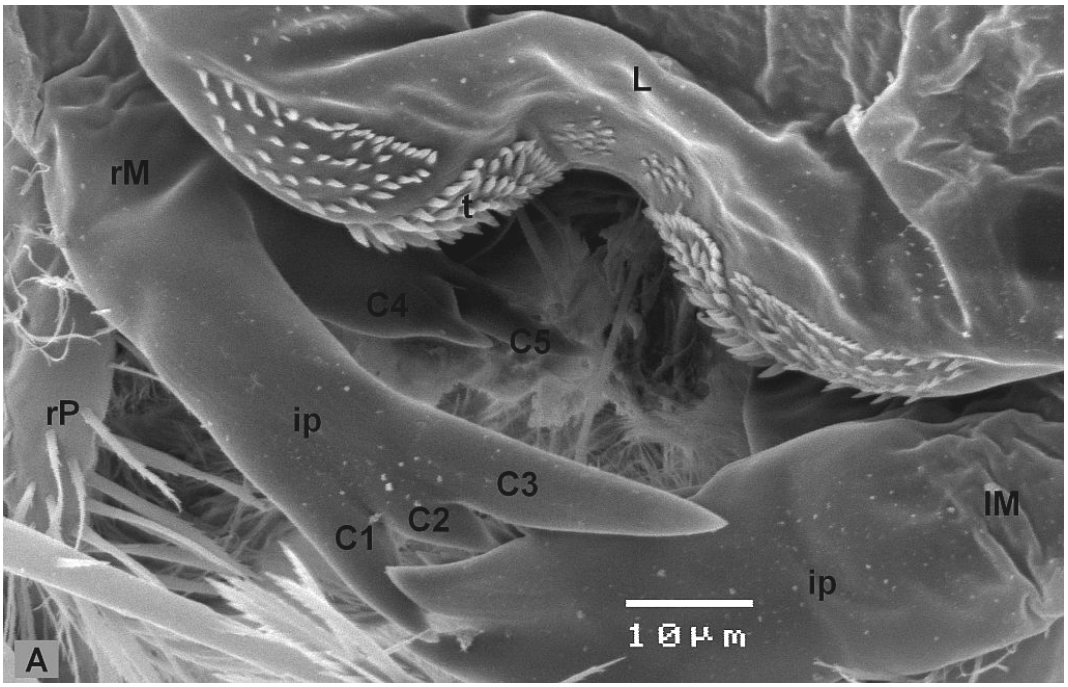


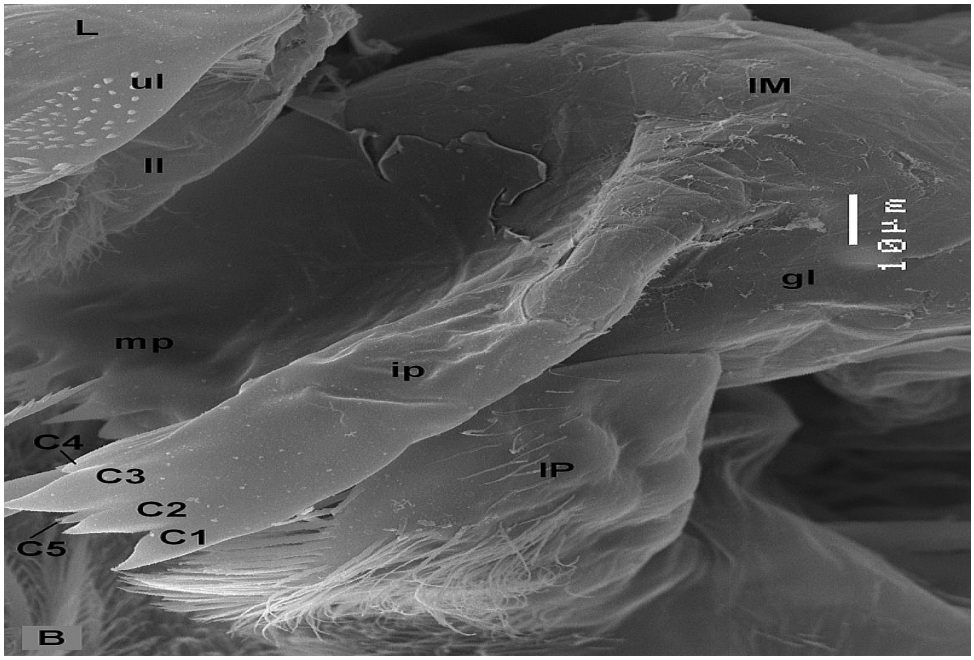
PLATE 7.4 SEM of the A. labrum of Z14, showing the position in the oral chamber. Insets: a) the serrulate setae of the lower lip, b) the two types of pores. The white arrow corresponds to inset a, the black arrow to inset b. B. upper lip of the labrum of Z1. Inset: the teeth. C. lower lip of the labrum of Z14. Abbreviations: ip, incisor process; L, labrum; lM, left mandible; lP, left paragnath; ll, lower lip; pp, plain pore; rM, right mandible; rP, right paragnath; rp, ring pore; serl, serrulate setae; t, teeth; ul, upper lip.

The mandibles of Z1 and Z14 were located posterior to the mouth and comprised a large gnathal lobe originating laterally and extending to the incisor process on the proximal end and the molar process on the inner distal end (Plate 7.5B; also Plate 7.2 and Plate 7.4A). The mandibles were asymmetrical, with regard to the armament of these processes.

The right incisor of both larval stages had five canine-like teeth: C1, C2 and C3 were in the same plane and of different size, C4 and C5 were located posteriorly, between the

incisor and molar processes (Plate 7.5A). The left incisor process of both Z1 and Z14 had five canine-like teeth: C1, C2 and C3 were similar-sized, C4 was smaller and C5 curved underneath the process (Plate 7.5B). During occlusion, the incisors overlapped. A small appendage articulated with the incisor process of only the left mandible of Z1 larvae was noted, believed to be the *lacinia mobilis* (Konishi and Kim, 2000; Richter *et al*., 2002) (Plate 7.5C). It was not segmented and was comprised of approximately 10 cusps. There was no evidence of the *lacinia mobilis* on Z14 larvae.





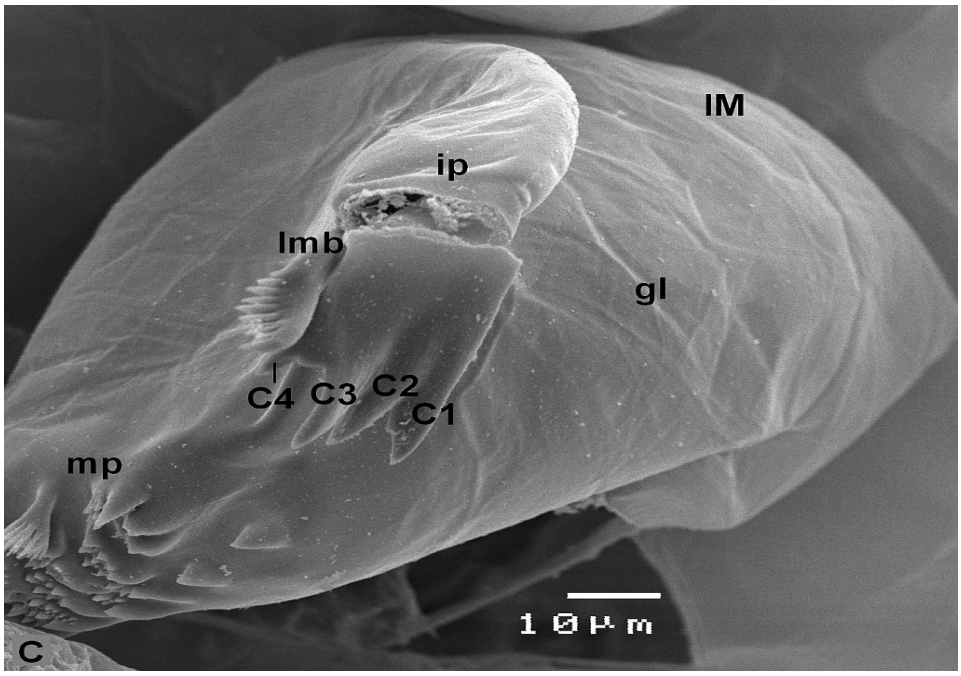


PLATE 7.5 SEM of the mandibles of Z1 A. right incisor process. B. left incisor process. C. *lacinia mobilis*. Abbreviations: C1; C2; C3; C4; C5, canine-like teeth 1-5; gl, gnathal lobe; ip, incisor process; L, labrum; lmb, *lacinia mobilis*; lM, left mandible; lP, left paragnath; ll, lower lip; mp, molar process; rM, right mandible; rP, right paragnath; serl, serrulate setae; t, teeth; ul, upper lip.

Both the right and left molar processes of Z1 consisted of slender teeth and rows of dorso-ventrally extending pointed denticles, with brush-like protrusions dorsal to the denticles (Plate 7.6). On the right molar process of Z14 (Plate 7.7A) there were at least 10 rows of antero-posteriorly directed, fully formed molars at the distal end and additional rows of growing molars at the proximal end. Various structures were observed on the right molar process of Z14, specifically knob-like projections and long spines dorsally to the molars, single cone-shaped teeth ventrally to the molars and brush-like protrusions to the proximal edge of the gnathal lobe (Plate 7.7A). The left molar process of Z14 (Plate 7.7B) had a triangular shape, with molars extending dorso-ventrally; at the base of the triangle long spines at either end and brush-like protrusions in the middle were observed, at the tip of the triangle the molars were less well-formed and more pointed (Plate 7.7B). The incisor and molar processes of the mandibles of Z14 were connected by a row of dentate spines (Plate 7.8). The opening of the esophagus in both Z1 and Z14 larvae was antero-dorsally to the molar process.

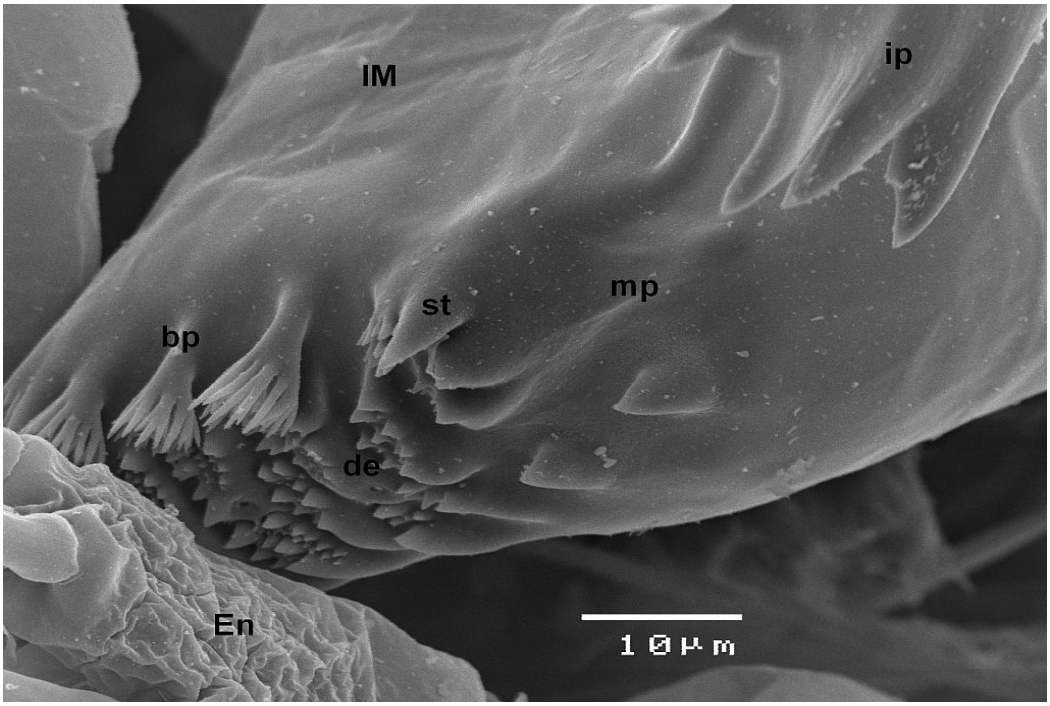
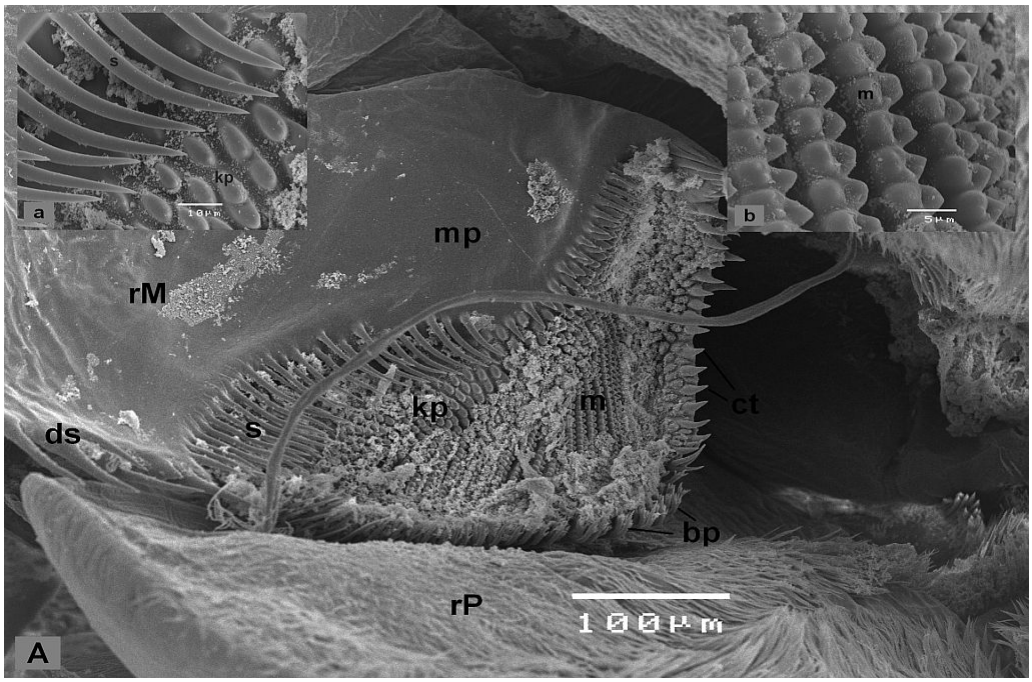


PLATE 7.6 SEM of the left mandible of Z1, showing the molar process. Abbreviations: bp, brush-like protrusions; de, denticles; En, endopodite; ip, incisor process; lM, left mandible; mp, molar process; st, slender teeth.



