Digestive enzyme dynamics during early life stages of the mud crab, *Scylla serrata* and the spiny lobster, *Panulirus ornatus*

Thesis submitted by

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ABSTRACT

The mud crab, *Scylla serrata* and the spiny lobster, *Panulirus ornatus,* are high value crustaceans in the tropics and sub-tropics of the Indo-Pacific region subject to intense fisheries pressure, particularly in Asia where no catch limits are imposed and fishery laws are often not strictly enforced. Because of increasing demand and dwindling fisheries landings, interest in aquaculture of both species has grown strongly over recent years. However, continued dependence on wild seed for stocking has been a major bottleneck for expansion and further development of aquaculture industries for both species. Hatchery techniques for these species have received significant research attention over recent years but considerable development is required to further improve survival and bring commercial viability to hatchery operations.

One of the most important yet poorly understood components of hatchery production of crustaceans is larval nutrition, particularly the aspects of larval nutritional requirements and digestive capacity. Larvae of aquatic animals, particularly early larvae, rely primarily on chemical digestion of ingested foods with the aid of enzymes. This study assayed the major digestive enzymes during larval development of both *S. serrata* and *P. ornatus* to assess larval capacity to digest major nutrients and to evaluate the relative utilization of these nutrients for energy: (a) during embryonic development and starvation of the newly hatched larvae (Chapter 3); (b) under different conditions of intermittent food availability (Chapter 4); (c) in response to different food quantity and quality (Chapter 5); and (d) at different stages of the moult cycle and larval ontogeny (Chapter 6).

Following the general introduction (Chapter 1) and general materials and methods (Chapter 2) sections, changes in activities of the three major digestive enzymes (amylase, protease and esterase) during embryonic development of *S. serrata* and *P. ornatus*, as well as in unfed newly hatched larvae, were examined in Chapter 3. For both species, esterase activities started to increase significantly during the early phase of embryonic development while amylase and protease activities remained at about the same levels, suggesting that lipids were the nutrients most heavily utilized during the early embryonic development in both species. However, towards the end of embryonic development, amylase and protease activities increased while esterase activities showed decreasing trends, suggesting that as lipid reserves were depleted and became insufficient to meet the increasing energy demand, protein reserves, and to some extent carbohydrates, were increasingly utilized. It was further shown that proteins continued to be the main energy source of newly hatched larvae during the initial phase of starvation for both *S. serrata* and *P. ornatus* as higher levels of protease compared to esterase and amylase were present in starved newly hatched larvae of both species.

Chapter 4 was designed to examine changes in the major digestive enzyme activities of first feeding larvae of *S. serrata* and *P. ornatus* subject to different food availability conditions: (a) when food was immediately available (fed) vs. when food was not immediately available (starved); (b) when food was initially available (fed) and then withdrawn; and (c) when initial feeding was delayed for different durations. These experiments were intended to obtain insights into how first feeding larvae manipulate their enzyme activities in order to adapt to various conditions of intermittent food availability likely to occur in their natural environment, which should provide useful information for the development of larval formulated diets and hatchery feeding protocols. The enzyme activity responses of first feeding zoeae of *S. serrata* suggested that protein reserves were the main

energy source while no food was available, but where food is available, first feeding zoeae spared proteins and utilized carbohydrates and lipids more extensively. In starved zoeae, protease activity, which was comparably much higher than amylase and esterase activities, remained high throughout the 72 h sampling duration. In contrast, protease activity in fed zoeae initially decreased sharply to very low levels although it eventually increased prior to moulting. Meanwhile, amylase and esterase activities gradually increased, suggesting that fed larvae were possibly building-up protein reserves.

The enzyme reponses of first feeding *P. ornatus* phyllosoma suggest their ability to utilize all three major nutrients, i.e., carbohydrates, proteins and lipids, but also highlight their capacity to prioritise the use of carbohydrates when fed. This was illustrated by the immediate and notable increase in amylase activity in fed phyllosoma, which remained high over following days, however such a phenomenon was not observed in starved larvae. When food was removed after the phyllosoma were fed for 24 h, amylase activity decreased back to low levels, suggesting that the phyllosoma quickly responsed to the withdrawal of food by substantially reducing their utilization of carbohydrates and shifting to greater utilization of proteins and lipids.

Newly hatched zoeae of *S. serrata* and phyllosoma of *P. ornatus* both showed an ability to compensate for delayed food availability by increasing amylase activity when initial feeding was delayed for varying durations. Both specific and total amylase activities of newly hatched zoeae where feeding was delayed for a period of 12 h to 36 h, were significantly higher than those of larvae fed immediately after hatching for the same 12 h duration. In newly hatched phyllosoma where feeding was delayed for a period of 24 h before being fed for 24 h, both specific and total amylase activities were also significantly higher than those fed immediately after hatching for the same 24 h period. However, such a response was no longer observed when feeding was delayed beyond 24 h, suggesting that the ability of newly

hatched phyllosoma to compensate for the delayed food intake diminished as the starvation period extended.

In Chapter 5, the digestive enzyme responses of larvae to quantity and quality of foods, particularly in terms of food density and food type were investigated. The results of this chapter clearly showed that both food quantity and quality significantly influenced larval digestive enzyme dynamics. Both *S. serrata* and *P. ornatus* larvae showed an ability to maximize the utilization of available food by increasing their digestive enzyme activities in response to increasing prey density. Comparison of enzyme activities of Zoea I *S. serrata* fed different densities of rotifers showed that the rotifer densities that resulted in maximal digestive enzyme activities fell within the range considered optimal for larval rearing of this species. A similar result was obtained for Stage I phyllosoma of *P. ornatus* fed different densities of *Artemia* nauplii. These results together suggest that digestive enzyme activity can be a good indicator of appropriate prey density used in larval rearing.

The digestive enzyme responses of Zoea II and megalopae of *S. serrata* to different types of food helped identify their relative nutritional values to the larvae. The digestive enzyme responses of Zoea II fed rotifers and *Artemia,* respectively, largely reflected the relative proximate contents of these two most commonly used hatchery prey. Similarly, the enzyme activities of megalopae fed either a microbound diet (MBD) developed in this laboratory or *Artemia* also indicated differences in digestibility and nutritional quality of *Artemia* compared to the formulated MBD. Huge differences in amylase activity detected between megalopae fed the MBD and those fed *Artemia* indicated a possible significant role of dietary carbohydrates in megalopal nutrition and a carbohydrate deficiency in the MBD used.

In Chapter 6, changes in digestive enzyme activities during the moult cycle and in the course of larval ontogeny of *S. serrata* and *P. ornatus* were examined. The results of activity changes in the major digestive enzymes related to the moult cycle of newly hatched Zoea I and Megalopa as the postlarvae of *S. serrata,* as well as Stage I and II phyllosoma of *P. ornatus,* provided insights into the utilization of major nutrients during the recurring episodes of feeding activity changes related to the moult cycle of larvae. During the moult cycle of *S. serrata* Zoea I, newly hatched zoeae appeared to initially spare proteins and relied more on carbohydrates and lipids for energy, but as the moult cycle progressed, all three major nutrients were utilized when the larvae were actively feeding during the inter-moult stage. On the other hand, newly moulted megalopae seemed to initially utilize carbohydrates and proteins heavily while sparing lipids, however, as the moult cycle advanced, all three major nutrients were utilized and, during the second half of the moult cycle, megalopae relied more on lipids.

During the moult cycle of *P. ornatus* Stage I phyllosoma, newly hatched larvae initially utilized carbohydrates and proteins to a greater extent, however as they developed towards moulting, there was a trend of gradually increasing lipid utilization. During the initial phase of the moult cycle of Stage II phyllosoma, utilization of carbohydrates and lipids was increased while protein utilization was slightly reduced. During the second half of the moult cycle, lipids were increasingly utilized as the phyllosoma approached moulting.

Changes in major digestive enzyme activities as larvae developed progressively into subsequent stages revealed ontogenetic improvements in the digestive capacity of both *S. serrata* and *P. ornatus* larvae. Comparison of results of specific and total activities showed that total activity appeared to be a better way of expressing changes in enzyme activities during larval ontogeny. In *S. serrata* larvae, the total activities of the major digestive enzymes clearly increased with larval development but more dramatic improvements

occurred at Zoea IV, Zoea V and Megalopal stages. During the development of *P. ornatus* phyllosoma from Stages I to V, the activities of all three major digestive enzymes also increased from one stage to next stage, but these increases were much more pronounced at Stage IV and V, where protease and esterase activities more than doubled. These results suggest that significant improvements in digestive capacity occur from Zoea IV onward for *S. serrata* and from Stage IV for *P. ornatus,* which may imply better chances of success in introducing formulated diets at, or beyond, these larval stages.

Chapter 7 summarizes the results of this study and offers general discussion within a broader context. Overall, the results of this research clearly demonstrated that analysis of digestive enzyme activities is a valuable method for the study of larval nutrition. For example, digestive enzyme activities could be used to indicate the relative utilization of various nutrients by the developing embryo and newly hatched larvae, providing useful information that could be used in the formulation of broodstock diets that could improve the quality of the newly hatched larvae. The enzyme responses of larvae to various conditions of food availability, food quality and quantity, as well as during recurring moulting cycles, also provides important information that could be used as the basis for devising appropriate larval feeding regimes and feeding protocols in hatcheries. Furthermore, changes in enzyme activities during larval ontogeny provide information on the digestive capacity of the various larval stages, providing clues regarding the suitable timing for introduction of formulated diets.

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STATEMENT ON SOURCES DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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CHAPTER 1

General Introduction

1.1 Prospects and constraints of *Scylla serrata* and *Panulirus ornatus* aquaculture production.

The mud crab, *Scylla serrata* and the spiny lobster (also known as tropical ornate rock lobster) *Panulirus ornatus* are emerging aquaculture species with huge economic potential in both Australia and the Indo-Pacific region (Figs. 1.1 and 1.2). *S. serrata* is the major mud crab species in Australia and is the largest and the fastest growing of the four species within the genus *Scylla* (Keenan, 1999). *P. ornatus* is likewise the fastest growing lobster within the family Palinuridae, and has the shortest larval phase among rock lobsters (Phillips *et al*., 1992; Johnston, 2006). These characteristics indicate suitability of *S. serrata* and *P. ornatus* as candidate species for aquaculture and, together with increasing global demand for both mud crabs and lobsters that continues to exceed supply from wild fisheries, have generated interest in developing an aquaculture sector for these species in tropical and sub-tropical countries around the world including Australia.

Fig. 1.0. Geographical distribution of mud crab, *Scylla* spp (FAO, 2015).

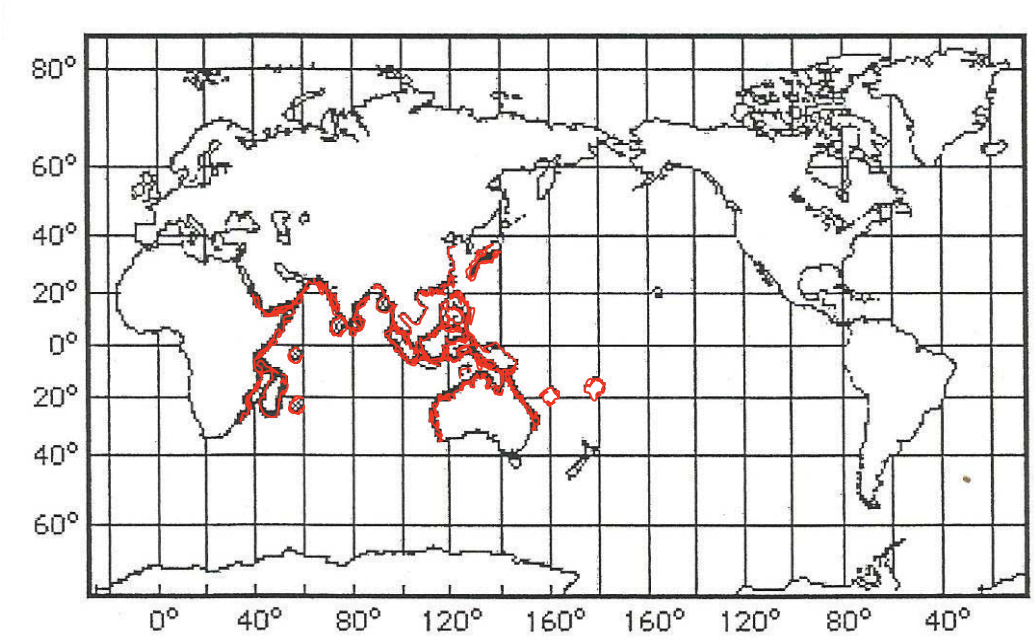

Fig. 1.2 Geographical distribution of *Panulirus ornatus* (from Holthuis, 1991)

Global aquaculture production of mud crabs increased from 6,882 tonnes in 1995 to 123,085 tonnes in 2005 (Shelley, 2008) and continued to increase to 179,536 tonnes in 2013 (FAO, 2015). However, continued, sustainable aquaculture production is a major challenge because the industry depends primarily on seed from the wild (Keenan, 1999; Shelley, 2008; Quinitio, 2015; Wang, 2015). In the case of spiny lobsters fisheries, more recent annual global production data is not available but production has been reported to fluctuate between 70,000 and 87,000 tonnes since 1983, except that in 2007, annual landings had the lowest level of 67,000 tonnes in 30 years (Jeffs, 2010). Considering that lobster aquaculture is still limited to the holding of juveniles and adults for weight gain (Johnston, 2006), world production data practically represent the total harvested stocks from the wild. While there may still be unexploited areas for harvesting wild crabs or lobsters, and fishing pressure could be increased further in some existing fishing grounds, the resulting increases in production will not be able to match increasing demand for these crustaceans. Aquaculture may have an important role in meeting growing demand for crabs and lobsters into the future,

but unless the current problem of seed supply is solved (i.e. through development of routine hatchery production of seed), further industry development cannot be sustained.

Hatchery production of *S. serrata* has received significant attention in several countries over the past decade[1], yet the technology is still largely confined to research institutions. While there are now a few commercial hatcheries for another 'mud crab' species (*Scylla paramamosain*) in Southeast Asia, the private sector is still hesitant to invest in hatchery production of *S. serrata* because of inconsistent production. Similarly, hatchery production of lobsters is considerably more difficult than that for other crustaceans because of their prolonged larval phase. For example, even though *P. ornatus* has the shortest larval phase among rock lobsters, it still takes 4 to 6 months to complete (Phillips *et al.*, 1992; Dennis *et al.*, 1997; Johnston, 2006). This extended larval phase makes completion of the larval stages in the hatchery particularly challenging with significant risks; these include substantially higher chances of acquiring diseases during the hatchery phase (Bourne *et al*., 2005; Payne *et al*., 2006). Significant improvement in larval survival of *P. ornatus* has been achieved after years of concerted effort by private companies and research institutions, but consistency of production and scaling-up of cultures systems remain as major challenges (Rogers *et al*., 2010).

1.2 The need for suitable formulated diets in hatchery production and the role of digestive enzyme studies in the development of larval diets.

Consistent high-volume hatchery production depends on the availability of suitable feeds that support rapid growth and good health of larvae. Formulated diets offer several advantages such as reduced feed costs, "off the shelf" convenience, short to medium term storage plus the possibility of adjusting the size of food particle and nutritional

[1] e.g., Australian Centre for International Agricultural Research - Mud Crab Project involving Australia and Philippines; Culture and Management of *Scylla* Project involving European countries, Vietnam and Philippines.

composition to suit the exact developmental stage of the larvae (Southgate & Partridge, 1998). In contrast, live foods, although routinely used in the majority of aquaculture hatcheries, have disadvantages including nutritional inconsistency (Bromley, 1978; Watanabe *et al.*, 1983; Lubzens *et al.*, 1989; Chaitanawisuti & Menasveta, 1989), high cost (Pearce, 1991; Rimmer & Rutledge, 1991), the need for specialized equipment and labour (Kanazawa *et al*., 1989), a requirement for dedicated hatchery space (Rodgers & Barlow, 1987), recurring culture 'crashes' (rapid, large scale mortality) and the potential as vectors for disease introduction (Person-Le Ruyet, 1990).

It is apparent that the current dependence of mud crab and lobster hatcheries on live food organisms as a larval food source, contributes significantly to their inconsistent production. For the mud crab, *S. serrata*, for example, high larval mortality is common when larvae moult from Zoea V to Megalopa, and from Megalopa into juvenile crabs (Holme *et al*., 2006b). Such mortality is often associated with 'moulting death syndrome' (MDS), a phenomenon where larvae are unable to completely shed their old carapace before the new one hardens (Williams *et al*., 1999). Although not fully understood, MDS is believed to be linked to inappropriate nutrition during larval development (Williams *et al*., 1999; Perry 2001).

Most mud crab hatcheries currently use 'traditional' live foods such as rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.) nauplii (Keenan, 1999) to feed larvae, but these prey species lack the essential highly unsaturated fatty acids (HUFA) required for developing marine larvae (Southgate, 2003). However, research has shown that *S. serrata* megalopae readily ingest formulated diet particles (Genodepa *et al.*, 2004a, 2004b; Holme *et al*., 2006a, 2006b), and these findings have provided the impetus for research to develop a nutritionally suitable, high quality microbound diet which will be an important step towards more effective hatchery production of mud crabs. Despite this seemingly promising

development, a bottleneck to further improvement of formulated diets for *S. serrata* larvae is our limited knowledge of their nutritional requirements and digestive capacity. Success in total replacement of live food with formulated diets during the early developmental stages of *S. serrata* were achieved with megalopae (Genodepa *et al*., 2004b) but not for the earlier larval stages (Holme *et al.,* 2006a). Furthermore, more information is required to fully understand the nutrient requirements of *S. serrata* larvae because available data are limited to studies conducted on late zoeal stages and megalopae (Suprayudi *et al*., 2002, 2004a, 2004b; Holme *et al*., 2006b, 2007a, 2007b, 2009a, 2009b).

In hatchery rearing of spiny lobsters, brine shrimp (*Artemia* spp.) nauplii are also provided to phyllosoma larvae because of their suitable size, movement and nutritional value (Inoue, 1965, 1978; Tong *et al*., 1997). Despite these favourable attributes of *Artemia*, survival of early phyllosoma stages is generally low (Phillips & Sastry, 1980; Kittaka, 1994; Kittaka & Abrunhosa, 1997; Johnston, 2006). The use of *Artemia* in combination with fish larvae and mussel meat or gonad has proven successful for rearing phyllosoma of many lobster species through puerulus (Kittaka, 1988, 1997; Ritar *et al*., 2002, 2003; Smith *et al*., 2009a), but the absolute dependence on *Artemia* and fresh foods is a limiting factor for commercial hatchery culture of spiny lobsters. Inherent problems still remain, including variable nutrient composition and availability, the high cost of labour and infrastructure and potential introduction of pathogens into the culture system (Ohs *et al*., 1998).

Development of suitable formulated diets for hatchery production of *P. ornatus* and other palinurids is being pursued aggressively by various sectors. While significant improvements have been reported (Smith *et al*., 2009a; Barnard *et al*., 2011), more information is still required to develop more efficient and cost-effective diets. An appropriate formulated diet can be developed most effectively when the ingestive and digestive morphology, physiology as well as feeding behaviour are fully understood (Johnston, 2006).

However, present knowledge on environmental conditions, natural prey and nutritional requirements of palinurid is limited (Philips & Sastry, 1980; Tong *et al*., 1997). Our limited understanding of the natural diet of phyllosoma, their nutritional requirements and digestive capabilities, stands as the major impediment to successful hatchery culture of palinurid phyllosoma (Macmillan *et al*., 1997; Johnston & Ritar, 2001; Nelson *et al*., 2002; Cox & Johnston, 2003, 2004).

Limited understanding of larval digestive capacity and nutrition is the major obstacle for the development of suitable formulated diets for hatchery production. Adults of potential aquaculture species are usually well studied and their digestive physiology quite well understood, but in most cases, very little is known about their larvae. Reviews of the digestive physiology of decapod crustaceans by several authors (Icely & Nott, 1992; Ceccaldi, 1997; Jones *et al.*, 1997a, 1997b) highlight that larval crustaceans are not as well equipped as the adults with regard to mechanical digestion of ingested food, and must rely primarily on chemical (enzymic) digestion. Enzymes are able to accelerate, or produce by catalytic action, some changes in a substrate for which they are specific (Gilbault, 1976). In decapod crustaceans, digestive enzymes are formed in the hepatopancreas by specialized cells known as F-cells (that look fibrous) and B-cells (that appear blister-like) (Gibson & Barker, 1979; Icely & Knott, 1992). Digestive enzymes play very important roles in the digestion of food by catalysing the chemical breakdown of food particles into forms that can be utilized for activity, maintenance and growth. Because of this close relationship between diet and the digestive enzymes produced to process the diet, qualitative and quantitative analysis of digestive enzymes has become an important tool in assessing the digestive ability of an organism.