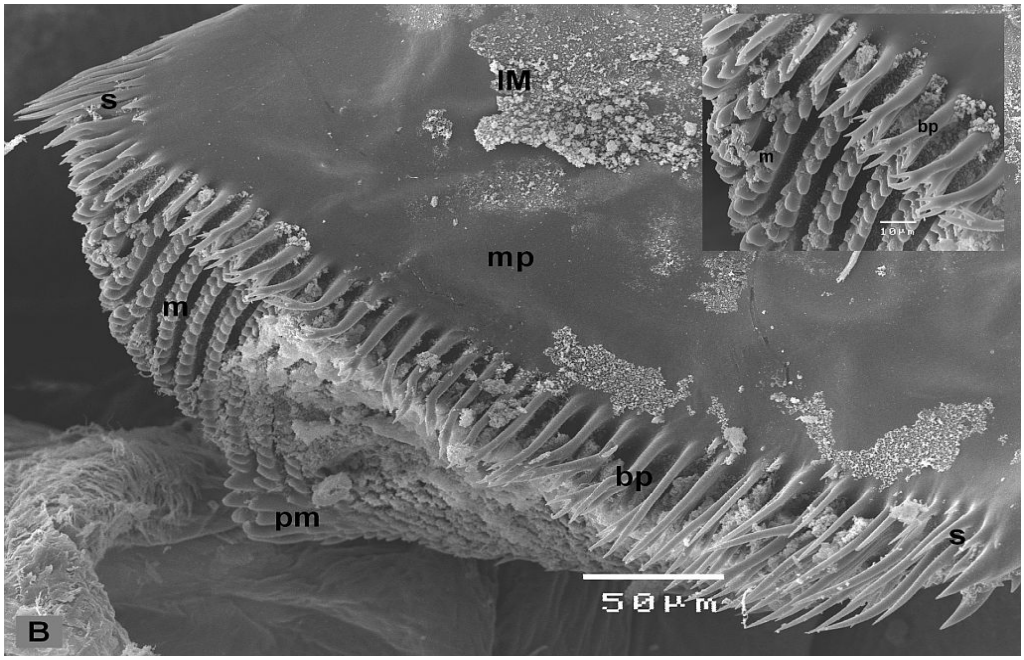


PLATE 7.7 SEM of the mandibles of Z14 A. right molar process. Insets: a) spines and knob-like projections, b) molars. B. left molar process. Inset: the molars and the brush-like protrusions. Abbreviations: bp, brush-like protrusions; ct, cone-shaped teeth; kp, knob-like projections; lM, left mandible; mp, molar process; m, molars; pm, pointed molars; rM, right mandible; rP, right paragnath; s, spines.

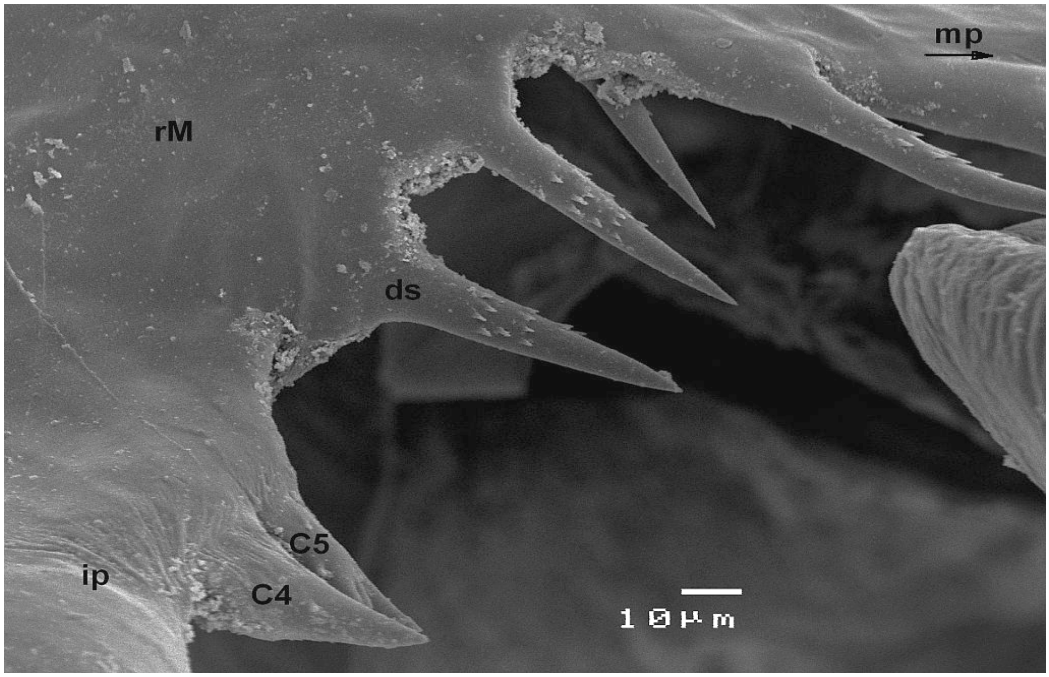


PLATE 7.8 SEM of the right mandible of Z14, showing the dentate spines between the incisor and molar processes. Abbreviations: C4; C5, canine-like teeth 4-5; ds, dentate spines; ip, incisor process; mp, molar process; rM, right mandible. Note: the arrow indicates direction towards the molar process.

Digestive System

The gut in *L. amboinensis* larvae was basically a tube, which opens anteriorly at the mouth and posteriorly at the anus.

The esophagus was a narrow vertical tube connecting mouth and stomach (Plate 7.9; also Plate 7.10A). In Z1, Z5, Z9 and Z14 larvae, the esophagus consisted of cubic epithelial cells and a chitinous cuticle, and setae projected from the lateral walls to the lumen. Beneath the cuticle were tegumental glands that secrete mucopolysaccharides

(Plate 7.9). As the larvae developed, the esophagus became wider and longer, and the setae denser.

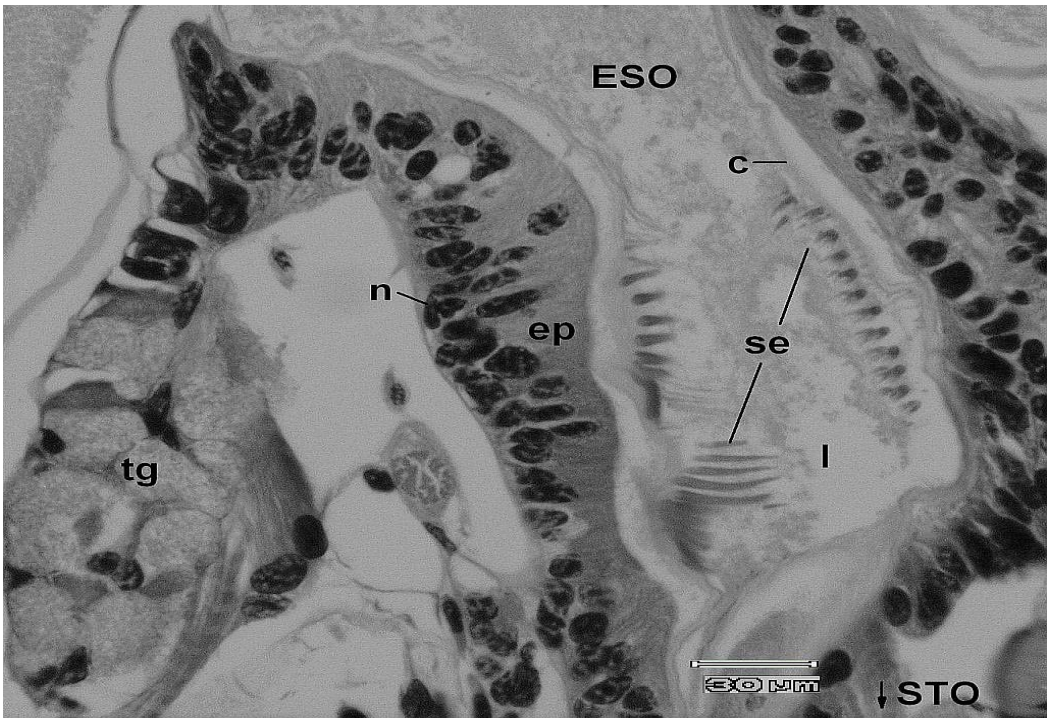
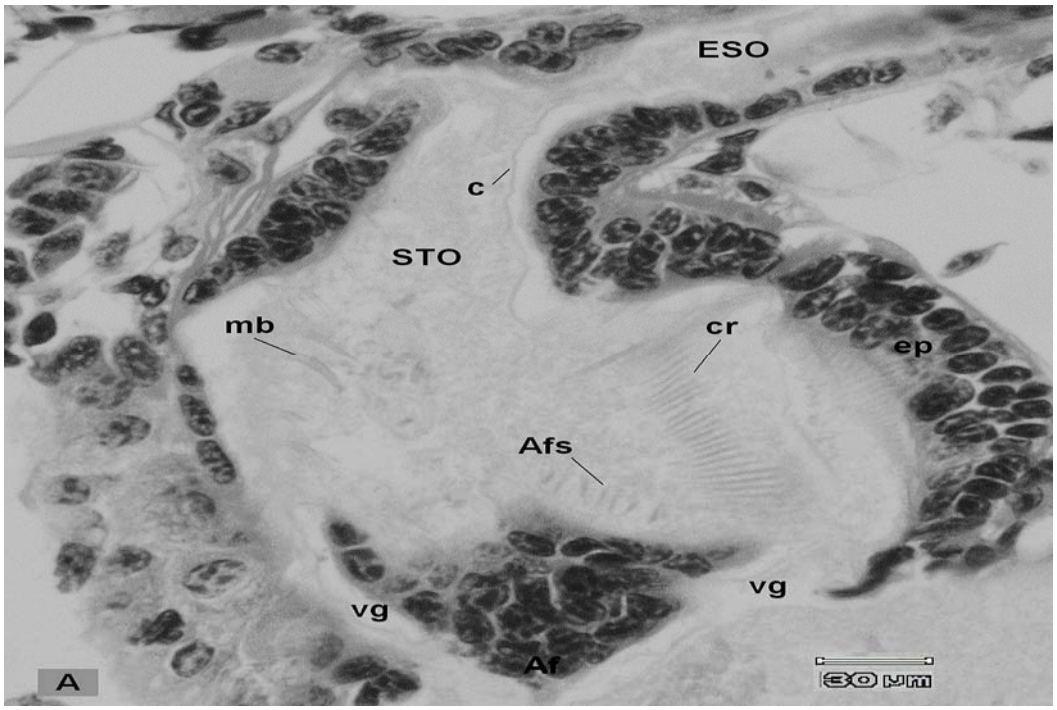


PLATE 7.9 Longitudinal section of the esophagus of Z9 *L. amboinensis*. The arrow indicates direction towards the stomach. Abbreviations: c, cuticle; ep, epithelium; ESO, esophagus; l, lumen; n, nuclei; se, setae; STO, stomach; tg, tegumental glands. H & E stain.

The stomach or proventriculus was immediately posterior to the esophagus. In all larval stages examined it was a simple chamber, consisting of columnar epithelial cells and a chitinous cuticle (Plates 7.10A and 7.10B). Lateral setae of the comb row projecting medially into the stomach were observed in Z1 larvae. In Z5 larvae, these became denser and elongated, and spines of the main brush had developed into the dorsal

chamber, projecting medially from the wall above the comb row (Plate 7.10A). Also in Z5, the anterior floor and ventral grooves were defined and anterior floor setae were observed (Plate 7.10A). The first indication of a filter press (equivalent to the gastric sieve, Bell and Lightner, 1988) was that of setae of the outer valve in the ventral chamber and was noted in Z9. By Z14 the filter press was complete, with the outer valve covered by a dense mat of setae, and the inner valve comprising the ampullary channels, also equipped with dorsally directed setae (Plates 7.10B and 7.10C).