The presence and concentration of digestive enzymes are known to indicate the relative importance of particular components of the diet (Van Wormhoudt, 1973; Galgani *et al*., 1984; Lee *et al*., 1984; Rodriguez *et al*., 1994; Johnston, 2003), as well as the digestive potential of an organism at various stages of ontogenetic development (Hammer *et al*., 2000; Lemos *et al*., 2000; Furne *et al*., 2005). Studies on digestive enzymes can help elucidate the ability of organisms to hydrolyse various dietary nutrient in response to different nutrient sources, levels, influx and secretory changes during ontogeny (Lee *et al.*,1984; Ocampo & Ezquerra, 2002). In crustacean larvae, changes in digestive enzyme activity during development (Galgani & Benyamin,1985; Hirche & Anger, 1987; Lovett & Felder, 1997a, 1997b) have been used by researchers as basis for determining the nutritional needs and feeding transition of larvae (Biesiot & Capuzzo, 1990; Kamarudin *et al*., 1994). Digestive enzyme activities of crustaceans have also been used to assess the feasibility of feeding with compound diets instead of live food, either partly or entirely, and to evaluate the nutritional value of diets containing different components (Teshima *et al*., 1986; Sheen & Huang, 1998; Brito & Chimal, 2000; Pan *et al.*, 2005). Data pertaining to digestive enzyme activity and profiles have helped overcome nutritional problems associated with formulation of artificial diets that meet the nutrient requirements of the target species (Furne *et al.*, 2005). They also helped define the inclusion limits for macronutrients such as dietary protein and carbohydrates (Pavasovic *et al*., 2007). A better understanding of the digestive abilities and nutritional requirements of *S. serrata* and *P. ornatus* larvae could eventually lead to the design of more appropriate diets and feeding regimes (Lee *et al.*, 1984) which are both required to improve survival and to support more efficient and reliable hatchery production.

1.3 Studies on digestive physiology and nutrition of larval mud crabs and lobsters

Knowledge of the developing digestive capabilities of early life stages of *S. serrata* is still very limited. The ontogenetic changes in mouthparts of *S. serrata* larvae was first described by Ong (1964), however, a later examination of some mouthparts of *S. serrata* by Jones *et al.* (2001) implied that the samples of Ong (1964) could have been from a different mud crab species. A detailed description of the functional morphology of the mouthparts, gut and digestive physiology of *S. serrata* were reported by Barker & Gibson (1978), but were limited only to adult crabs. A more recent description of the ontogeny of mouthparts and foregut of *S. serrata* larvae reported by Lumasag *et al.* (2007) provided relevant information on the mechanical digestion of food. However, additional knowledge of the enzymes found in larvae would provide a better idea of larval digestive capability. Earlier studies on digestive enzymes of *S. serrata* were limited to juvenile (Rutledge, 1999; Pavasovic, 2004) and adult crabs (Barker & Gibson, 1978), but recently, there were reports on the ontogeny of endogenous and exogenous amylase, and total protease activities in mud crab larvae fed live foods (Serrano, 2012) as well as chymotrypsin and carboxypeptidases A and B (Serrano, 2013). But still the available information is very limited and more research is required to shed new light on the digestive capabilities of *S. serrata* larvae.

Studies on the digestive capacity of spiny lobsters have primarily focused on the morphology of mouthparts and digestive tract of adults and juveniles; the few studies on larval lobsters failed to include samples from all development stages (Johnston & Ritar, 2001). Studies to correlate mouthpart and foregut structure with function during ontogeny for all phyllosoma stages have been reported only for *Panulirus argus* (Wolfe & Felgenhauer, 1991) and *Jasus edwardsii* (Johnston & Ritar, 2001). An attempt to do a similar study with *P. ornatus* (Johnston, 2006) was constrained by limited availability of samples of several phyllosoma stages.

Published reports on the digestive enzymes present during the early life stages of spiny lobsters are likewise limited and most research has been conducted with *J. edwardsii*. Developmental changes in digestive enzyme activities of puerulus, post-puerulus, juvenile and adult *J. edwardsii* were studied by Johnston (2003) in order to assess capacity to utilise dietary components and to develop diets that would meet nutritional requirements. Digestive enzyme activities of cultured (Stage I to VI) and wild (Stage V to XI) phyllosoma larvae of *J. edwardsii* were also investigated by Johnston *et al*. (2004b) to provide an indication of digestive capabilities and nutritional requirements, and to help identify the characteristics of their natural prey. The impact of starvation on digestive activities of *J. edwardsii* Stage I to IV phyllosoma were also assessed by Johnston *et al*. (2004a) to identify nutrients metabolized or conserved during food deprivation, highlighting critical energy reserves.

Reports on digestive enzyme activities of *P. ornatus* are limited to studies conducted by Johnston (2006). Among the highlights of this research was the presence of a suite of digestive enzymes (proteases, carbohydrases and lipases) documented at day 0 and at day 5 in Stage I phyllosoma plus the presence of enzyme secreting F-cells, which was interpreted to indicate capability to digest prey from the onset of feeding. A general increase in the number of tubules and enzyme-secreting F-cells in the digestive gland was also reported, suggesting capability to modulate enzyme activity based on quality of food. Considering that the study of Johnston (2006) involved only a few stages of *P. ornatus*, further investigation of the morphological structure of the digestive gland and enzyme activities throughout development of the species was recommended.

1.9 Research aims and thesis structure

The general aim of this thesis was to examine changes in major digestive enzyme activities of the mud crab, *S. serrata*, and the spiny lobster, *P. ornatus* during their early stages of development, including the factors that affect these changes. This aim was pursued in an attempt to provide information which could serve as basis for development of suitable diets and feeding protocols for hatchery rearing of these species. The analysis of digestive enzymes was chosen as a tool to indicate the nutrient requirements and digestive capacity of larvae in this study.

This thesis consists of seven chapters including this general introduction (Chapter 1). The following chapter (Chapter 2) describes the general materials and methods used in the four research chapters (Chapter 3 to Chapter 6). This approach was deemed necessary to avoid repetition of some methods. Each data chapter has its own set of objectives which were pursued by conducting two or more experiments on both *S. serrata* and *P. ornatus*. The final chapter (Chapter 7) wraps up the study by presenting general conclusions. The experimental chapters of this thesis and their specific objectives were as follows:

Chapter 3 - Changes in digestive enzyme activities and nutrient utilization during embryonic development and starvation of the newly hatched larvae of *Scylla serrata* and *Panulirus ornatus.*

Objectives:

- To determine the levels of the major digestive enzymes at various stages of embryonic development and starvation of the newly hatched larvae;
- To determine which nutrients serve as critical energy reserves during embryonic development and during starvation of newly hatched larvae;

Chapter 4 - Digestive enzyme responses to intermittent food availability in first feeding larvae of *Scylla serrata* and *Panulirus ornatus.*

Objectives:

- To determine the enzyme response of first feeding larvae of *S. serrata* and *P. ornatus* to various conditions of intermittent food availability and determine how larvae adapt to such conditions: (1) when food was immediately available (fed) versus when food was not immediately available (starved); (2) when food was initially available (fed) and then withdrawn; and (3) when initial feeding was delayed for different durations.

Chapter 5 – Digestive enzyme responses of *Scylla serrata* and *Panulirus ornatus* larvae to quantity and quality of feeds: the effects of food density and food type

Objectives:

- To determine and compare the enzyme responses of first feeding *S. serrata* and *P. ornatus* larvae to different densities of rotifers and *Artemia* nauplii, respectively;
- To determine and compare the enzyme response of Zoea II *S. serrata* fed either with rotifers or *Artemia* nauplii;
- To determine and compare the enzyme response of *S. serrata* megalopae fed either *Artemia* or a formulated diet.

Chapter 6 - Changes in digestive enzyme activities related to the moulting cycle and larval ontogeny of *Scylla serrata* and *Panulirus ornatus*

Objectives:

- To examine changes in the activities of major digestive enzymes related to the moulting cycle of selected larval stages of *S. serrata* and *P. ornatus*.
- To examine ontogenetic changes in digestive enzyme activities throughout larval development (from Zoea 1 to Crab Instar I) of *S. serrata* and (from Stage I to V phyllosoma) of *P. ornatus*.

CHAPTER 2

General Methodology

2.1 Sources of Experimental Animals

2.1.1 Source of Mud Crab Eggs and Larvae

Mud crab, *S. serrata*, larvae were produced from spawners collected using baited traps from estuaries around Townsville, north Queensland, Australia, and reared in the aquarium facility of James Cook University (JCU) until they spawned. Species identity was confirmed according to Keenan *et al.* (1998). Spawners were held in 5 units 1000-L outdoor tanks with re-circulating seawater (28–36 ‰ salinity) and fed once daily with squid, mussel, shrimp and fish meat at a rate of 5 to 8% body weight. Berried crabs were disinfected using 50–80 mg L^{-1} formalin solution for 6 h and transferred to a 300-L indoor tank for egg incubation and hatching. The tank was provided with re-circulating seawater (at an exchange rate of 1.5 L min^{-1}) subject to mechanical filtration (to 1 µm) and UV treatment. Salinity in the incubation tank ranged from 32 to 36 ‰ while temperature ranged from 27 to 29 ºC.

The newly hatched larvae were reared up to crab stage using the protocol developed at JCU (Genodepa, 2003; Fig. 2.1). Briefly, the active larvae were attracted to a light source and collected using a scoop bowl soon after hatching and stocked into 300-L rearing tanks at a density of 100-150 larvae L^{-1}. Newly hatched larvae were transferred directly from the hatching salinity of 32–36 ‰ to 20–22 ‰, which was the rearing salinity used for Zoea I. Salinity in the larval culture tanks was gradually increased to between 25–28 ‰ as the larvae developed. The larvae were fed with rotifers (*Brachionus* sp.) and brine shrimp (*Artemia* sp.) nauplii. Rotifers were introduced once only at the rate of 40–60 individuals mL^{-1} on the first day of larval culture and maintained in the larval rearing tank by

daily addition of microalgae, *Nannochloropsis* sp. As the larvae grew older, the rotifer density was reduced gradually to a negligible level by the time they had become Zoea V. *Artemia* were first introduced into larval rearing tanks (at a density of 0.5 individuals mL^{-1}) on the second day after larvae have moulted to Zoea II, then gradually increased to 3–5 individuals mL^{-1} by the time the larvae reached Zoea V stage. At Megalopa stage, only *Artemia* nauplii were fed at a density 3–5 individuals mL^{-1}. Daily water exchange in larval culture tanks ranged from 15 to 25% at Zoea I and Zoea II stages to 30–50% from Zoea III onwards.

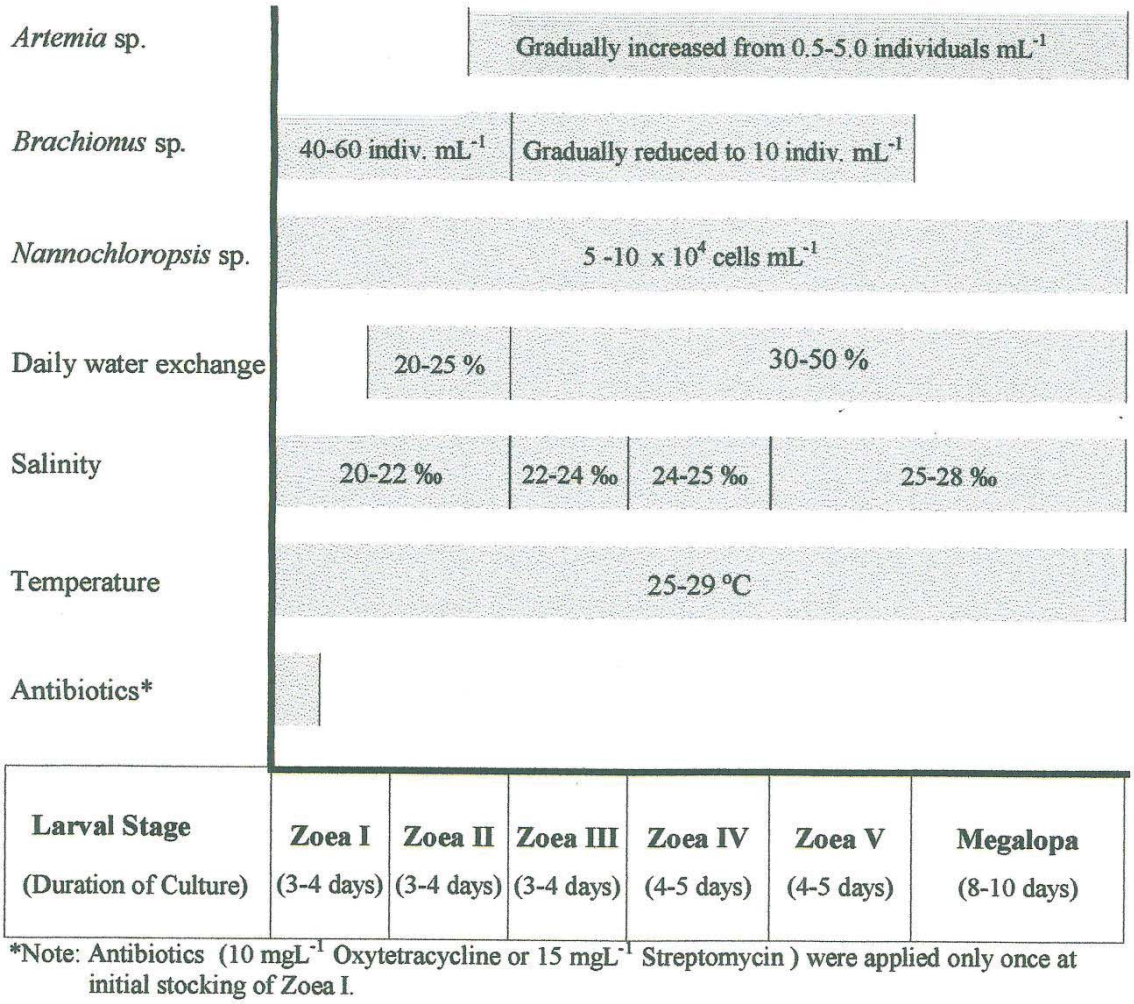

Fig. 2.1. Protocol developed for rearing of mud crab, *S. serrata* larvae at James Cook University (from Genodepa, 2003).

2.1.2 Source of Lobster Eggs and Larvae

Egg and phyllosoma samples were from wild-caught tropical rock lobster, *P. ornatus*, broodstock reared at the Tropical Aquaculture facility of the Australian Institute of Marine Science (AIMS), Townsville, Queensland, Australia. Details of broodstock maintenance and spawning of *P. ornatus* were reported by Bourne *et al.* (2004). Briefly, the broodstock were maintained in indoor 12,000-L circular tanks with flow-through seawater (34-35‰ salinity; 27-28 ºC temperature) under a 14L:10D photoperiod, and fed alternatively on a proprietary wet feed, blue mussel (*Mytilus galloprovincialis*) and squid (*Loligo opalescensat*) at a rate of 5-8% total body weight, adjusted daily on the basis of observed food consumption. Berried females with eggs estimated to be within 1-3 days of hatching were removed from broodstock tanks and held in a 200-L tub for 4 h and treated with 25 mg L^{-1} formalin, 8 mg L^{-1} oxytetracycline, 9 mg L^{-1} erythromycin and 20 mg L^{-1} streptomycin for 4 h to partially surface sterilize the female and eggs. After treatment, berried females were held in a 1000-L hatching tank with flow through seawater at a turnover rate of 100% h^{-1}. Berried females were not fed in the hatching tank and faeces together with any discarded eggs were siphoned daily. On the day of hatching, active and photopositive phyllosoma were collected from the surface of the hatching tank and transferred into a 20-L container of clean seawater for estimation larvae numbers before the newly hatched phyllosoma were volumetrically dispensed into mass culture vessels.

Production of larvae was based on the standard rearing protocol for *P. ornatus* at AIMS described by Smith *et al*. (2009). Briefly, newly hatched phyllosoma were stocked at a density of 30 larvae L^{-1} in rearing vessels with a flow-through of treated seawater (34-35‰ salinity, 27-28 °C temperature) and fed according to the general rearing protocol summarized in Table 2.1.

Table 2.1 General feeding protocol developed for ornate rock lobster, *P. ornatus*, larvae at the Australian Institute of Marine Science.

Larval stage	*Live foods regime*	*Average instar duration*
Stage I-III (Instar 1-5)	Newly hatched *Artemia*, 1.5 -4 nauplli mL^{-1} day^{-1}	5-6 weeks
Stage IV-VI (Instar6-11)	Enriched Day 6-7 *Artemia* (body length 1.5-2.3mm), 1 $mL^{-1}day^{-1}$ + chopped mussel gonad, 20mg L^{-1} day^{-1}	6-7 weeks
Stage VII-VIII (Instar12-18)	Enriched Day 9-10 *Artemia* (body length 2.5-3.1mm), 1 $mL^{-1}day^{-1}$ + chopped mussel gonad, 20mg $L^{-1}day^{-1}$	7-8 weeks
Stage IX-XI (Instar19-24)	Enriched Day 12-13 *Artemia* (body length > 3.1 mm), 1$mL^{-1}day^{-1}$ + chopped mussel gonad 20mg L^{-1} day^{-1}	6-7 weeks

2.2 Enzyme Assays

2.2.1 Sample Preparation

Egg and larval samples collected from the experiments in this study were placed in 1.7 mL Eppendorf tubes and stored in an ultra-cold freezer (-70°C) until they were assayed for digestive enzymes. Samples for enzyme assay were diluted in ice-cold deionized water (50x dilution, weight: volume) and homogenized using a handheld homogenizer, then centrifuged at 4ºC for 10 min at 12,000g. The supernatant was then transferred into new Eppendorf tubes and further diluted in buffers specific for each enzyme assay.

2.2.2 Quantification of Enzyme Activity

Fluorescence-based assay techniques were used to detect protease, amylase and esterase activities. The assays were done in black 96-well plates (SIGMA, P8741) using duplicate or triplicate wells per sample and four replicates per treatment. Fluorescence was measured in a Victor-Wallac Multi-label Counter equipped with standard fluorescein filters.

Assay of α-amylase was carried out using the EnzCheck® Ultra Amylase Assay Kit by Molecular Probes/Invitrogen (Product Code E33651). The kit contains a starch derivative "DQ ™ starch substrate" that is labelled with BODIPY®FL Dye to such a degree that the fluorescence was quenched. This substrate is efficiently degraded by amylase; digestion relieves the quenching and yield highly fluorescent fragments. The accompanying increase in fluorescence is proportional to amylase activity and can be monitored with a fluorometer or microplate reader using standard fluorescein filters.

The sample supernatants were diluted in kit buffer (100 mM MOPS, pH 6.9) and then 50 µL from each sample was titrated into wells of a 96-well plate. Fifty µL of background (buffer-only control) and standard curve (different concentrations of amylase) samples were also placed in the same well plate as sample supernatants and then 50 µL of 200 µg mL^{-1} DQ starch substrate was added into each well as quickly as possible using a multi-channel pipette. The mixture was incubated at temperature of 23ºC protected from light for 30 minutes. Fluorescence was then measured at excitation of 485 nm and emission of 535 nm. The true fluorescence increase due to amylase activity was determined by subtracting the fluorescence from the background. Fluorescence values were plotted against the concentrations of the enzyme standards to create a standard curve and the linear range of the standard curve was used to determine the concentration of amylase in the samples. *Bacillus* sp. α-amylase (SIGMA Product Code A6380) was used enzyme as enzyme standard. One

enzyme unit is defined as the amount of enzyme required to liberate 1 mg of maltose from starch in 3 min at 20°C, pH 6.9.

Assay of protease activity was carried out using the EnzCheck® Protease Assay Kit by Molecular Probes/ Invitrogen (Product Code E6638). The kit contains casein derivatives that are heavily labelled with a pH-insensitive green fluorescent BODIPY® FL dye. Protease-catalysed hydrolysis releases highly fluorescent dye-labelled peptides which can be measured with a fluorometer or microplate reader, giving a fluorescence that is proportional to protease activity.

The sample supernatants were diluted in kit buffer (10 mM Tris-HCl, pH 7.8) and then 50 µL from each sample was titrated into a 96-well plate. Fifty µL of the buffer-only control (background sample) and different concentrations of a protease (standard curve samples) were also placed into the same plate as sample supernatants and then 50 µL of the 10 µg mL^{-1} BODIPY casein working solution was added into each well as quickly as possible using a multi-channel pipette. The mixture was incubated at temperature of 23ºC and protected from light for one hour before fluorescence was measured at excitation of 485 nm and emission of 535 nm. True fluorescence due to protease activity of the samples was determined by subtracting the fluorescence from the background sample. Fluorescence values were plotted against the concentrations of the enzyme standards to create a standard curve and the linear range of the standard curve was used to determine the concentration of protease in the samples. Trypsin from porcine pancreas (SIGMA Product Code T5266; 1 BAEE unit will produce a ΔA_{253} of 0.001 per min at pH 7.6 at 25°C using BAEE as substrate; reaction volume = 3.2 m, 1 cm light path) was used as the protease standard and protease activity was expressed as “total trypsin-like proteases”.