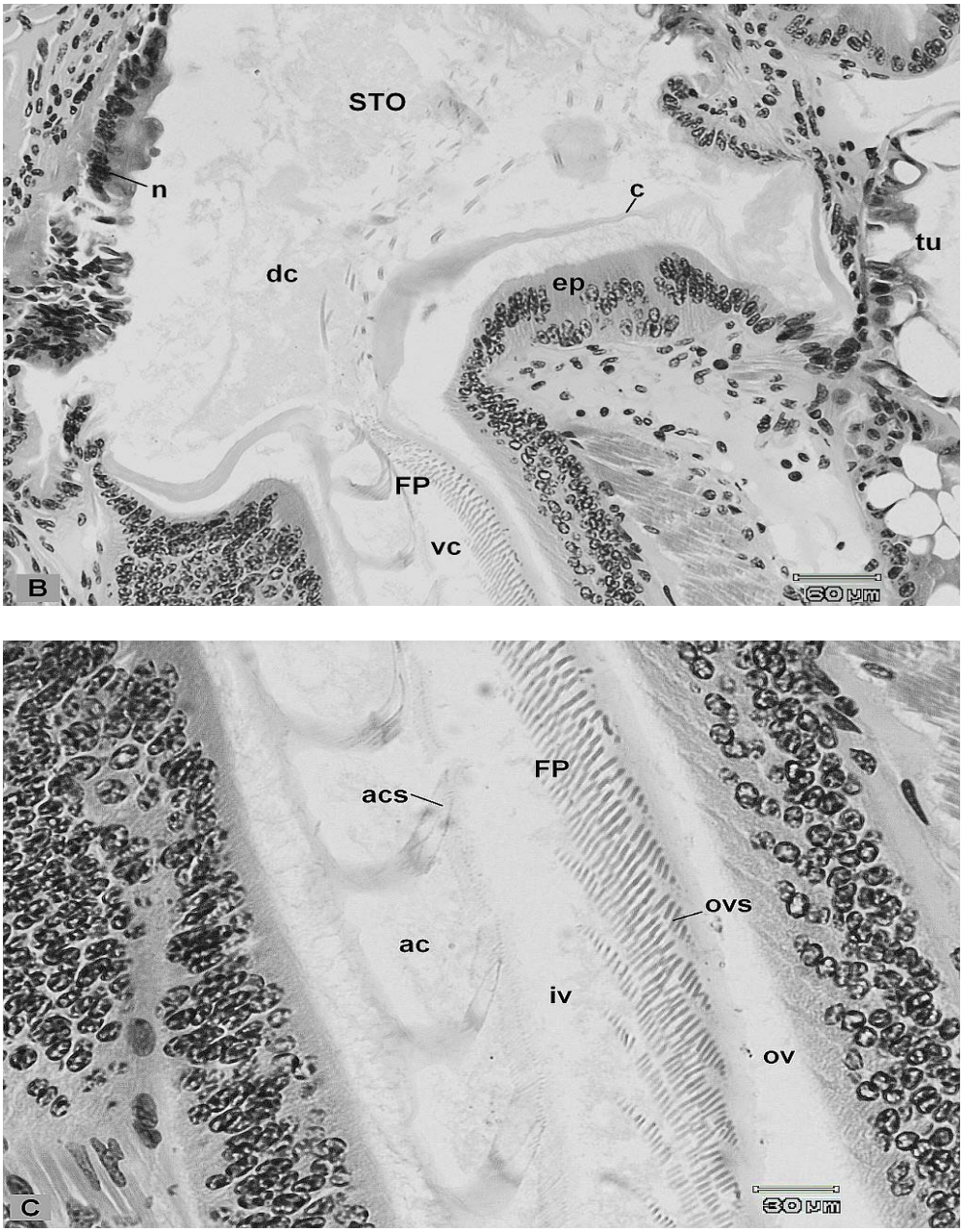


PLATE 7.10 Longitudinal sections of the A. Z5 stomach. B Z14 stomach. C. Z14 filter press. Abbreviations: ac, ampullary channel; acs, ampullary channel setae; Af, anterior floor; Afs, anterior floor setae; cr, comb row; c, cuticle; dc, dorsal chamber; ep, epithelium; ESO, esophagus; FP, filter press; iv, inner valve; mb, main brush; n, nuclei; ov, outer valve; ovs, outer valve setae; STO, stomach; tu, hepatopancreatic tubules; vc, ventral chamber; vg, ventral groove. H & E stain.

In all larval stages examined, the hepatopancreas was extensive, occupying most of the cephalothorax. The hepatopancreatic tubules (Plate 7.11A), ragged in appearance owing to their finger-like shape, connected to the stomach and the midgut. In the epithelium of the tubules there were five cell types (Plate 7.11B) lying in the basophilic membrane, the cell types did not change with larval development. The *embryonic* E-cells occupied the distal part of the tubule. The *fibrilar* F-cells were located in the medial reaches of the tubules between R and B-cells. The *resorptive* R-cells were the most abundant type in the hepatopancreatic tissue. They are multi-vacuolate, containing spherical globules of lipids and, occasionally, glycogen (Mikami *et al*., 1994). The *blister-like* B-cells with the relatively large round vacuole bordered the tubule cavity, to release their contents. Also *myoepithelial* M-cells laid in the distal epithelial layer, surrounding each tubule (Bell and Lightner, 1988) (Plate 7.11B). In early stages the hepatopancreas consisted of a few large tubules, whereas older larvae showed numerous hepatopancreatic tubules that completely surrounded the stomach and midgut.

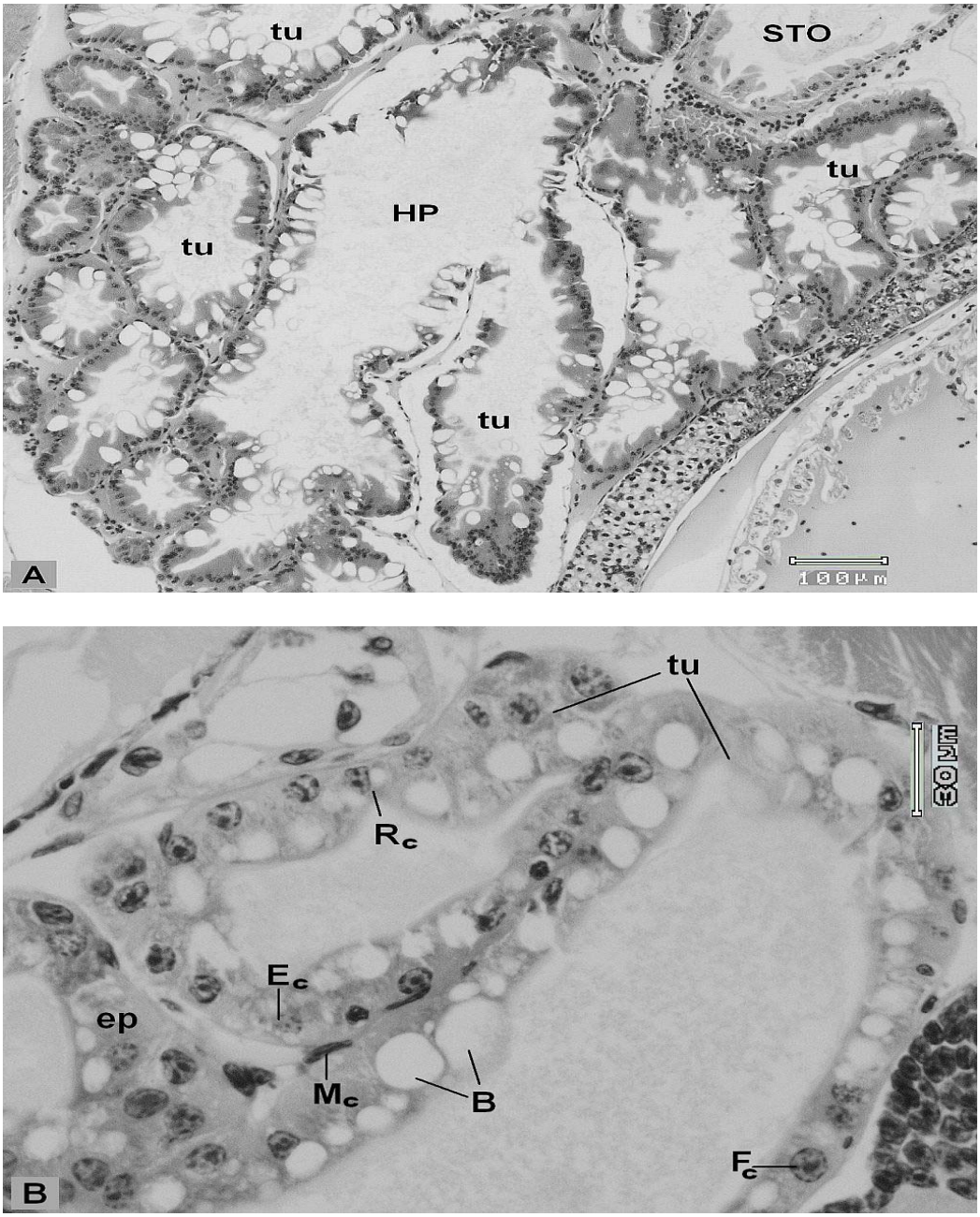
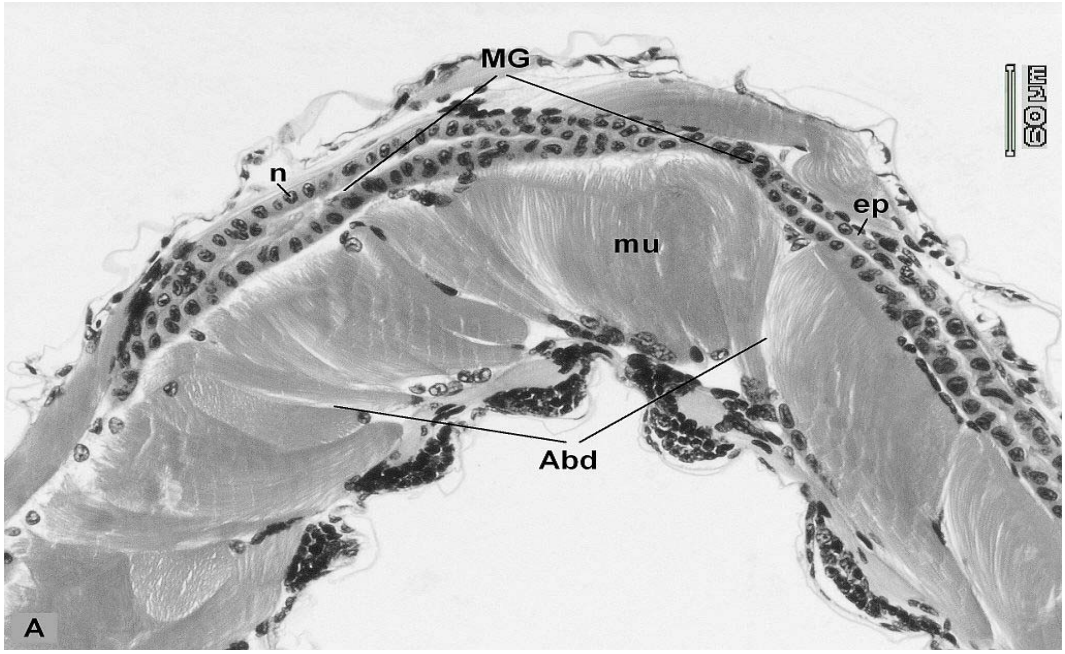


PLATE 7.11 Longitudinal sections of the A. hepatopancreas of Z14. B. hepatopancreatic tubules of Z5 showing the cell types. Abbreviations: B, blister-like cells; E_c, embryonic cells; F_c, fibrilar cells; HP, hepatopancreas; M_c, myoepithelial cells; R_c, resorptive cells; STO, stomach; tu, tubules. H & E stain.

The midgut in early and late larval stages started in the cephalothoracic region, passed between the dorsal and ventral hepatopancreatic lobes and run dorsally along the length of the abdomen (Plate 7.12A). In Z1, Z5, Z9 and Z14, the epithelium of the midgut was thick, made up of elongated columnar cells that extended from the basement membrane to the lumen, with central nuclei and a microvillous brush border at the lumen-facing surface (Plates 7.12A and 7.12B). The structure of these epithelial cells was similar to the R-cells of the hepatopancreas (Mikami *et al*., 1994), confirmed by the spherical globules observed after feeding of the larvae (Plates 7.12B and 7.12C). Beneath the basement membrane, in all stages examined, there was circular musculature (Plate 7.12A) and within the midgut lumen food matter appeared to be enveloped in a peritrophic membrane (Plate 7.12C). The midgut increased in length as the larvae increased in size with development.



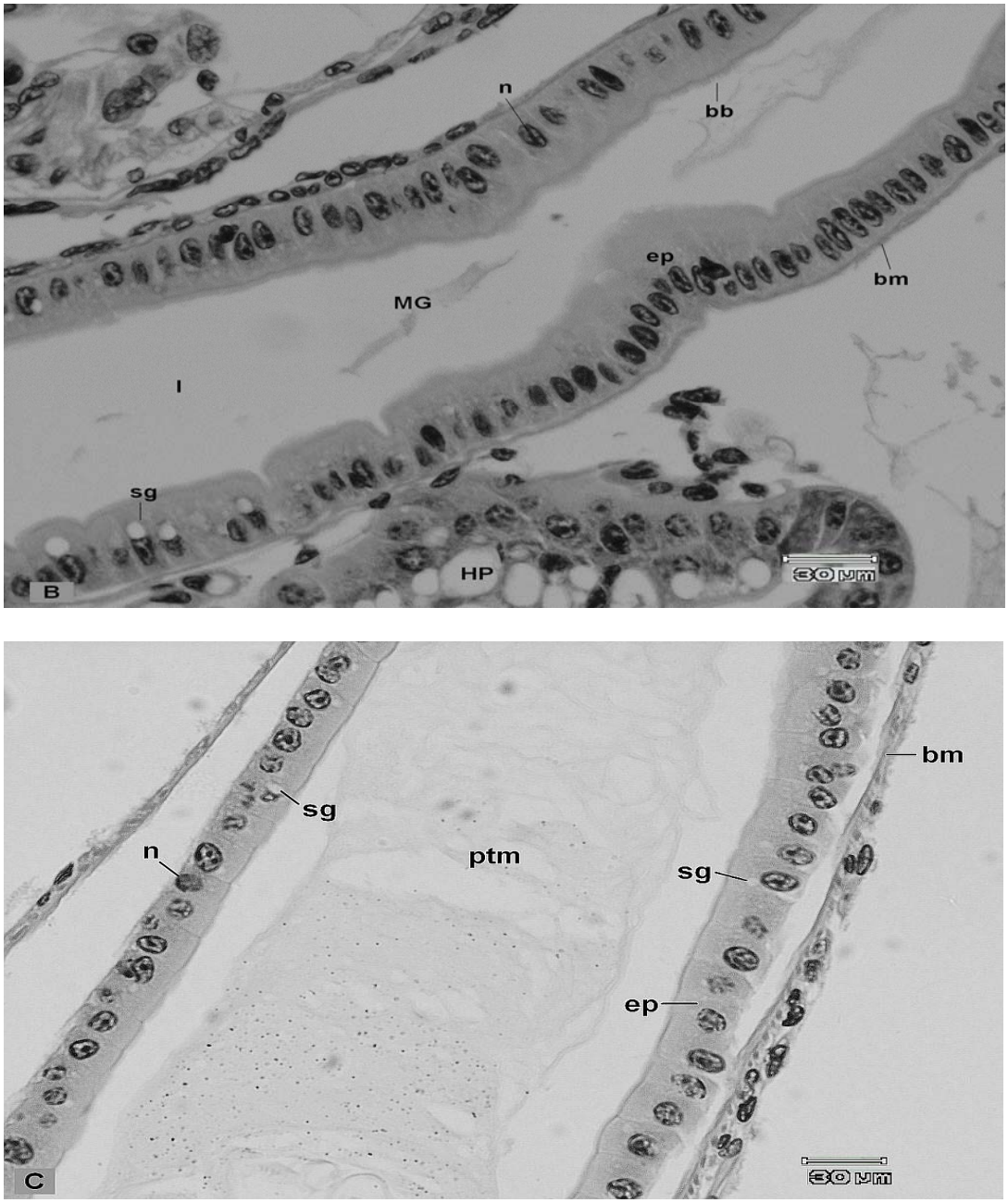
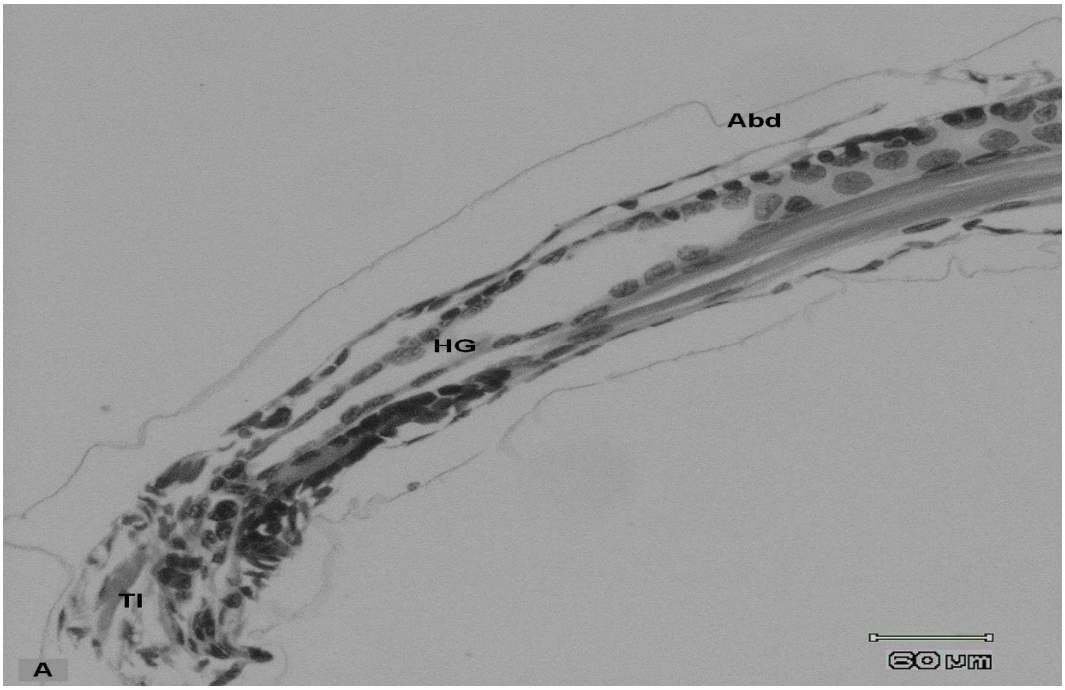