Assays of non-specific esterase were conducted using a fluorogenic substrate, 4-methylumbelliferyl butarate (MUB) (SIGMA Product Code 19362) following the method of Rotllant *et al*. (2008) which was a modified from Vaneechoutte *et al*. (1988). A stock solution was prepared by dissolving 100 mg of MUB in 10 mL of dimethyl sulfoxide (DMSO) to which 100 µL of Triton X-100 was added and stored in ultra-cold freezer (-70ºC) in 250 µL aliquots. The stock solution was diluted 100 times in phosphate buffer, pH 7.0 to obtain a final concentration of 0.4 mM MUB which was used in the assays. Ten µL of the sample supernatants were titrated into wells of a 96-well plate and then 250 µL of the 0.4 mM MUB was quickly added using a multi-channel pipette. Fluorescence was measured at 355 nm excitation and 460 nm emission at 30ºC, reading every 10 seconds for 4 minutes.

2.2.3 Protein Determination

Protein in the sample homogenates were measured using a FluoroProfile® Protein Quantification (FPQ) Kit (SIGMA Product Code FP0010). The kit consists of bovine serum albumin (BSA) protein standard (SIGMA Product Code 5619), FluoroProfile® Fluorescent Reagent (Sigma Catalog Number Q5054) and Quantification Buffer (Sigma Catalog Number Q0509). Using deionized water as diluent, four-fold serial dilutions of the BSA was made in order to have protein standard samples ranging between 40 µg mL^{-1} and ~50 ng mL^{-1}. Several dilutions of the sample homogenates were also prepared in order to have samples that will fall within the range of the protein standards. A working reagent was then prepared by mixing water/sample buffer, FluoroProfile® Fluorescent Reagent and Quantification Buffer in an 8:1:1 ratio. Fifty µL of the protein standard samples, sample homogenates and background (buffer-only control) samples were titrated into wells of a 96-well plate and an equal volume of working reagent was quickly added using a multi-channel pipette. The mixture was incubated for 30 minutes at room temperature (23-25ºC) and

fluorescence was measured at excitation of 485 nm and emission of 615 nm. True fluorescence of the samples was determined by subtracting the fluorescence from the background and then the fluorescence values were plotted against the concentrations of the enzyme standards to create a standard curve. The linear range of the standard curve was used to determine the concentration of protein in the sample.

CHAPTER 3

Changes in digestive enzyme activities and nutrient utilization during embryonic development and starvation of the newly hatched larvae of *Scylla serrata* and *Panulirus ornatus*

3.1 Introduction

Grow-out culture of the mud crab, *Scylla serrata* and the tropical rock lobster, *Panulirus ornatus* is seen to have huge economic potential because of their premium market value. However, increasing or merely sustaining aquaculture production is a huge challenge for both species because the culture industry continues to depend on wild seed for stocking because of inconsistent larval production from commercial hatcheries (Keenan, 1999; Johnston 2006; Shelley, 2008; Jeffs, 2010; Rogers *et al*., 2010; Quinitio, 2015; Wang 2015). Our limited understanding of the complex life histories of *S. serrata* and *P. ornatus*, which include certain stages that are nutritionally sensitive, is among the factors that contribute to the slow development of techniques supporting consistent and reliable hatchery production. Lack of information on nutritional requirements and digestive physiology of larval stages of these crustaceans has prevented the development and use of feeds that meet their requirements. Considering that the analysis of digestive enzymes is one of major techniques used to gain an understanding of larval nutrition (Icely & Nott, 1992; Jones *et al*., 1997a, 1997b; Anger, 2001), this method can also be adapted to investigate energy sources of the developing embryo and newly hatched larvae.

Changes in digestive enzymes in eggs of *S. serrata* and *P. ornatus* during embryonic development, as well as in starved newly hatched larvae, can reveal nutrients that serve as energy sources for developing embryos and early larvae. Understanding the relative importance of these nutrients at these development stages will be useful in the formulation of diets for broodstock and first feeding larvae (Holland, 1978; Wehrtmann & Graeve, 1998; Rosa *et al*., 2003; Yao *et al*., 2006) that better match their requirements.

The research reported in this Chapter examined the activities of α-amylase, trypsin-like proteases and non-specific esterase in *S. serrata* and *P. ornatus* eggs at various stages of embryonic development, and in newly hatched larvae at various periods of starvation, in order to determine the mechanisms of enzyme regulation during these early development stages. Specifically, this research used the activity patterns of digestive enzymes to: (1) determine levels of the major digestive enzymes at various stages of embryonic development and starvation of newly hatched larvae of *S. serrata* and *P. ornatus*; and (2) determine which nutrients serve as critical energy reserves during embryonic development and when no food is available to newly hatched larvae of both species.

3. 2 Materials and Methods

3.2.1 Collection of Mud Crab Egg and Larval Samples

Mud crab, *S. serrata* egg and larval samples were sourced from broodstock reared at the aquarium facilities of JCU following the methods described in *Section 2.1.1.* Samples of eggs used for determination of changes in enzyme activities during embryonic development were collected from a berried crab every 3 days from day 0 to day 9, then daily from day 9 onward in order to ensure that egg samples included those on the day prior to hatching. Different batches of eggs were examined using a compound microscope to determine the stage of embryonic development and weights of samples were measured using

an electronic balance (Cahn C-33 micro-balance, precision = 1 μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) before being kept in 1.7 mL Eppendorf vials and stored in a freezer at -70ºC until assay.

Samples used to determine changes in enzymes activity in newly hatched zoea that were reared without any food were from the same broodstock whose eggs were sampled for enzyme activities. Samples of larvae were collected immediately after hatch (0 h), then every 4 h up to 16 h, then at 24, 36, 48, and 60 h after hatch. To ensure accurate determination of the time of hatching, the berried crab was closely monitored for mass hatching of eggs, which occurs before sunrise, and is characterized by the vigorous swimming activity of the female crab. As soon as vigorous swimming activity and mass hatching of eggs was observed, the female was transferred into a new hatching tank to release some of her larvae for 30 minutes (from 03:30 h to 04:00 h), before being removed and returned to her former tank. The larvae that were synchronously hatched within 30 minutes were collected and stocked into 300 L tanks at a density of 100 larvae L^{-1} and were reared without food until sampled for analysis. Larval samples were measured and weighed as described above, and retained inside 1.7 mL Eppendorf vials in a freezer (-70 ºC) until they were assayed for enzyme content.

3.2.2 Collection of Lobster Egg and Larval Samples

Egg and larval samples from *P. ornatus* were collected from broodstock reared at AIMS as described in *Section 2.1.2*. Egg samples for analysis of enzyme changes during embryonic development were collected from two berried females. Samples from the first female were collected weekly and then a few days prior to hatching (i.e., day 6, 13, 20 and 23). Samples from the second female were collected one day after eggs were extruded, then every seven days for the first two weeks and every two days starting from the appearance of

eye pigment (i.e., day 1, 7, 14, 16, 18, 20, 22, and 24); samples of the newly hatched larvae were taken on day 26. Samples for determination of enzyme changes in the unfed newly hatched phyllosoma were also taken from the second female broodstock and were collected every two days from day 1 until all the larvae died (i.e., day 1, 3, 5, 7, and 9). A compound microscope was used to determine the stages of embryonic development of egg samples and the total number of larvae in larval samples. A Cahn C-33 microbalance was used to determine egg and larval weights from respective samples as described above, and all samples were held inside 1.7 mL Eppendorf tubes and stored at -70 ºC until assay of digestive enzymes.

3.2.3. Assay of Enzymes

Egg and larval samples were assayed for α-amylase, trypsin-like protease and non-specific esterase activities following the methods described in *Section 2.2.1* and *Section 2.2.2*. Mud crab egg sample extracts for assay of amylase activity were diluted 1:100 (sample: buffer) while those for protease and esterase assays were diluted 1:50. Mud crab larval sample extracts for assay of amylase, protease and esterase were all diluted 1:50. Lobster egg sample extracts were diluted 1:100 for both amylase and protease assays and 1:50 for esterase assay, while lobster larval sample extracts were diluted 1:150 for amylase and protease assays and 1:100 for esterase assay. Enzyme activities of eggs were expressed as "specific activity", which is based on protein content (mU mg^{-1} protein), while those of larvae were expressed both as specific activity and as "total activity" or activity per individual larva (mU ind^{-1}).

3.2.4 Statistical Analyses

Statistical analyses of resulting data were performed using SPSS for Windows Version 12.0. Each data set was tested for homogeneity of variance using Levine's test and, when necessary, arcsine square-root or logarithmic transformation was performed to make the data normal and homogeneous. One-way ANOVA was performed to test for significant differences among various enzyme levels at various stages of embryonic development and between enzyme levels of larvae at various ages from hatching. Duncan's multiple range test was also used for multiple comparisons to determine specific differences among treatments. Data are presented as mean, plus or minus (±) standard error (SEM), and results were considered significantly different at $P \leq 0.05$.

3.3 Results

3.3.1 Mud crab (S. serrata)

Based on the embryonic development staging by Ates *et al.* (2012) for *S. serrata*, the stages of embryonic development corresponding to each sampling point of this study is shown in Table 3.1. Under the incubation conditions in this study, the embryonic development of *S. serrata* lasted for 13 days and hatching occurred on day 14. Embryonic eyespots were first seen on day 8 while heartbeat was observed from day 11 onward.

Table 3.1 Days of egg sampling (day 0 as day of oviposition) and corresponding stages of embryonic development of *S. serrata* based on descriptions by Ates *et al*. (2012).

Sampling Days	Stage of Development
day 0	cleavage
day 3	gastrula
day 6	naupliar
day 9	eye formation
day 10	thoracico-abdominal
day 11	heartbeat
day 12	heartbeat
day 13	pre-hatch
day 14	pre-hatch

The changes in specific activities of amylase, proteases and esterase during embryonic development are shown in Fig 3.1. Amylase activity was initially very low at around 4 *mU* mg^{-1} protein during early embryonic development but began to increase rapidly ($P<0.05$) from day 10 (corresponding to thoracico-abdominal stage) onward and reached the highest level of 148 *mU* mg^{-1} protein on day 14 (Fig. 3.1A), the day the larvae were hatched. Similarly, protease activity during embryonic development was not detected from day 0 to day 10 but increased significantly ($P<0.05$) from day 11 (corresponding to heartbeat stage) onward, reaching a peak of 181,000 *mU* mg^{-1} protein on day 14, just hours prior to hatching (Fig. 3.1B). On the other hand, esterase activity showed a different pattern of changes as it initially remained comparatively stable between day 0 to 3 (cleavage to gastrula stage) at 16.5 and 15.0 mU mg^{-1} protein, respectively, but showed a decrease from 15.0 to 7.8 *mU* mg^{-1}

protein beginning day 3 to day 6 (gastrula to naupliar stage), before increasing significantly ($P<0.05$) to reach a peak of 59.8 *mU* mg^{-1} protein on day 11. However, it subsequently decreased sharply to 25.7 *mU* mg^{-1} protein on day 14 when the eggs were about to hatch (Fig. 1C).

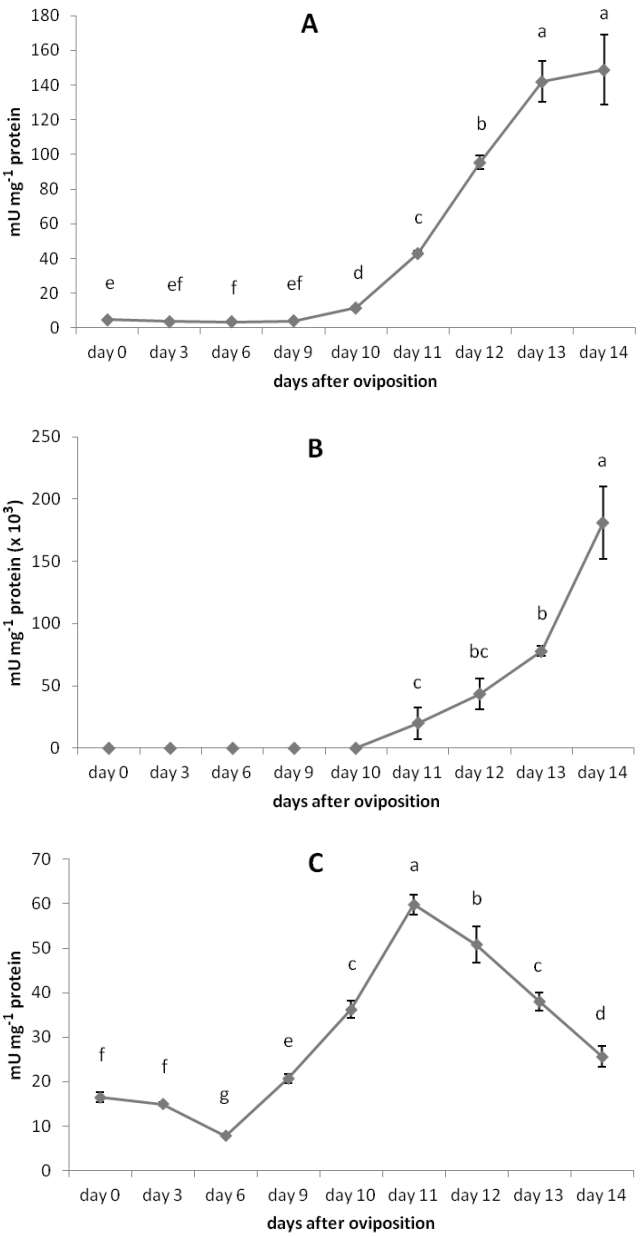

Fig. 3.1 Mean (±SEM, n=4) specific activities of amylase (graph A), protease (graph B) and esterase (graph C) of eggs samples during embryonic development of *S. serrata*. Means with the same letters above are not significantly different ($P>0.05$).

The specific and total activites of amylase, protease and esterase in unfed newly hatched larvae are presented in Fig.3.2. The specific activities of amylase and protease detected in the embryos just prior to hatching (as seen on day 14 of Fig. 3.1 A and Fig. 3.1 B, respectively) increased further after hatch (as seen at 0 h in Figs. 3.2 A and Fig. 3.2 C, respectively) but these increases were not significant ($P>0.05$). In contrast, specific esterase activity continued to decrease during the final stages of embryonic development and was significantly lower in newly hatched larvae (Fig. 3.2 E) compared to the level detected in embryos on the day of hatching (Fig. 3.1 C) ($P<0.05$).

Some of the larvae were able to survive up to 72 h without food but the samples collected at 72 h were not sufficient for all enzyme assays, hence measurement of enzyme activities was possible up to 60 h only. The specific activity of amylase significantly dropped from around 218 *mU* mg^{-1} protein at 0 h to around 127 *mU* mg^{-1} protein at 4 h after hatching ($P<0.05$), then slightly fluctuated before gradually increasing to the highest level of around 270 *mU* mg^{-1} protein after 48 h and then sharply decreasing to the lowest level of around 86 *mU* mg^{-1} protein at 60 h ($P<0.05$) (Fig. 3.2A). Specific protease activity remained at about the same level and did not differ significantly from the time the larvae hatched (Fig. 3.2 C), while esterase specific activity initially dropped significantly to the lowest level at 4 h after hatch ($P<0.05$), then fluctuated slightly, but the changes in levels up to the final sampling at 60 h were mostly not significantly different. The absence of food for newly hatched larvae resulted in a clear trend of decreasing total activities of the three major digestive enzymes. Total amylase activity decreased significantly after 4 h from hatching, then slightly fluctuated from 4 h to 48 h, before decreasing significantly at 60 h ($P<0.05$) (Fig. 3.2 B). Total protease and esterase activities also dropped significantly after 4 h ($P<0.05$), then continued to decrease until 60 h; but the changes within adjacent sampling periods did not differ significantly (Figs. 3.2 D and F).

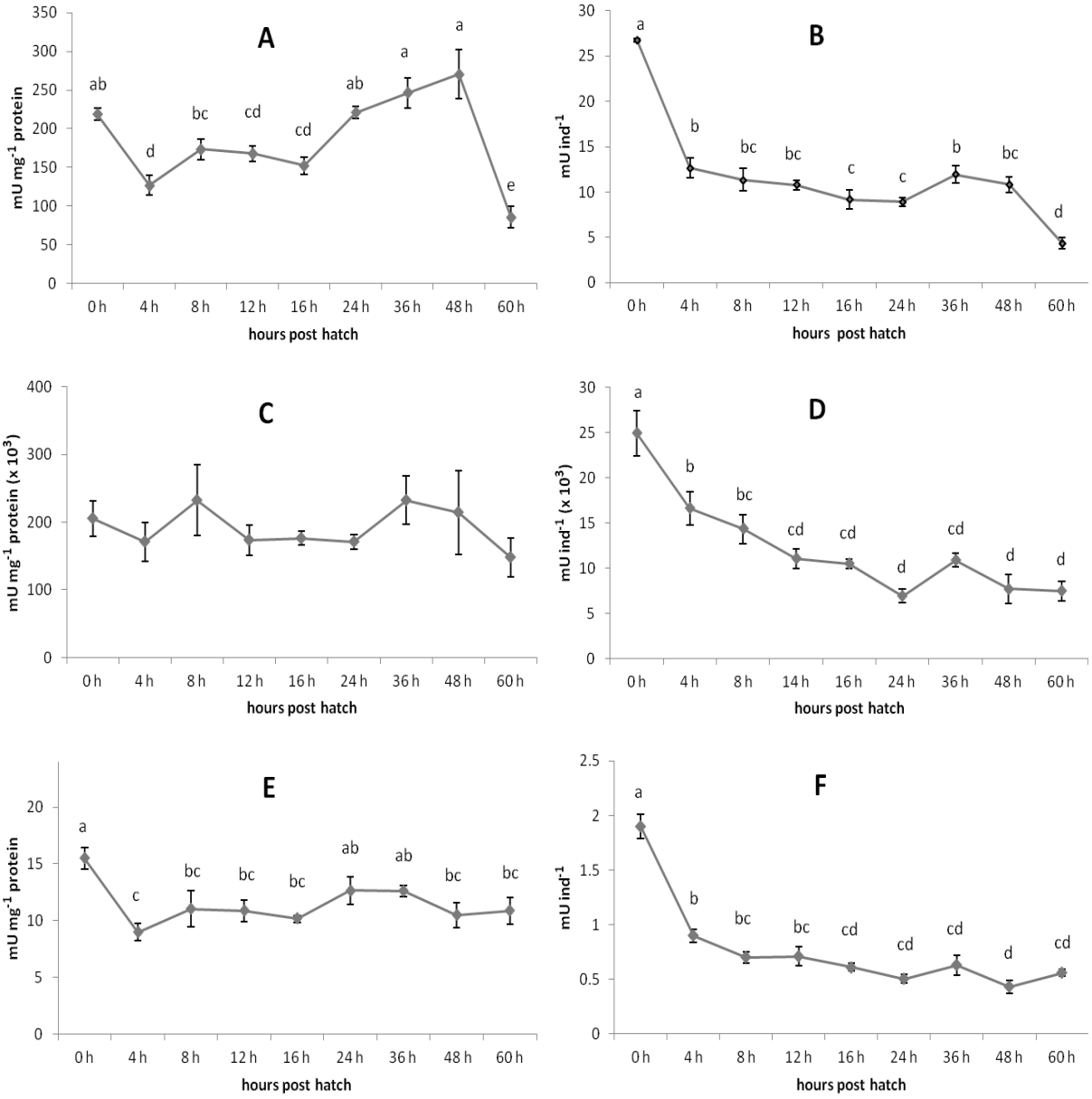

Fig. 3.2 Mean (±SEM, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (graphs A and B), protease (graphs C and D) and esterase (graphs E and F) in the newly hatched (Zoea 1) larvae of *S. serrata* reared without food. Means with the same letters above are not significantly different (P>0.05).

The protein content of *S. serrata* eggs during embryonic development and that of the newly hatched zoeae expressed as percent wet weight of sample are presented in Fig. 3.3A and B respectively. Protein content of eggs decreased from 22.5% on day 0 to 5.5% on day 14 while that of larvae dropped significantly from about 5.2 % at 0 h to 4.3 and 2.8 % at 4 h and 8 h, respectively ($P<0.05$), then continued to decrease, reaching the lowest level of about 1.7% at 24 h; however, the changes within adjacent sampling periods no longer differed significantly. Protein levels of larvae monitored from 24 h until the last sampling at 60 h fluctuated slightly but did not differ significantly ($P<0.05$; Fig. 3.3B).