PLATE 7.12 Longitudinal sections of the A. Z5 midgut. B. Z9 midgut. C. Z14 peritrophic membran. Note: the separation of the epithelium from the basement membrane is artifactual. Abbreviations: Abd, abdomen; bm, basement membrane; bb, brush border; ep, epithelium; HP, hepatopancreas; l, lumen; MG, midgut; mu, muscle; n, nuclei; ptm, peritrophic membrane; sg, spherical globules. H & E stain.

In *L. amboinensis* larvae, the hindgut occupied the last part of the abdomen and opened to the anus at the telson (Plate 7.13A). The epithelial cells of the cuticularized hindgut of Z1, Z5, Z9 and Z14 contained less cytoplasm, compared to the midgut cells, and the central nuclei laid on a thin basement membrane (Plate 7.13B). In Z9 and Z14 (Plates 7.13B and 7.13C), there were numerous infoldings of the previously straight hindgut walls of the early stages (Plate 7.13A). Alcian blue sections revealed traces of mucopolysaccharide material associated with the epithelium (Plate 7.13C).



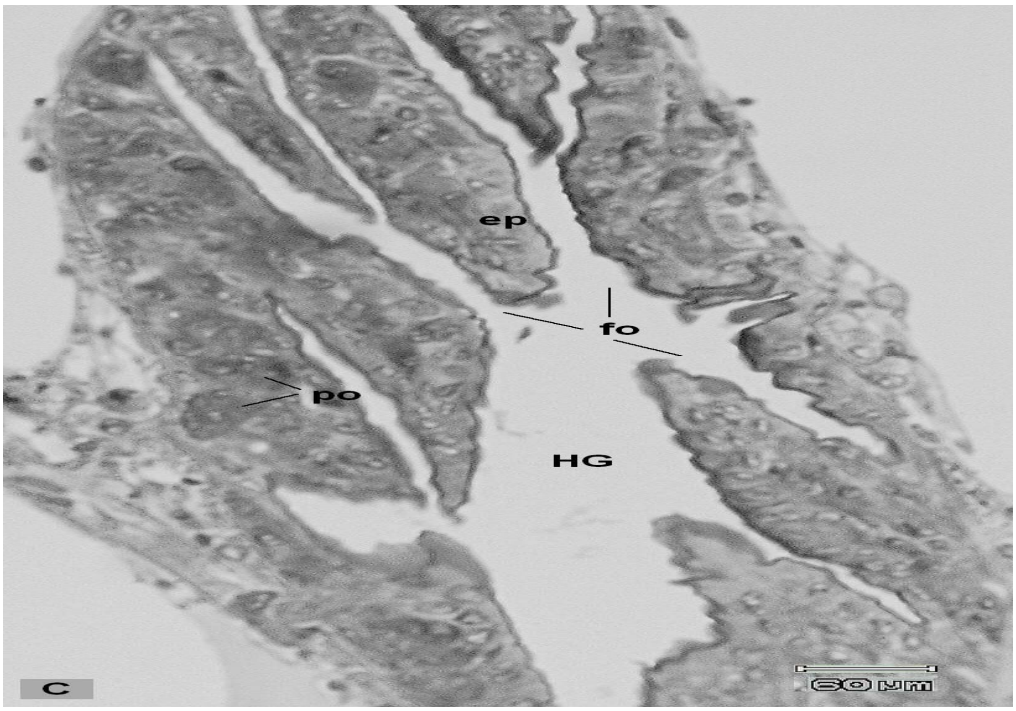


PLATE 7.13 Longitudinal sections of the A. Z1 hindgut B. Z14 hindgut wall folds. C. Z14 mucopolysaccharide secretions. Abbreviations: Abd, abdomen; bm, basement membrane; c, cuticle; ep, epithelium; fo, folds; HG, hindgut; po, mucopolysaccharides; n, nuclei; Tl, telson. A and B. H & E stain, C. Alcian Blue-P.A.S.

7.4 Discussion

Feeding studies using *L. amboinensis* demonstrated that early stage larvae readily accept live feeds, such as rotifers and *Artemia* nauplii (Calado *et al*. 2008a; Cunha *et al*. 2008). During larval development, more fleshy feeds, such as fresh squid, mussel gonad and adult *Artemia*, are consumed (pers. obs.). Changes in decapod feeding preferences and capability have been associated with changes to the structure and function of ingestive and digestive organs (Nishida *et al*. 1990; Cox and Johnston 2004).

The potential role of the feeding appendages can be deduced from their form and the type of setae or spines. Accordingly, in *L. amboinensis* early and late stage larvae, the primary function of the maxillae may be a respiratory one, with the long plumose setae on the scaphognathite creating a current flow over the developing gills. The shorter plumose setae on the endites may act as a net to prevent entry of particles to the branchial chamber (Farmer 1974; Factor 1978; Crain 1999).

The elevated position of the endopodites of the maxillules of Z1 and Z14 suggests a role in preventing prey from escaping, while the basal endites with their cuspidate setae grasp and manipulate the captured prey (Crain 1999). The plumodenticulate and pappose setae on the endopodite and coxal endite of the maxillules of both larval stages could serve in trapping and returning small food fragments that have escaped from the oral chamber (Hunt *et al*. 1992; Crain 1999). Furthermore, the serrated setae of the basal endite of the Z1 and Z14 maxillules may clean appendages post-feeding (Johnston and Alexander 1999). This may be particularly important in older *L. amboinensis* larvae,

which feed on mussel tissue as well as *Artemia*. Indeed, in Z14, the number of serrated setae had increased notably. In *Jasus verreauxi*, the mouthpart appendages of phyllosomata fed on mussel gonad became fouled quickly, and the larvae were observed cleaning for an extended period, a behavior not recorded when fed solely upon *Artemia* (Nelson *et al*. 2002b).

The role of the paragnaths seems to differ between early and late stage *L. amboinensis* larvae. In the early stages, the paragnaths may act principally as a barrier to keep the food in the mouth region, as evidenced by their highly curved nature and the dense pads of long serrulate setae adapted to grip small particles without clogging (Alexander 1988; Hunt *et al*. 1992; Crain 1999). In the late stage larvae, the prominent feature of the paragnaths is the cuticular pores, suggested to secrete agglutinating substances to facilitate binding and ingestion of food particles (Alexander 1988; Hunt *et al*. 1992).

In both early and late larval stages, the labrum with its numerous teeth would provide a rigid abrasive surface for prey holding and processing during external mastication (Hunt *et al*. 1992; Crain 1999; Cox and Johnston 2004). In crustaceans, the labrum is reported to secrete mucus to further bind the food prior to ingestion (Hunt *et al*. 1992). The pores observed on the labrum of *L. amboinensis* larvae would suggest a similar function. Yet, the fact that the newly hatched larvae have only one pore may signify that this additional function of the labrum develops gradually, as the larvae mature. Further investigation would be needed to ascertain if both types of labral pores recorded in the older larvae are involved in the secretion of agglutinating substances. However, based on similar reports on *J. verreauxi* this would be likely (Cox and Johnston 2003).

In predators such as *L. amboinensis*, the mandibles appear elongated and oriented in a vertical plane (Steele and Steele 1993; Watling 1993). The asymmetry of the left and right mandibles and the diversity of structures on the molar processes of *L. amboinensis* larvae would provide a range of masticating surfaces. The incisors of both early and late stage larvae would cut/tear food into small pieces suitable for ingestion and may assist the labrum to pass the masticated food into the esophagus (Caine 1975). An additional small tooth at the C4 position of the left incisor process reported in a study on *L. amboinensis* larval development (Wunsch 1996) was not observed in this study. The role of the *lacinia mobilis* of Z1 is not clear, as this appendage has been mainly used as a diagnostic tool in arthropod systematics (Richter *et al*. 2002). However, it has been hypothesized it may function as a biting element or it may help guide the incisor into the right plane and lock it in its final position (Richter *et al*. 2002). In Z1, the molar process consisted of pointed teeth, suggesting a linear cutting action of the mandibles in early stages, as has been proposed for spiny lobster larvae (Cox and Johnston 2003). Older *L. amboinensis* larvae would be able to grind food, as evidenced by the substantial molars that had developed on the molar process of Z14, thus enabling them to feed on fibrous preys.

When food has been macerated by the mouthparts, it passes into the anterior section of the foregut, the esophagus (Anger 2001). In early and late stage larvae, movement of the food from the esophagus towards the proventriculus may be facilitated by the setae, as they brush against each other when the muscles contract (Caine 1975; Cox and Johnston 2004), and by mucus secretions from the underlaying tegumental glands (Barker and Gibson 1978; Sousa and Petriella 2006). Additionally, mucus secretions may protect the

un-calcified walls from damage by ingested particles (Barker and Gibson 1978; Mikami *et al*. 1994).

Lysmata amboinensis is similar to other caridean larvae in that it does not possess a gastric mill for internal cutting and crushing of prey items (Anger 2001), contrary to its presence in many other decapods (Caine 1975; Barker and Gibson 1978; Nishida *et al*. 1990; Johnston and Alexander 1999). Gastric mill efficiency has been inversely correlated to mandible efficiency (Pathwardan 1935). In *L. amboinensis* larvae, the mandibles are well equipped for cutting food from hatch, thus compensating for the absence of a gastric mill.

Cox and Johnston (2004) proposed that some food mastication takes place in the foregut as lateral setae of opposing walls brush against each other. This may also occur, to a limited extent, in *L. amboinensis* larvae by the lateral setae in the esophagus and stomach. The main role of the setae and grooves in the stomach would, however, be to sort and filter masticated food particles (Mikami and Takashima 1993). The main brush and the setose barrier to the ventral grooves, formed by the lateral setae of the comb row and the anterior floor setae overlapping, would pose two levels of filtration (Johnston and Ritar 2001). In early stages of *L. amboinensis* it is likely that filtration ability in the stomach is limited, due to the simple structure of the main brush and comb row. Therefore, a suitable food may be of gelatinous or soft texture. In later stages, filtration ability in the stomach of the larvae would be improved, due to an increase in the density and length of setae and the development of the filter press, which might facilitate the ingestion and sorting of fleshier prey items. The typical structure of a filter press has outer valve setae that press the smallest particles to the inner valve and through the

ampullary channels to the hepatopancreas, whereas coarse particles bypass the filter and are directed to the midgut lumen (Sousa and Petriella 2006).

The core phase of digestion takes place in the hepatopancreas. The various cell types found in the hepatopancreas of early and late stage *L. amboinensis* larvae would be involved in secretion of digestive enzymes and absorption of nutrients. The E-cells, being un-differentiated and un-specialized, are used in tubule growth and possibly in generating other cell types (Mikami *et al*. 1994). Synthesis and storage of digestive enzymes occurs in the F-cells (Barker and Gibson 1978). The B-cells absorb nutrients of large molecular size from the hepatopancreas lumen and store them in vacuoles (Barker and Gibson 1978; Mikami *et al*. 1994). The R-cells facilitate final absorption of digested nutrients, including carbohydrates, amino acids and lipids, by selective transportation through the cell membrane and intracellular metabolism (as opposed to extracellular by the B-cells) (Mikami *et al*. 1994; Anger 2001). Increased tubule density in the hepatopancreas of older larvae suggests a potential to deal with prey of complex nutritional profile.

The midgut of early and late stage larvae was dominated by cells similar to the R cells of the hepatopancreas, with a structure that suggests absorptive functions (Anger 2001). Furthermore, the midgut is thought to be involved in the production of the peritrophic membrane, which may serve to surround the semi-fluid food mass ending in the gut and protect the gut lining from mechanical abrasion (Johnson 1980; Martin *et al*. 2006). A longer midgut in the older larvae would enable a longer passage time to process a diverse range of preys.