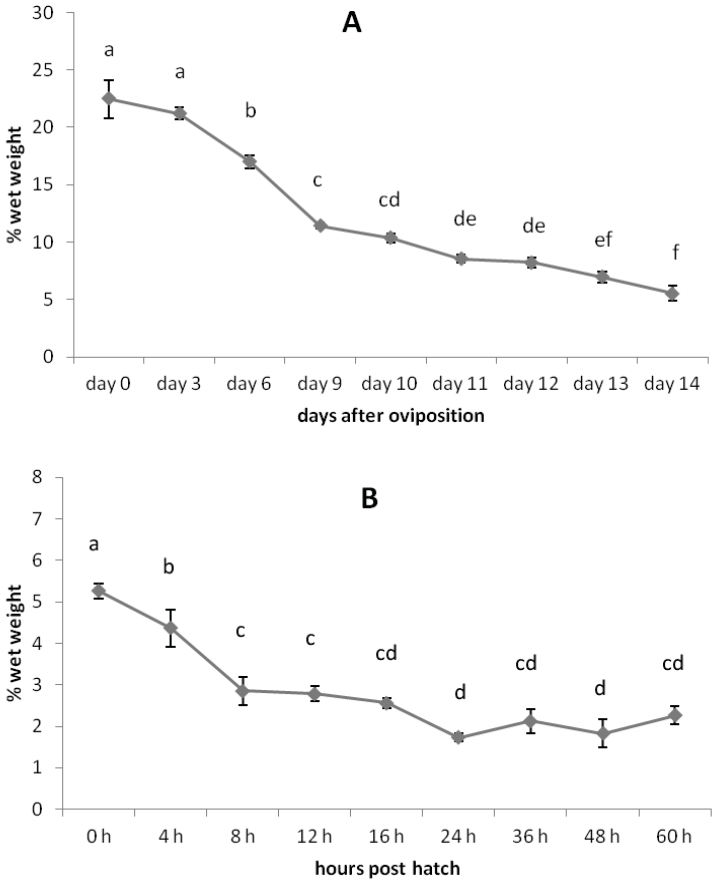

Fig. 3.3. Mean (±SEM, n=4) protein content of *S. serrata* egg samples during embryonic development (A) and newly hatched (Zoea I) larvae of reared without food (B). Means with the same letters above are not significantly different ($P>0.05$).

3.3.2Lobster (P. ornatus)

The presence of eggs in the first lobster female was not immediately noticed during the routine daily check of the broodstock tanks because the eggs were few and almost completely hidden from view, hence initial collection of egg samples was delayed. Eventually all the eggs were dropped making it necessary to collect eggs samples from a second female. The second female had a good number of eggs which were successfully incubated and hatched, allowing collection of sufficient samples for enzyme assay. Embryonic eyespot was noted after 13 and 14 days of incubation after oviposition in the first and second female, respectively, and the hatching of the eggs from the second female occurred after 26 days of incubation. Considering that except for differences in development time, the embryonic development of *P. ornatus* was very similar to that of *J. edwardsii* as described by Tong *et al.* (2000), the classification of the embryonic stages of *P. ornatus* was based on that of *J. edwardsii* (Tong *et al*., 2000) and is presented in Table 3.2 with the corresponding development time (days).

The specific activities of amylase, protease and esterase during embryonic development are shown in Fig 3.4. A trend of increasing activity of the three enzymes was observed in both batches of egg samples, with dramatic increases in amylase and protease activities occurring towards the later stages of embryonic development when heartbeat became visible but started early in the case of esterase activity. In particular, significant increases in the specific activities of amylase (Fig. 3.4 A-1 & A-2) and protease (Fig 3.4 B-1 & B-2) began about three weeks after oviposition and both continued to increase further until hatching ($P<0.05$). In the case of esterase (Fig. 3.4 C-1 & C-2), significant weekly increases in specific activities occurred within the first three weeks ($P<0.05$), then further activity changes occurring after the third week until hatching, no longer varied significantly ($P>0.05$; Fig 3.4 C-2).

Table 3.2. Description *P. ornatus* egg stages with corresponding development time at 34-35‰ salinity and water temperature of 27-28°C (based on the description of the spiny lobster, *J. edwardsii* egg stages by Tong *et al*., 2000).

Egg Stages	Description	Days from Ovoposition
Stage 1	Eggs darken from bright red to red orange. Cell borders become visible in the amorphous yolk and then a small, transparent, yolk-free area becomes visible inside the eggs.	Day 0-12
Stage 2	Eye spots become visible and dark pigmented median eye appears	Day 13-14
Stage 3	Eye spots enlarge, appendages visible and pigmentation increases. Egg colour start to turn brown.	Day 15-17
Stage 4	Red chromatophores become visible on extremities of appendages	Day 18-20
Stage 5	Heartbeat visible. Chromatophores extend along length of appendages.	Day 21-23
Stage 6	Chromatophores enlarge. Heartbeat getting faster. Embryos begin to move inside the egg.	Day 24-26

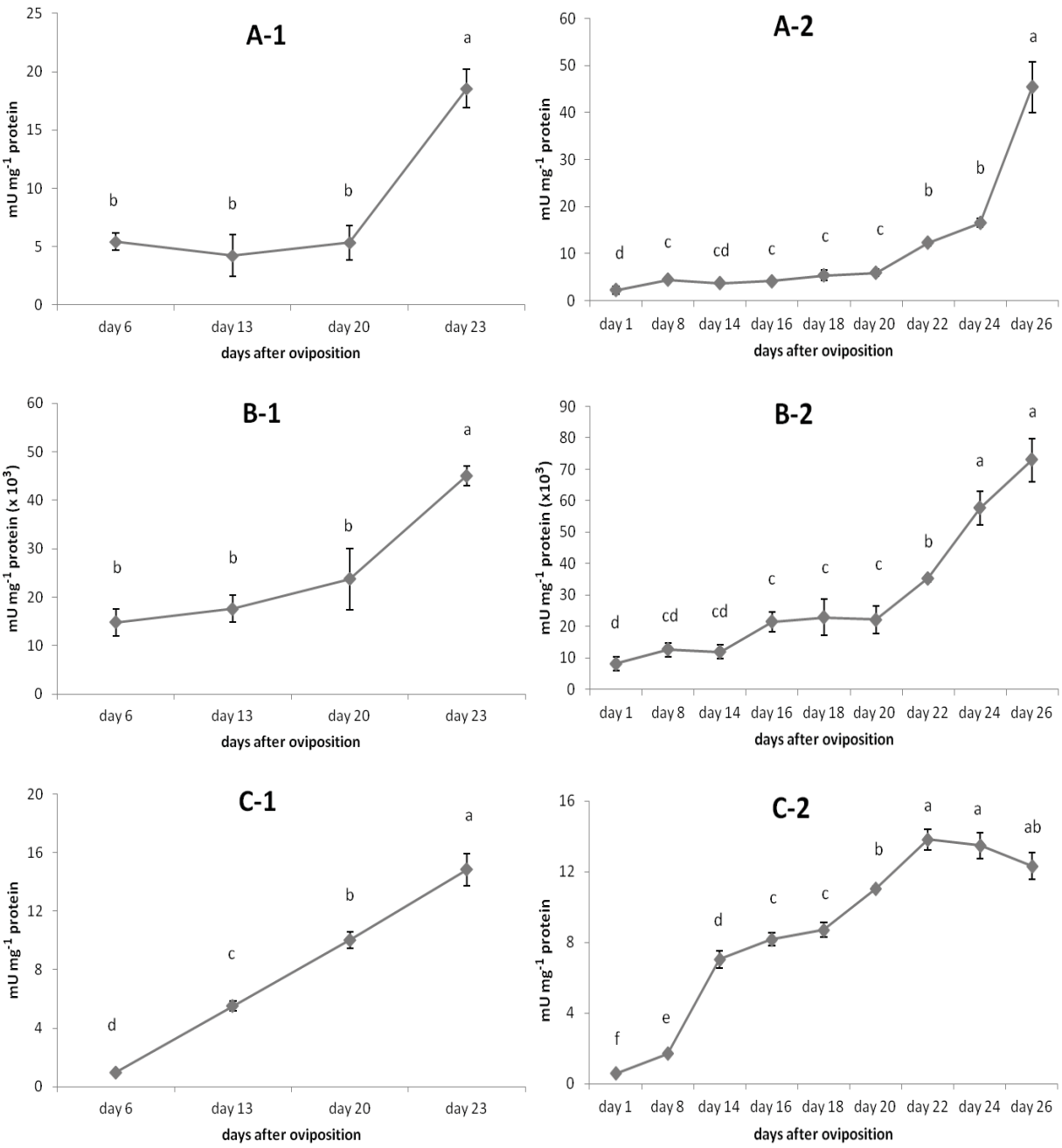

Fig. 3.4 Mean (±SEM, n=4) specific activities of amylase (graphs A-1 and A-2), protease (graphs B-1 and B-2) and esterase (graphs C-1 and C-2) during embryonic development of *P. ornatus*. Results for the first set of eggs are shown in Figs. A-1, B-1 & C-1 while those for the second set are shown in Figs. A-2, B-2 & C-2. Means with the same letters above are not significantly different (P>0.05).

Changes in protein levels of the first and the second set of *P. ornatus* eggs during embryonic development are shown in Fig. 3.5 A and 3.5 B, respectively. The protein content of the first set of eggs, which were eventually dropped, were lower compared to the second set that were successfully hatched, but both showed decreasing protein content during embryonic development. In particular, the percent wet weight of protein in eggs gradually decreased during embryonic development from about 31.8 to 16.2 % from day 6 to 23 in the first set and from 43.68 to 14.67 % from day 1 to 26 in the second set, with significant reduction in levels occurring every week ($P<0.01$).

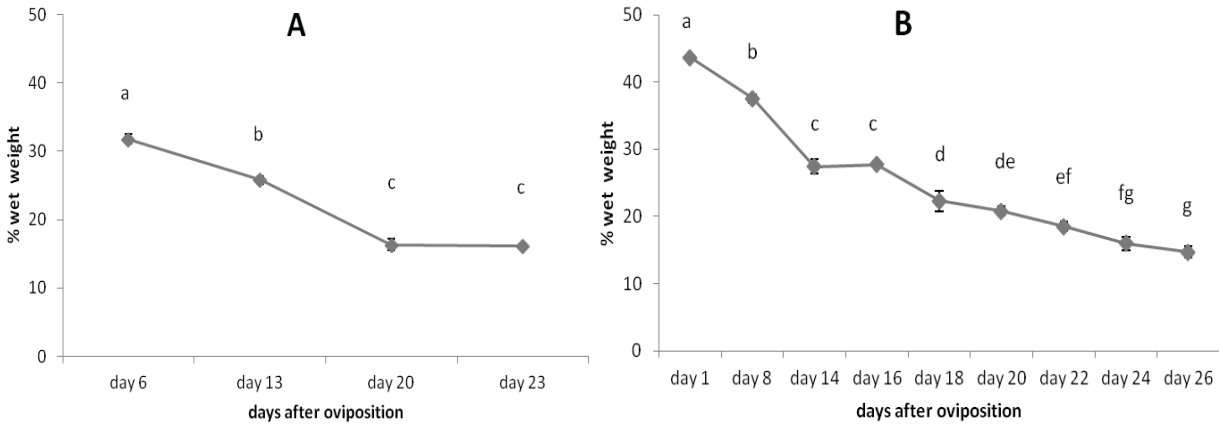

Fig.3.5 Mean (±SEM, n=4) protein content of egg samples during embryonic development of *P. ornatus* from two sets of samples (Set 1- Fig. A; Set 2- Fig. B). Means with the same letters above are not significantly different ($P>0.05$).