Posterior to the midgut is the last section of the digestive tract, the hindgut. In the mud crab, *S. serrata*, the midgut and hindgut occupy 40-50 and 50-60 % of the intestinal tract,

respectively (Barker and Gibson 1978). In the prawn *Palaemonetes argentinus*, the midgut is the longest part of the digestive tract (Sousa and Petriella 2006). In *L. amboinensis*, it is difficult to pinpoint where the midgut ends and the hindgut starts, this being the case for all larval stages examined and similarly to reports on other crustaceans (Johnson 1980). Accordingly, the cuticle of the hindgut may be very thin and transparent, and stain poorly or not at all, making it nearly invisible under light microscopy and resulting in the tissue incorrectly identified as the un-cuticularized midgut (Johnson 1980). Gland secretions in the hindgut of decapods have been suggested to have a dual role, protecting the epithelium from abrasion and binding the fecal matter (Barker and Gibson 1978; Mikami *et al*. 1994). However, in *L. amboinensis* larvae, the food mass may be enclosed in the peritrophic membrane before reaching the hindgut, thus it may be that mucus secretions would act as lubricants. The hindgut walls of early and late stage larvae exhibited anti-peristaltic contractions (pers. obs.), presumed to facilitate movement of indigestible matter through to the anus (Sousa and Petriella 2006). In the older larvae the wall folds of the hindgut may allow for changes in food volume.

In summary, a range of morphological changes in the mouthparts and gut of *L. amboinensis* were observed during larval development. The mouthparts increased in size, the mandibular canine-like teeth became more robust, the molars became better equipped for grinding and spines developed between the corresponding processes. The setation of maxillae and maxillules increased, and numerous pores appeared on the labrum and paragnaths. In addition, the filter press developed in the proventriculus and the number of tubules in the hepatopancreas increased. The midgut increased in length and the hindgut

developed infoldings, which increase the surface area. Such changes imply a shift in prey processing ability with increasing size/age of the larvae.

Chapter 8 – General Discussion

The main objective behind all experiments conducted during this project was to improve the larviculture of *L. amboinensis* which, at present, hinders the commercial aquaculture potential of this popular aquarium species. To experiment on larvae however, one must ensure their consistent and plentiful supply, and to do so, highly productive pairs in captivity need to be established. *Lysmata spp*. are PSHs, eventually fulfilling the reproductive role of both a male and a female partner (note they cannot self-fertilize) (Bauer 2000). Although this is a shared trait in the genus, the onset of sexual maturity or sex phase change is not fixed.

Commonly, juvenile *L. amboinensis* brought in from the wild are MPs. It would be important to identify if and when they can produce adequate sperm to fertilize an SH, at which size they would become SHs and if there are ways to induce or, at least influence, the timing of such phase change (Chapter 2). MPs of just 32.6 mm TL could successfully fertilize a much larger SH, with actual larval production dependent on the size of the SH. Given that embryo volume in *L. amboinensis* was similar, regardless of SH size, differences were due to the number of eggs laid and to the available abdominal area for attachment. Accordingly, the larger the SH, the higher the reproductive output.

MPs exhibited sex-ratio, size-ratio and size-assortative strategies, depending on pair circumstances. In male pairs, one MP partner would change sex phase the earliest possible, to have at least one SH in the pair. Moreover, in the case of size asymmetry, it would be the larger MP that changed first; their partner would then continue to grow and become an SH at a size matching that of their pair. What was rather surprising was the

response of those MPs paired form the start with a large SH. Since the female role was already realized by the SH, the MPs could continue reproducing as males, until they reached an "optimum" size to assume female function as well. Instead, the MPs changed to SHs at a smaller size than expected. Perhaps the reason lies in their ecology, as in nature *L. amboinensis* pairs consist, predominantly, of SHs, allowing both individuals to exercise their dual function. Regardless of the underlying mechanisms, the fact remains that social environment affects sex phase change in *L. amboinensis* shrimp.

While conducting the aforementioned experiments on the sexual biology of *L. amboinensis* it was noted that the berried SHs would lose a portion of their spawn during the incubation period. This is actually not uncommon among crustaceans that brood the embryos until they have developed and are ready to hatch (Kuris 1991; Clarke 1993; Calado and Narciso 2003). However, the loss was greater than would be expected if the main factor in play was a natural increase in egg volume. Through the method of elimination, it was concluded that the administered maintenance diet of squid and mussel was the most likely cause of the observed egg mass loss. This assumption was tested in a comparison of various maturation diets for *L. amboinensis* broodstock (Chapter 3).

Broodstock diets affect sperm production of males, gonadal maturation and fecundity of females, and the biochemical composition of offspring. Research on these topics has been extensive, although limited to commercially important prawns and lobsters (Bray and Lawrence 1992; Cahu *et al*. 1995; Palacios *et al*. 1998; Wouters *et al*. 2002; Perez-Velazquez *et al*. 2003; Smith *et al*. 2004). In comparison, marine ornamental culture is still in its infancy, and in corresponding hatcheries, managers have simply adopted the

practises used in the farming of food species, with dietary regime being one such example (Dhert *et al*. 1997; Sales and Janssens 2003).

The diets chosen to be tested for their suitability for *L. amboinensis* broodstock were among those widely applied in aquaculture operations. The results indicated that, indeed, a maturation diet comprised of squid and mussel yields sub-optimum larval production. Feeding *L. amboinensis* solely on frozen adult *Artemia* biomass, on-grown on a mix of algae, also resulted in low numbers of larvae. Despite a similar reproductive outcome, the reason for each diet's performance differed. In the case of squid/mussel, poor egg mass retention caused the variable, and generally low, viable fecundity. In the case of *Artemia* biomass, low larval numbers were attributed to poor embryo hatchability, given that egg loss was low. Nevertheless, the three most common food items were unsuitable as maturation diets for *L. amboinensis*.

Examining the FA profile of the diets provides some possible explanations. C_{18} MUFAs and PUFAs were abundant in *Artemia* but not in the squid/mussel diet. Thus it appears there may be a requirement for C_{18} FAs, especially PUFAs, for satisfactory egg development in *L. amboinensis*. High shrimp embryo hatching rate has been linked to increased dietary DHA concentration (Xu *et al*. 1994; Cavalli *et al*. 1999). The *Artemia* were deficient in DHA. On the other hand, excessively high DHA levels, like those recorded in squid, have caused adverse effects in penaeids' reproduction (Bray *et al*. 1990). It must be noted that feeding the cleaner shrimp a combination of squid, mussel and *Artemia* (sequentially) improved egg and larval production. This may have occurred because under this feeding regime MUFA, PUFA and HUFA levels in the tissues of the shrimp would balance over time.

The best reproductive performance was achieved with the Kuruma dry feed. Feeding crustacean broodstock entirely on artificial or formulated diets would be ideal for many aquaculture farm owners and for researchers. Despite numerous attempts, complete replacement of fresh (frozen) feeds with artificial diets has, generally, been unsuccessful. The finding that *L. amboinensis* can be fed on a pelleted diet, without negative consequences on survival and growth, and with positive effects on reproduction, is promising. The profile of the Kuruma feed revealed a high MUFA content, a high PUFA content (although lower than the *Artemia* diet) and a high HUFA content (although lower than the squid/mussel diet). Perhaps a diet that provides a rich pool of FAs of different saturation degree is the model for *L. amboinensis* broodstock, yielding high egg production, retention and embryo hatching.

It would be worth investigating if other dry feeds would achieve a similar outcome as the Kuruma diet, and also look methodically for those components of the pellet that made it a suitable maturation diet for *L. amboinensis*. When considering reproductive processes, the type and amount of lipids supplied is often associated with the physiological condition of spawners and resulting larvae (Palacios *et al*. 1998; Wouters *et al*. 2001; Racotta *et al*. 2003). That is not to say though, that they are the only nutritional elements to be considered. There are other macro-nutrients, and especially, micro-nutrients, such as vitamins and carotenoids, that can influence various aspects of crustacean reproduction (Mengqing *et al*. 2004; Williams 2007). The success of the Kuruma diet may be equally related to these other nutrients.

To further investigate the role of FAs in the reproduction of *L. amboinensis* and re-assess the suitability of *Artemia* after enrichment, as maturation diet for the cleaner

shrimp, Algamac-3000 was selected to boost the nutritional value of the prey (Chapter 4). As discussed earlier, C_{18} FAs may shape spawning performance in *L. amboinensis*, further supported by the fact all *Artemia* diets (enriched and un-enriched) had a high MUFA and PUFA content and all displayed insignificant brood losses during incubation. The importance of DHA was confirmed through increased embryo hatchability, and thus reproductive output, of *L. amboinensis* fed diets containing high quantities of this HUFA. Broodstock fed Algamac enriched *Artemia* were of a mean TL of 46.8 mm, those fed Kuruma pellets were 43.6 mm; in either case, the mean larval production was similar. Taking into consideration fecundity in *L. amboinensis* increases with size/age, one could argue that the Kuruma diet outperformed the enriched *Artemia* in reproductive terms. Un-enriched *Artemia* were dominated by FAs of the n-6 series. Although enriching the *Artemia* with Algamac-3000 reduced the relative proportion of n-6 FAs, n-3 FAs occupied, at best, half of the n-6 levels. On the contrary, the n-3/n-6 ratio in the Kuruma diet was 3.4, with both high EPA and DHA levels contributing to this result, substantiating the importance of HUFAs of the n-3 series in the reproduction of the cleaner shrimp, as reported for many marine animals (Sargent *et al*. 1997; Narciso and Morais 2001).

Combining the findings from this and the previous study on maturation, it would be recommended that for *L. amboinensis*

- Dietary provision of C_{18} MUFAs and PUFAs may lead to enhanced egg production and egg mass retention.
- Dietary provision of n-3 HUFAs, for example EPA and DHA, may improve embryo hatchability.

- A DHA inclusion level of between 11-14% of total dietary FAs may be best for increased larval output.
- A n-3/n-6 ratio of 3.4, as recorded in the Kuruma pellet diet, should serve the reproductive needs of the broodstock.

Observations and suggestions thus far relate to the dietary regime of the cleaner shrimp. A low PUFA or HUFA content in experimental diets may not necessarily be reflected in the animal's lipid profile, as selective retention of certain FAs has been reported (Cahu *et al*. 1995; Calado *et al*. 2005a, b). Ideally, the FA profile of the shrimp fed the different maturation diets should have been generated, to reveal the metabolic pathways involved in oocyte production. Within the economic and time constraints of this study, it was not possible to do so at that point. But, as part of the experiment on the FA profile of newly hatched *L. amboinensis* larvae, broodstock tissues were also analyzed (Chapter 6). From this information some general observations can be made; for example, HUFAs were indeed abundant in broodstock tissues, with EPA and DHA highest, perhaps an indication of preferential sequestering from the diet. A DHA/EPA ratio of 1.5 was recorded in the hepatopancreas and ovaries, similar to the ratio in the pellet diet used. FAs of the n-3 series were more abundant than n-6 FAs. MUFAs were also relatively abundant, but PUFAs were not. Whether PUFAs were continuously utilized, as also assumed for the larvae, or dietary provision was not adequate, cannot be ascertained from this study. Projects seeking to quantitatively define the FA requirements of *L. amboinensis* broodstock and larvae would clarify these issues.

A nutritionally sound maturation diet would not only satisfy the needs of the broodstock, but supply the embryos with sufficient endogenous reserves, if prey

availability is poor at the time of hatch. In general, caridean larvae are planktonic carnivores, able to feed immediately after hatching, but they are also FPLs, an inherited trait that allows them to survive and develop to Z2 in the absence of food (Anger 2001). This is a safety mechanism to account for potential patchiness in zooplankton, encountered even in the most productive environments (Le Vay *et al*. 2001). Previous studies by Calado *et al*. (2007; 2008a) reported that newly hatched *L. amboinensis* are not FPLs, contradicting suggestions by Cunha *et al*. (2008) of signs of FPL in the species. In the current study, the suspicions of Cunha *et al*. (2008) were confirmed. Newly hatched larvae were able to moult to Z2 under starvation (Chapter 5).

Parental provision of EFAs and other micro-nutrients can determine larval quality and affect survival during the first larval stage in shrimp culture. It appears, however, the egg yolk is depleted quickly and energy accumulated through prey consumption becomes the fuel for larval development (Calado *et al*. 2007). Despite the ability to tolerate early starvation, providing nutritious diets to larvae immediately after hatch increases their chances to reach settlement (Simoes *et al*. 2002; Calado *et al*. 2005a; Calado *et al*. 2007; Palmtag and Holt 2007). Diet suitability is influenced by factors such as, larval size , the predator's feeding mechanisms and behavior, the prey's size and swimming behavior and digestibility and assimilation efficiency of the prey; and these properties are likely to change during ontogeny of predator and/or prey (Kumlu 1999; Ruscoe *et al*. 2004).

Examining *L. amboinensis* larval survival, growth and development in response to prey size, density and profile showed that

- Survival was affected by prey size, with the smallest prey, i.e. rotifers, becoming less suitable as the larvae progressed.

- Growth was not affected by any of these parameters.
- Development was affected by prey profile, with DHA enriched *Artemia* achieving the highest rate to Z4.

Rotifers, *Artemia* nauplii and *Artemia* metanauplii are all acceptable first feeds for newly hatched *L. amboinensis*. Rotifers would be the diet of choice for Z1, if survival at each larval stage was considered independently. However, given larviculture is a continuous and dynamic process, such an approach would be incorrect. In that aspect, *Artemia* would be most suitable feed from hatch to Z4, since dietary carry-over effects to the next stage were noted that improved survival.

Prey density was not a seminal factor for early larval stages cultured under constant environmental conditions. Food availability *per se* suffices for *L. amboinensis* larvae to survive and progress. Thus, in the case of *Artemia*, distributing 1 nauplius or metanauplius ml^{-1}, instead of 3 ml^{-1}, would be equally effective and would reduce feeding costs. However, results from scientific small-scale trials may not directly apply in a commercial hatchery operation, where, for example, competition among con-specifics for resources could call for higher prey densities.

It must be noted that when maturation diets were considered, survival and growth of the broodstock were also unaffected. Perhaps these findings, combined, verify the plasticity of *Lysmata* species, larvae and adults (Calado *et al*. 2005a). And, just like maturation diets affected reproduction, larval diets affect development in *L. amboinensis*. Developmental rate increased and a more synchronous progression to subsequent zoeal stages was induced when larvae were fed Algamac-3000 enriched 1-day old *Artemia*, compared to other commercial products or live algae. Faulk and Holt (2005) also came to

a similar conclusion regarding Algamac preparations and live algae as *Artemia* enrichments, for feeding to cobia larvae.

Thus, DHA (prominent in Algamac-3000) once again proved essential, this time for larval development. DHA levels in the enriched *Artemia* metanauplii were not as high as those recorded for Algamac-3000 enriched adult *Artemia* fed to the shrimp broodstock (6% as opposed to 11% in total FAs, respectively); the metanauplii catabolize DHA faster than later *Artemia* stages (Ritar *et al*. 2004). But, regardless of the concentration in the metanauplii, DHA was preferentially retained and was higher in the shrimp larval tissues than in the diet.

The profile of the larvae reflected dietary inputs only to a small degree. Some FAs were sequestered and stored and other FAs were used up continuously to satisfy the energy demands of the larvae. Monounsaturates belong to the latter, with MUFA levels in *L. amboinensis* slightly lower than in *Artemia*. HUFAs, other than DHA, were higher than corresponding amounts in the diets, suggesting selective accumulation and preservation. This was the case for the n-3 EPA and the n-6 docosapentaenoic (DPA). The n-6 ARA was also preferentially sequestered but a threshold must exist, since the greatly elevated ARA levels in the *Artemia* fed Algamac-ARA did not result in an equal increase in larval ARA concentration. The diet with the highest DHA/EPA/ARA (1:0.6:0.3) and n-3/n-6 (2.2) ratios improved developmental rate in *L. amboinensis* larvae.

The aforementioned results were obtained by analyzing Z4 larvae fed various *Artemia* diets. When compared to the profile of Z1 larvae fed rotifers (Chapter 6) we begin to see a pattern in larval nutrient requirements. Accordingly, larvae tend to both accumulate and utilize PUFAs, if available in their diet. PUFAs are employed at times of rapid membrane

proliferation (Rainuzzo *et al*. 1992); presumably, one such time would be when the larvae are near moult, which is when larvae in either experiment were collected for analysis. This may explain why, although PUFAs increased in response to dietary provision, they did not reach the levels recorded in the rotifers or *Artemia*, as they may have been preferentially used up. HUFAs in Z1 larvae, on the other hand, closely matched the equivalent levels in the rotifers. That may be because HUFA levels in the rotifers were already high, thus satisfying larval requirements. On the contrary, HUFA levels in the *Artemia* diets fed to Z4 were much lower than in rotifers. Still, Z4 larvae had high HUFA levels in their profile, which verifies that FAs from this group are preferentially retained. A reliable way to assess nutrient importance is to starve newly hatched larvae and observe the changes in their profile. Using this approach for *L. amboinensis* the significance of the HUFAs ARA, EPA and DHA was established.

Generally speaking, the FA profile of Z1 *L. amboinensis* is rather predefined and in close approximation to the ovary, tail muscle and hepatopancreas of the adults, with differences primarily in absolute amounts. The dominant FA groups in broodstock and larvae were SFAs and HUFAs. SFAs, in particular palmitic and stearic, are primarily used to store energy, whereas HUFAs are involved in regulating immunology and neurophysiology (Brett and Muller-Navarra 1997). In the newly hatched larvae, the DHA/EPA ratio was approximately 1.0, likely indicating these EFAs should be balanced in Z1 *L. amboinensis*. The relationship changed as the larvae developed, with DHA assuming a greater value, since the Z4 larvae with the fastest and most uniform progression had a DHA/EPA ratio of 1.7. In the ovaries and hepatopancreas of the

broodstock DHA was also higher than EPA, whereas in the tail muscle the two FAs were of similar concentration.

In summary, by combining the results from Chapters 5 and 6 the following conclusions can be drawn in regards to the larval FA profile and possible larval diet requirements

- SFAs are inherently high in newly hatched larvae, and similar to broodstock tissues. Even as the larvae progress SFAs continue to be abundant, likely accumulated through the diet, as seen by the Z4 profile.
- MUFAs in newly hatched larvae tend to be slightly lower than those in broodstock tissues, and may be catabolized preferentially to satisfy the metabolic requirements of the larvae, as seen by the starved larvae profile.
- If adequate PUFAs and HUFAs are provided through dietary sources, then larvae tend to use PUFAs for development and store HUFAs. That was observed for both Z1 and Z4 larvae.
- HUFAs are the most important group of FAs for *L. amboinensis* larvae, as ascertained by the starved larvae profile.
- DHA and EPA are of equal importance in Z1 larvae, but this relationship may change as the larvae develop, with DHA becoming more essential, as seen by the Z4 profile.
- ARA appears to be of less importance than either DHA or EPA, for broodstock and larvae alike. Furthermore, there may be a threshold requirement for ARA, above which this FA will not increase, regardless of dietary amounts present, as seen in Z4 larvae.

- Although the ratio of n-3/n-6 FAs varied across treatments, overall, FAs of the n-3 family were more abundant than FAs of the n-6 family, the expected outcome for marine shrimp.

The lipid/FA profile of late stage larvae should also be generated to provide an insight on required energy substrates and EFAs at a crucial time, close to metamorphosis. For now, this is rather unattainable, since survival to those developmental stages does not yield the numbers and biomass necessary for the corresponding analysis, but as survival of the early stages improves, the odds of conducting similar experiments with late stage *L. amboinensis* larvae should increase.

All *Lysmata* larvae studied to date experience a delay in development at one or more points in their life cycle, due to mark time moulting (Fletcher *et al*. 1995; Palmtag and Holt 2001; Rhyne and Lin 2004; Calado *et al*. 2005a). These critical periods may be connected to major physiological changes that require a shift in nutrition to be overcome. Observed differences in the morphology of mouthparts and gut in *L. amboinensis* during larval development would lead to progressive changes in food preference and processing ability of the species (Chapter 7).

Increased size, setation and spination of individual appendages accompanied larval growth, suggesting larger prey handling. Teeth on the molar process of Z1 *L. amboinensis* rather resembled pre-molars, due to their pointy shape, compared to the perfectly formed molars of Z14. Early stages would likely cut flesh with the incisors, break up the food pieces further at the molar region and ingest. Feed fragments would be kept in the oral cavity mainly by the paragnaths, as evidenced by their position and shape in young larvae. The development of grinding surfaces on the mandibular structures adds

to the specialization of the molar area and indicates that older larvae would be able to deal with hard and fibrous foods (i.e. crushing and grinding), expanding the range of prey types they can consume. The numerous mucus-secreting pores on the labrum would help bind the food bolus prior to ingestion. A similar function has probably been assumed by the paragnaths in late larval stages, given these structures no longer enclose the oral region and have instead developed pores, which secret agglutinating substances.

The appearance of the filter press in the stomach and an increase in the density of tubules in the hepatopancreas should have significantly improved the filtering and digestive capabilities of older *L. amboinensis* larvae. That would translate to larvae being able to process (i.e. separate, digest and assimilate) nutritionally complex feeds more efficiently. A proportionally long midgut, as seen in all larval stages examined, may imply increased gut retention, allowing the larvae to assimilate a higher percentage of energy from the prey (Le Vay *et al*. 2001). The formation of folds in the hindgut of older larvae would compensate for volume changes in the food bolus, allowing larger prey passage at later stages.

Early larval stages of *L. amboinensis* possess adequate armature to capture, manipulate and ingest small soft feeds. Survival of young larvae on rotifers and *Artemia* has been satisfactory; however, dietary alternatives of softer items like medusae, ctenophores, chaetognaths and polychaetes should also be examined. More substantial preys (larger, firmer, fleshier) would benefit older *L. amboinensis* larvae; adult *Artemia*, gammarids, squid, mussel, clams, dry pellets are good candidates for later larval development.

Although it is rather early to discuss artificial diets for *L. amboinensis*, given a lot more issues need to be resolved to fully understand culture principles, only through

formulating an acceptable, water-stable larval diet will it be possible to define absolute nutritional requirements for the species. A few steps towards that direction have been made here, by examining the development of ingestive and digestive structures, by comparing maternal and dietary provision of energy resources to young *L. amboinensis* larvae and by identifying the role of specific nutrients in larval survival, growth and development.

In general, based on the structural characteristics of the early larval stages, the formulated diet should be soft, perhaps presented as gelatinous or semi-moist pellets, with a high lipid content, rich in PUFAs and HUFAs, especially EPA and DHA, and with a greater proportion of n-3 rather than n-6 FAs. Furthermore, it should come in discrete pieces that can be easily manipulated by the young larvae. As the profile of late larval stages was not examined, it is difficult to speculate on nutrient composition, other than to say that, given their ability to not only cut but grind food and, an improved filtering capacity in the stomach, more muscular and tough preys can be incorporated in the preparation and small dry pellets may be appropriate. For the broodstock, a dry artificial diet composed of approx. 75% protein and 15% fat, rich in HUFAs and C18 MUFAs and PUFAs, and with moderate levels of DHA and EPA and high n-3/n-6 ratio, seems to suffice for maturation and good larval production. The data presented in the current study provide the foundation for succeeding cleaner shrimp studies and may be a starting point for making aquaculture production of this marine ornamental a profitable and sustainable activity in the future.

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Appendix

The key morphological features characterizing the individual larval stages of *L. amboinensis* (pers. obs.) are listed in Table A1. Whilst this does not constitute a complete description of the larval development, it serves as a guide to stage the larvae during the study, as these traits can be readily observed under a dissection microscope. In the metamorphic moult from zoea to PL the external appearance resembles the adult, and within a few months the PL becomes a juvenile (Plates A.15, A.16 and A.17).

TABLE A.1 Key characters for the identification of the larval stages of *L. amboinensis*.

Zoeal stage	**Morphological features**	**Plate**
Z1	sessile eyes, three pairs of maxillipeds	A.1
Z2	eyestalks, 1st pereiopod biramous bud	A.2a, b
Z3	1st pereiopod functional, 5th pereiopod uniramous bud, uropods develop	A.3a, b
Z4	5th pereiopod functional, 2nd pereiopod biramous bud, endopod of uropod shorter than telson	A.4a, b
Z5	2nd pereiopod functional, 3rd pereiopod biramous bud, endopod same length as telson	A.5a, b
Z6	3rd pereiopod functional, 4th pereiopod biramous bud, 1 dorsal rostral tooth	A.6
Z7	all pereiopods functional, antennal flagellum triangular	A.7
Z8	antennal flagellum elongated, more slender	A.8
Z9	antennal flagellum 2-segmented	A.9
Z10	antennal flagellum 7-segmented, as long as scaphocerite, plepods develop, small spine on rostrum	A.10a, b
Z11	antennal flagellum longer than scaphocerite, pleopods biramous buds (exopods rudimentary)	A.11
Z12	endopods of pleopods < 10 setae, exopods 1 or 2 setae (except from 1st pleopod exopod, no seta)	A.12
Z13	endopods > 10 setae, exopods < 10 setae (1st pleopod exopod only 1 seta)	A.13
Z14	both exo- and endo- pods > 20 setae (1st pleopod exopod unchanged), endopods of pleopods 2-5 with *ai*, telson triangular, rostrum > 5 spines dorsally	A.14a, b

PLATE A.1 *Lysmata amboinensis* Z1, showing sessile eyes and the maxillipeds. Abbreviations: an, antennae; ant, antennules; E, eyes; mxp2, 2nd maxillipeds; mxp3, 3rd maxillipeds. Note: all appendages are paired but for display purposes are only labelled on one side of the shrimp.

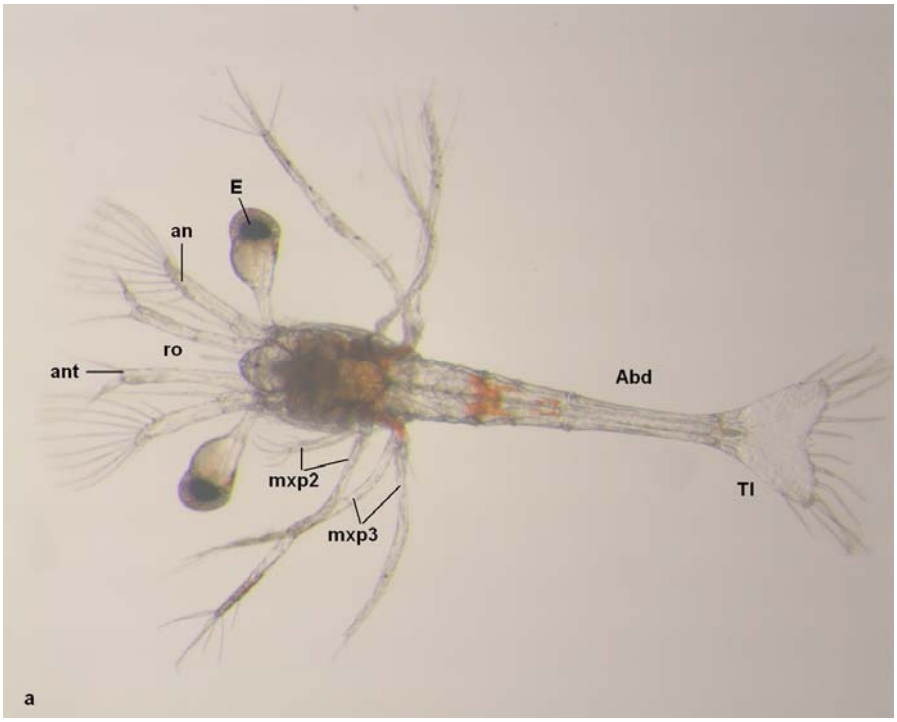


PLATE A.2 *Lysmata amboinensis* Z2, showing a) eyes on stalks and the maxillipeds, b) biramous bud of 1st pereiopod. Abbreviations: Abd, abdomen; an, antennae; ant, antennules; E, eyes; mxp2, 2nd maxillipeds; mxp3, 3rd maxillipeds; prb1, 1st pereiopod bud; ro, rostrum; Tl, telson.

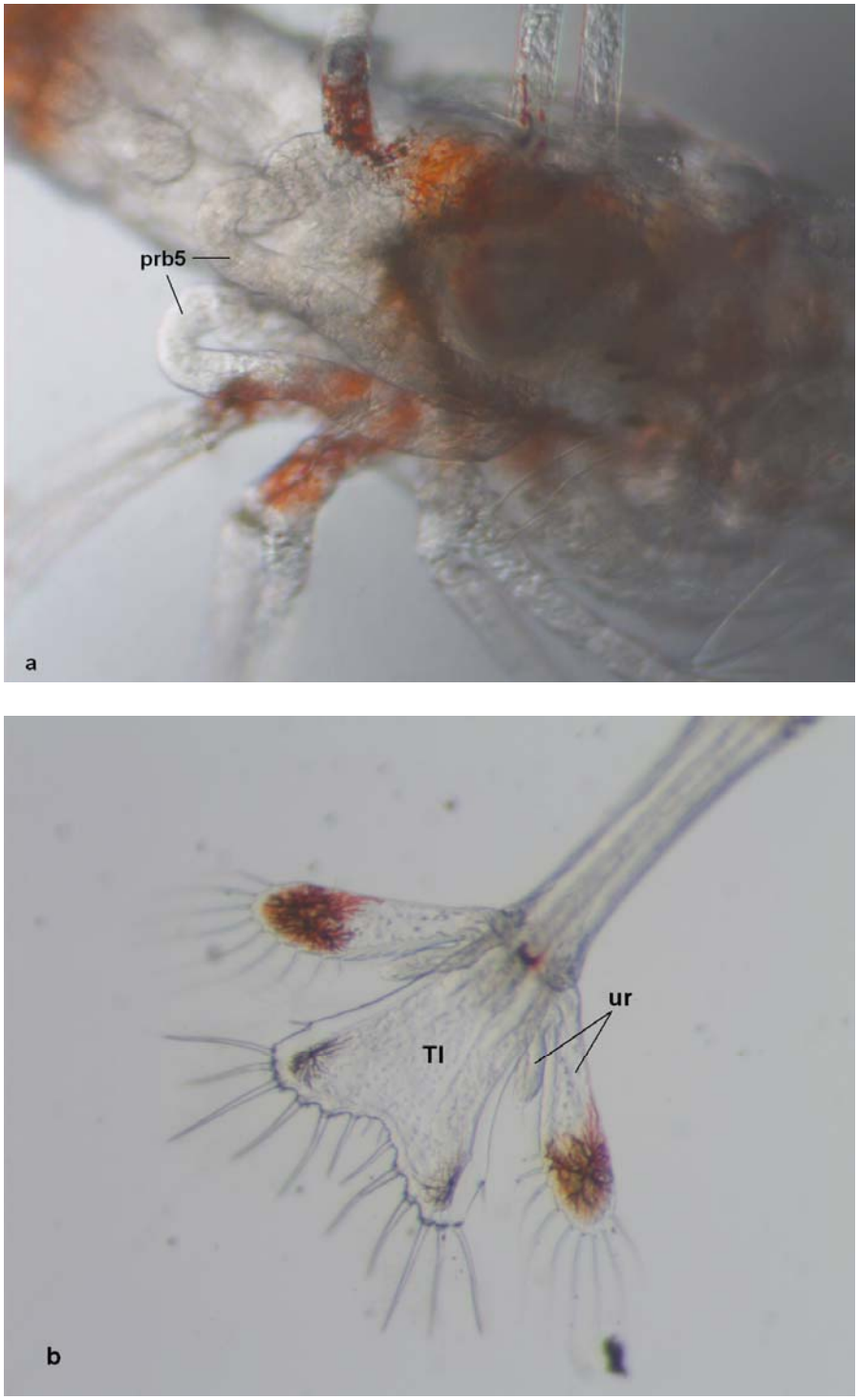


PLATE A.3 *Lysmata amboinensis* Z3, showing a) uniramous bud of 5th pereiopod and b) biramous uropods developing on telson. Abbreviations: prb5, 5th pereiopod bud; Tl, telson; ur, uropods.

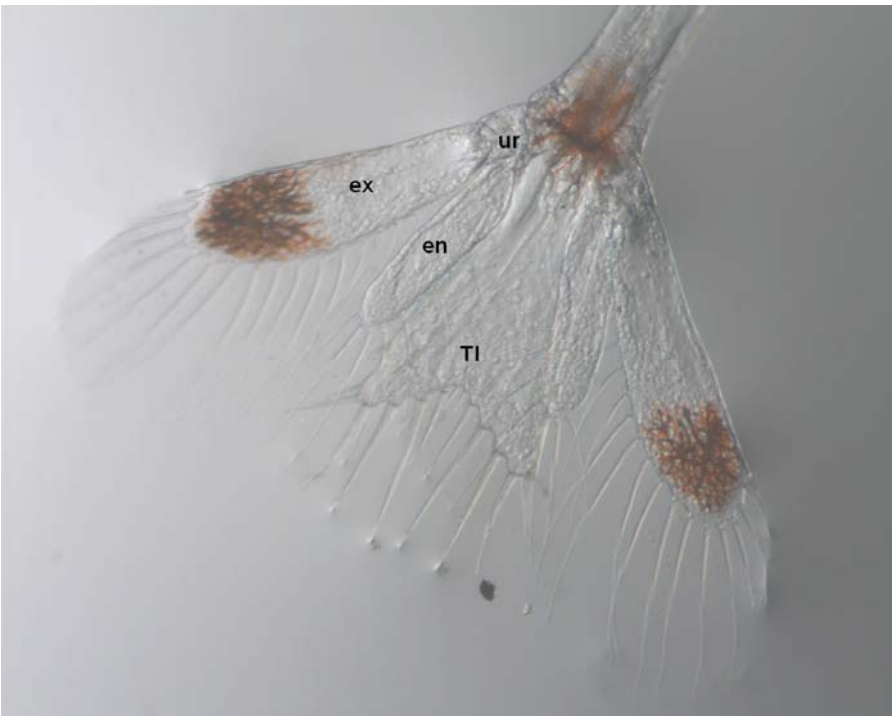


PLATE A.4 *Lysmata amboinensis* Z4, showing the exopod and endopod of uropods. Abbreviations: en, endopod; ex, exopod; Tl, telson; ur, uropods.

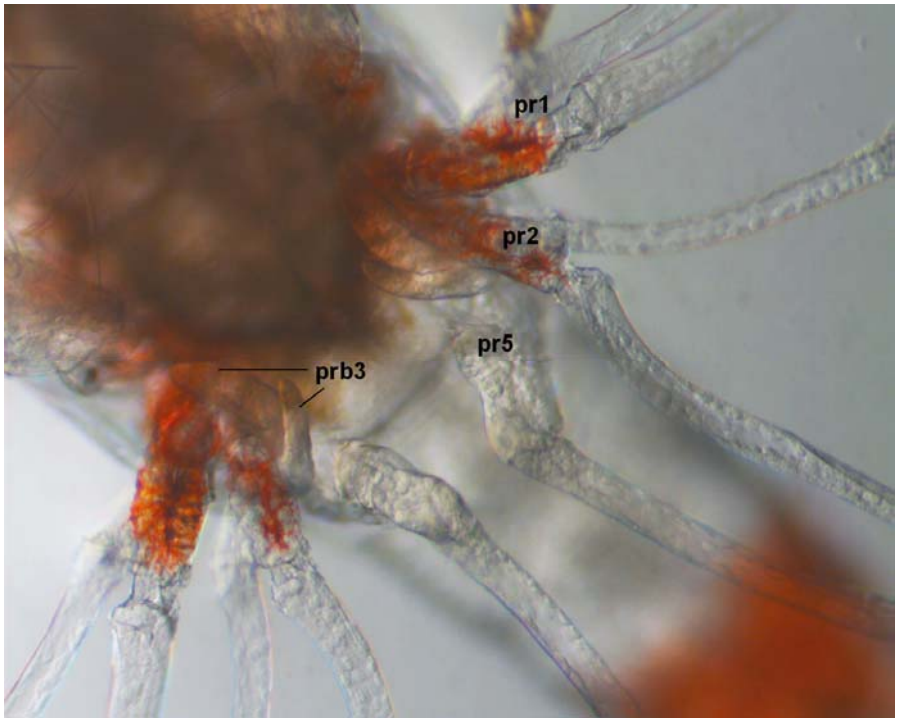


PLATE A.5 *Lysmata amboinensis* Z5, showing the pereiopods. Abbreviations: pr1, 1st pereiopod; pr2, 2nd pereiopod; pr5, 5th pereiopod; prb3, 3rd pereiopod bud.

PLATE A.6 *Lysmata amboinensis* Z6, showing the maxillipeds and pereiopods. Abbreviations: mxp2, 2nd maxillipeds; mxp3, 3rd maxillipeds; pr1, 1st pereiopod; pr2, 2nd pereiopod; pr3, 3rd pereiopod; pr5, 5th pereiopod.

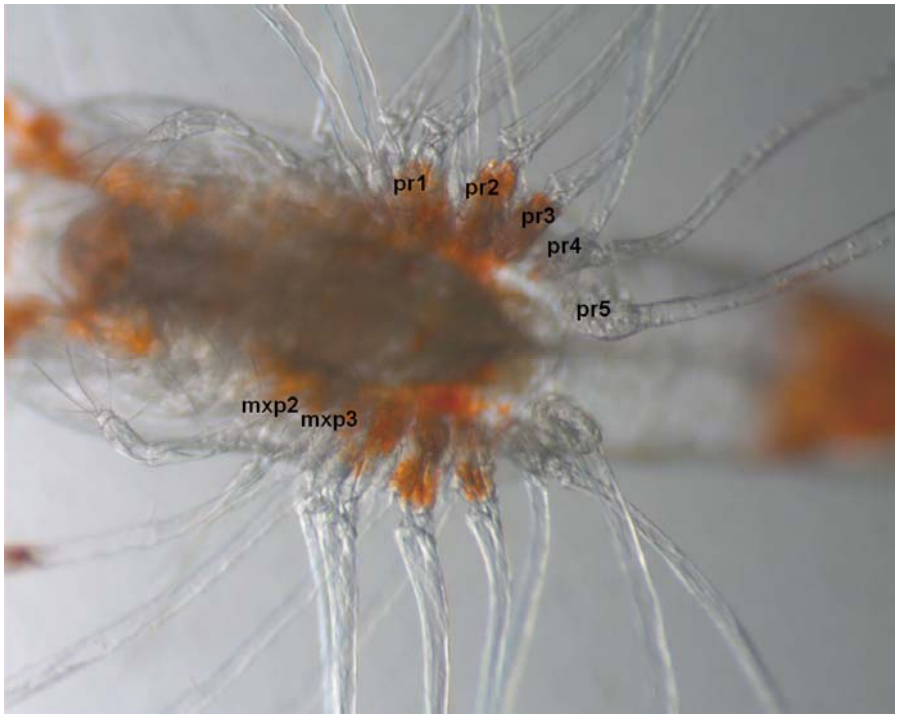


PLATE A.7 *Lysmata amboinensis* Z7, showing the maxillipeds and pereiopods. Abbreviations: mxp2, 2nd maxillipeds; mxp3, 3rd maxillipeds; pr1, 1st pereiopod; pr2, 2nd pereiopod; pr3, 3rd pereiopod; pr4, 4th pereiopod; pr5, 5th pereiopod.

PLATE A.8 *Lysmata amboinensis* Z8, showing the scaphocerite and flagellum of the antennae and the peduncle of the antennules. Abbreviations: E, eye; f, flagellum; pe, peduncle; scc, scaphocerite.

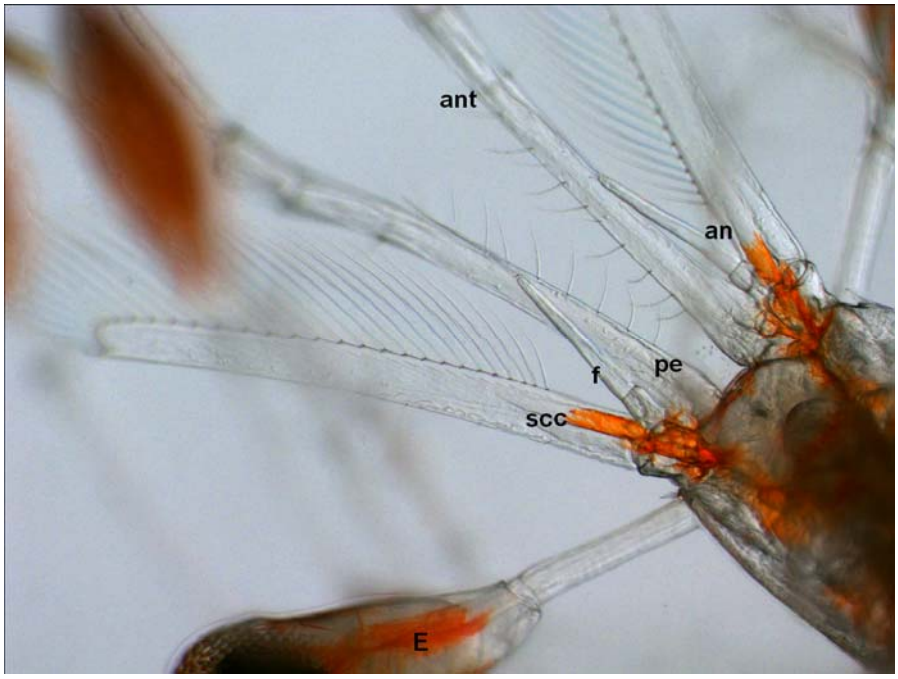


PLATE A.9 *Lysmata amboinensis* Z9, showing the scaphocerite, 2-segmented flagellum of the antennae and the peduncle of the antennules. Abbreviations: an, antennae; ant, antennules; E, eye; f, flagellum; pe, peduncle; scc, scaphocerite.

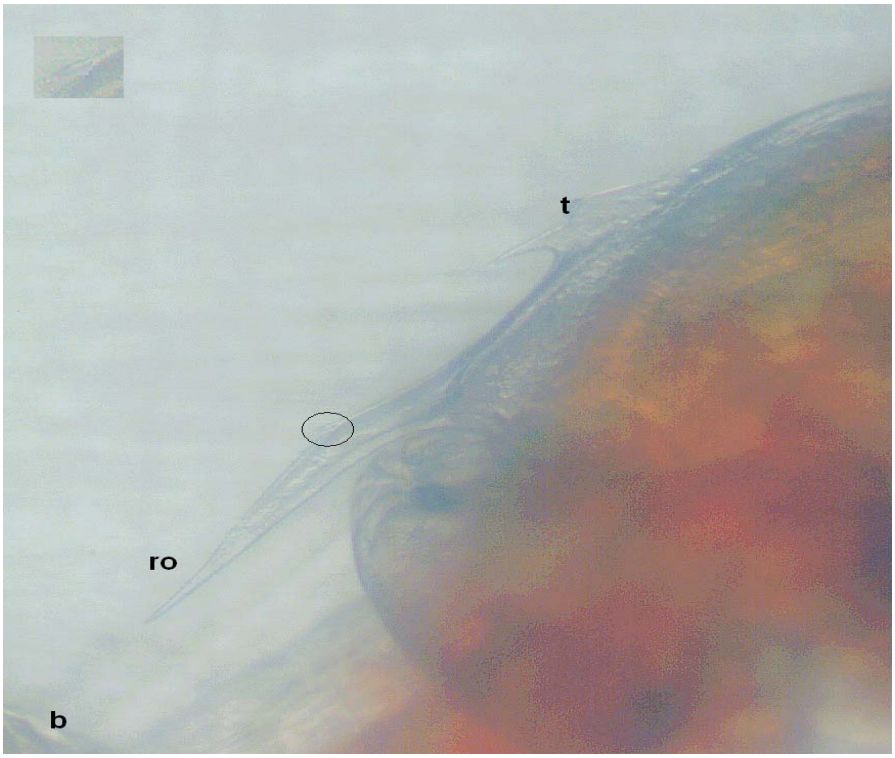


PLATE A.10 *Lysmata amboinensis* Z10, showing a) pleopods developing on the abdomen, b) rostrum with small spine (circled area) and tooth. Inset: magnification of the spine. Abbreviations: Abd, abdomen; pl, pleopod; ro, rostrum; t, tooth.

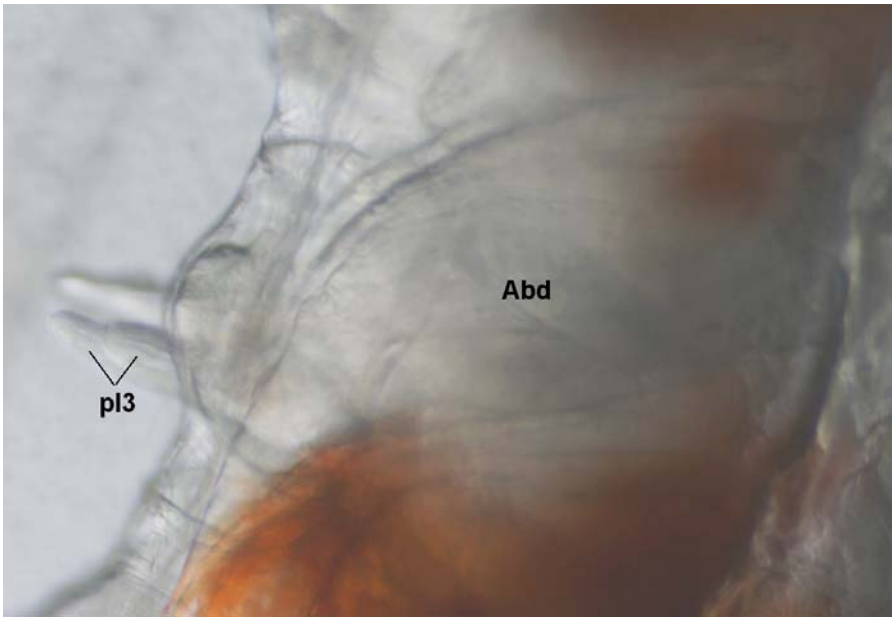


PLATE A.11 *Lysmata amboinensis* Z11, showing the biramous bud of the 3rd pleopod. Abbreviations: Abd, abdomen; pl3, 3rd pleopod.

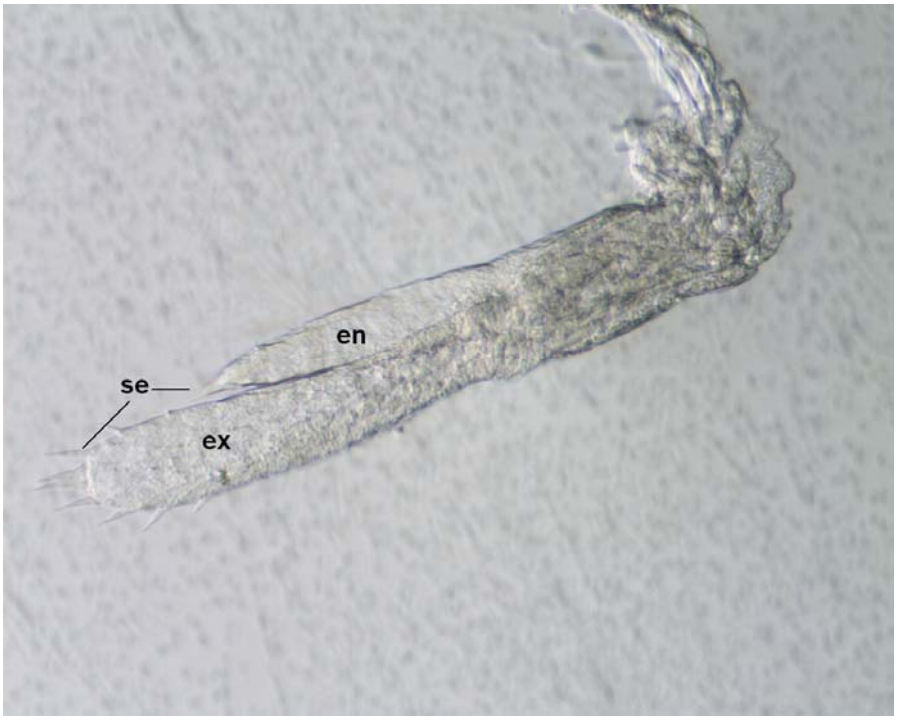
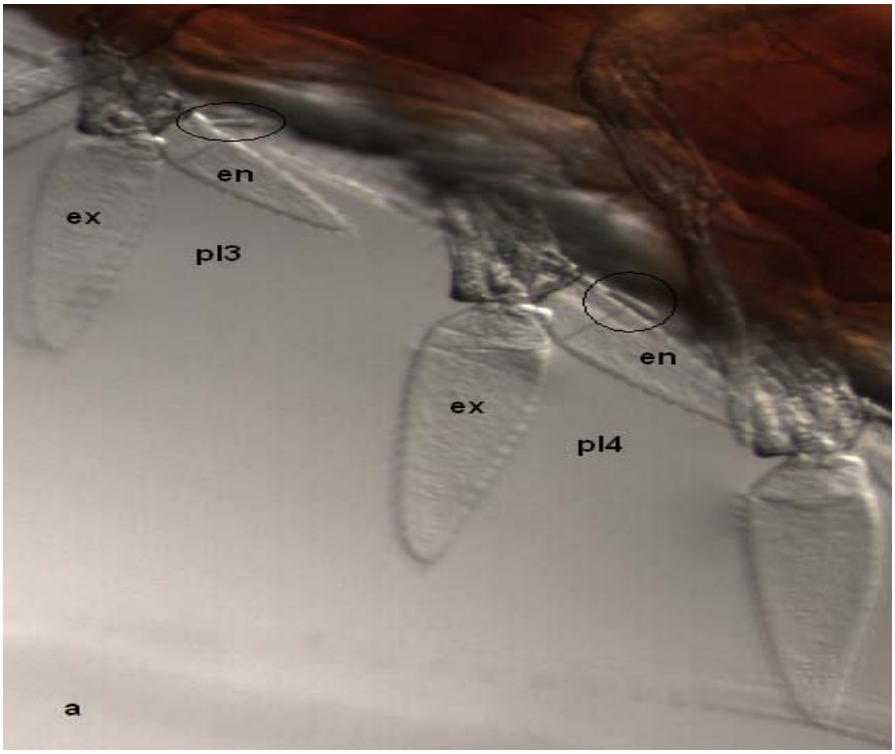


PLATE A.12 *Lysmata amboinensis* Z12, showing setae on the endo- and exo- pod of the 4th pleopod. Abbreviations: en, endopod; ex, exopod; se, setae.

PLATE A.13 *Lysmata amboinensis* Z13, showing setae on the endo- and exo- pod of pleopods. Abbreviations: se, setae; en, endopod; ex, exopod.



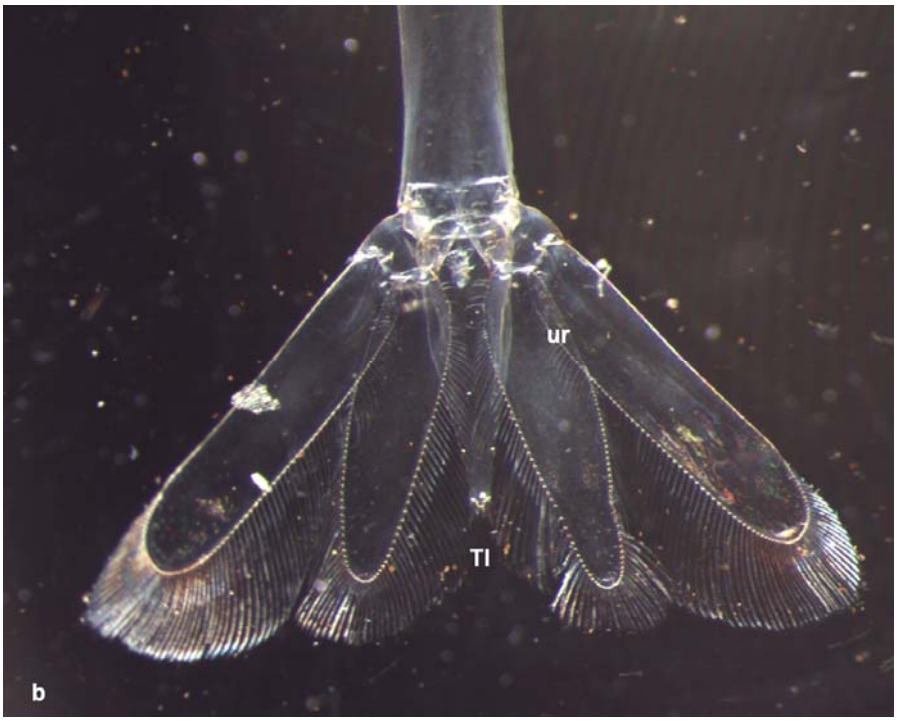


PLATE A.14 *Lysmata amboinensis* Z14, showing a) *appendix interna* (circled area) on the endopods of the 3rd and 4th pleopods and b) uropods and telson. Abbreviations: en, endopod; ex, exopod; pl3, 3rd pleopod; pl4, 4th pleopod; Tl, telson; ur, uropods.

PLATE A.15 Three late stage *L. amboinensis* larvae, feeding on juvenile *Artemia*.

PLATE A.16 Newly settled *L. amboinensis* post-larva, feeding on adult *Artemia*.

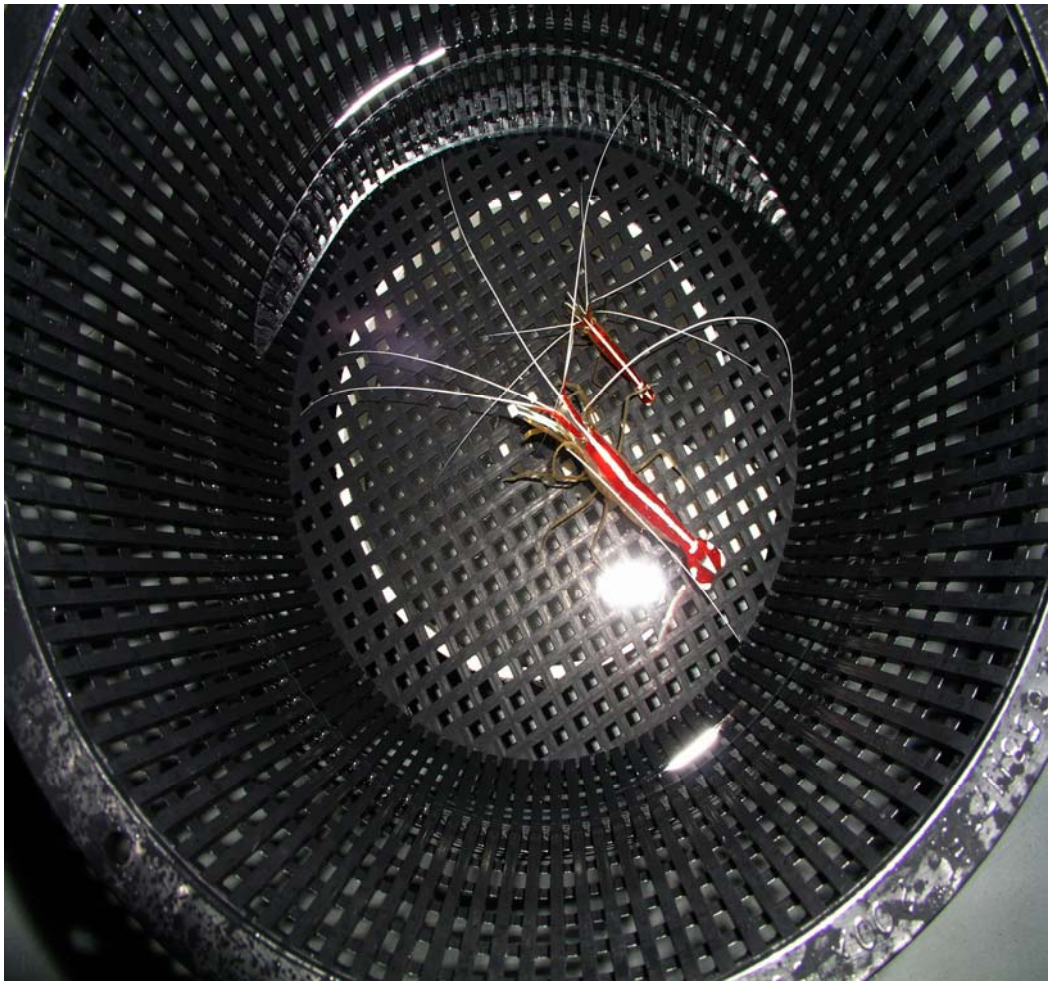


PLATE A.17 Captive *L. amboinensis* pair, comprised of the juvenile male (smaller individual) and the hermaphrodite adult (larger individual).