Changes in specific and total activities of amylase, protease and esterase in the starved newly hatched phyllosoma are presented in Fig. 3.6. Some of the larvae were able to survive up to ten days without food but sufficient samples for enzyme assay were available only until day 9. Specific and total amylase activities showed alternate increases and decreases from day 1 to day 9 but the levels did not vary ($P>0.05$) (Fig. 3.6 A & B). Specific and total protease activities both showed decreasing trends with increasing starvation period but significant reduction in activity levels in both occurred only from day 3 to 5 ($P<0.01$) and from day 5 to 7 ($P<0.05$) (Fig. 3.6 C & D); it did not differ significantly from day 1 to 3 or from day 7 to 9 ($P>0.05$). Specific esterase activity increased as the period of starvation extended; it increased significantly from day 1 to 3 ($P<0.05$), then maintained about the same levels from day 3 to 7, before increasing further at day 9. However, the changes in activity levels between the adjacent sampling periods from day 3 to day 9 did not differ ($P>0.05$) (Fig. 3.6 E). Total esterase activity initially increased slightly from day 1 to 3, then decreased significantly at day 5 ($P<0.05$) and maintained about the same levels until day 9, but all the changes during the entire period of starvation did not differ significantly from the level at day 1 (Fig. 3.6 F).

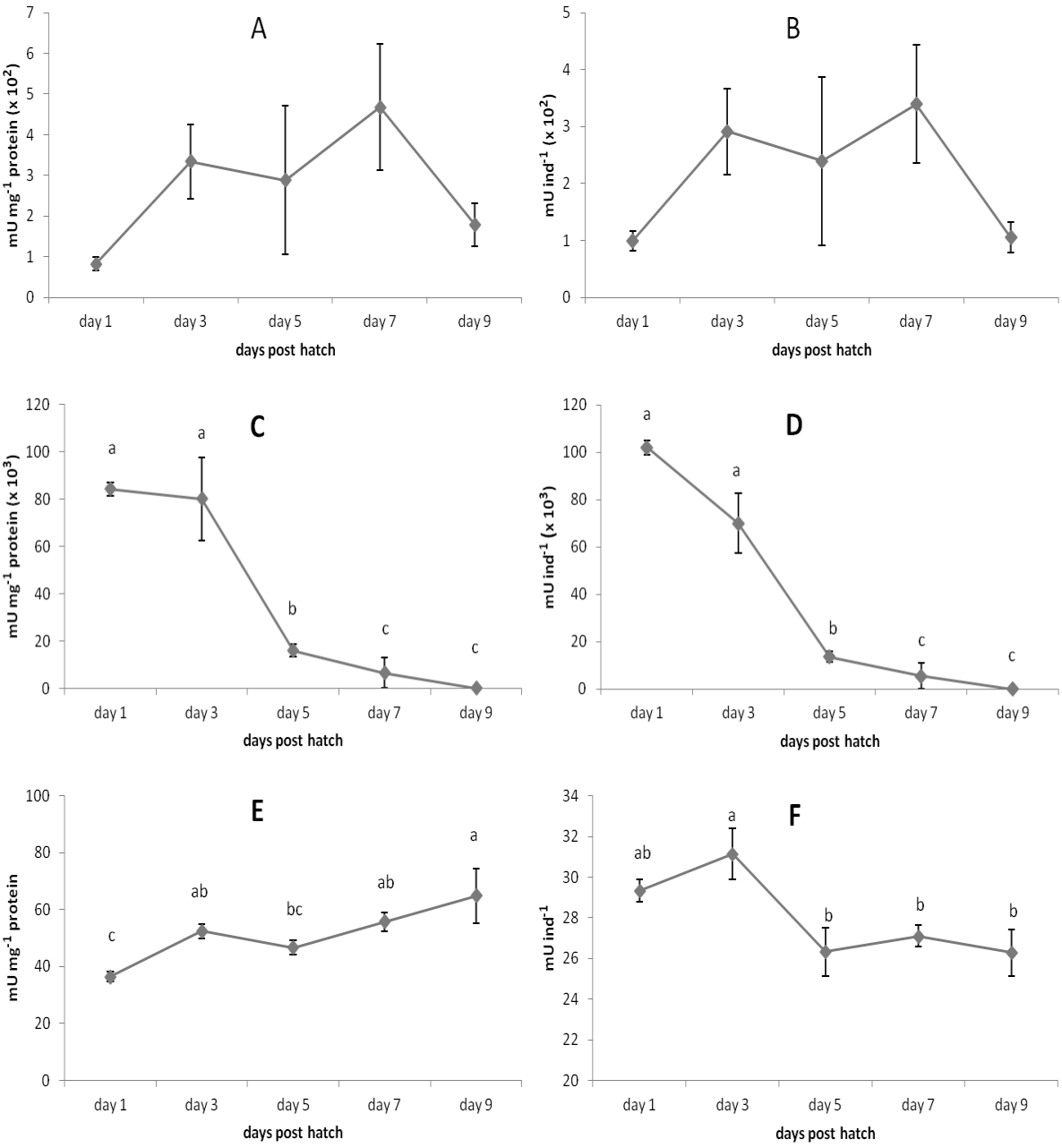

Fig.3.6. Mean (±SEM, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (graphs A and B), protease (graphs C and D) and esterase (graphs E and F) in newly hatched (Stage 1) *P. ornatus* phyllosoma reared without food. Means with the same letters above are not significantly different (P>0.05).

The protein content of the newly hatched phyllosoma after various periods of starvation is presented in Fig. 3.7. Larval protein content significantly decreased from around 6.44% wet weight on day 1 to only 3.35% at day 9 ($P<0.01$). A significant decline in protein level occurred during the early phase of starvation, particularly from day 1 to day 3 ($P<0.01$), followed by gradual decreases up to day 9 that did not differ significantly from the prior sampling point.

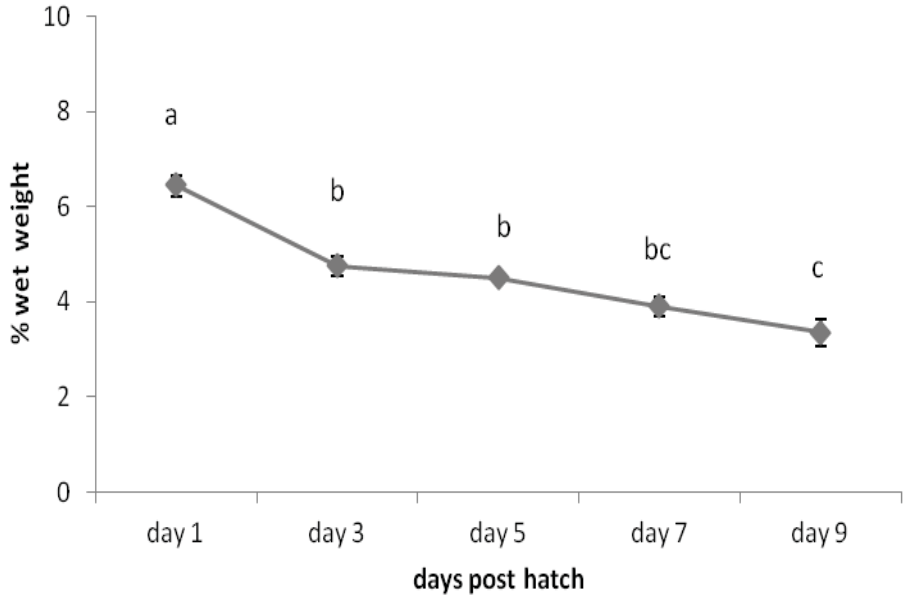

Fig. 3.7 Mean (±SEM, n=4) protein content of newly hatched (Stage 1) *P. ornatus* phyllosoma reared without food. Means with the same letters above are not significantly different ($P>0.01$).

3.4 Discussion

Changes in digestive enzyme activity during embryonic development and starvation of newly hatched larvae can provide useful information on the sources of energy supply for the crucial period of early development because digestive enzymes are responsible for the breakdown of nutrient reserves to provide energy for maintenance, development and growth (Icely & Nott, 1992; Ceccaldi, 1997; Jones *et al.*,1997a, 1997b). For example, a high protease (trypsin) activity indicates protein catabolism, lipase (esterase) activity is indicative of lipid use, and amylase activity suggests carbohydrate (i.e. starch and glycogen) catabolism (Kamarudin *et al*., 1994; Rodriguez *et al*., 1994; Johnston, 2003).

A wide range of units have been used by authors to describe crustacean digestive enzyme activity, which has been expressed relative to the weight of the hepatopancreas, protein content of the hepatopancreas, soluble or whole body protein content, and whole body wet or dry weight (Jones *et al*., 1997a, 1997b; Puello-Cruz *et al*., 2002), as well as per larva (Munilla-Moran *et al*., 1990; Johnston *et al.,* 2004a, 2004b). Although the protein content of the hepatopancreas is considered the most suitable base for specific enzyme activity values, unfortunately, this is impractical in larval stages (Anger, 2001). In this particular study, enzyme activities were expressed in relation to both the protein content (specific activity) and per larva (total activity) for a better comparison of activity patterns in larvae considering that larval protein content was influenced by the period of starvation. Enzyme activity during embryonic development, however, was only reported in relation to the protein content of the egg samples as it was impractical to count the eggs because they stuck together.

Typical of Brachyuran crabs, mud crab eggs remain attached to pleopods of females during the full period of embryonic development, which lasts for various durations depending on water temperature and species (Furota, 1996; Nagao *et al*., 1999; Hamasaki, 2003; Zeng, 2007; Ates *et al*., 2012). In this study, *S. serrata* eggs were incubated at the

lower end of the range of suitable incubation temperature of between 25-26ºC. The enzyme activities of *S. serrata* during the early phase of embryonic development (from cleavage to eye formation stage) suggest that the energy requirements were minimal and protein was initially conserved since esterase activity was low, amylase activity even lower and protease activity was undetected. Since esterase activity was first to increase significantly and the levels were comparably higher than those of amylase and protease activities, lipids were likely the main energy source utilized first by the developing embryo. The gradual decrease in the amount of yolk observed during the early stages of embryonic development of mud crabs observed by Ates *et al*. (2012) supports the idea that energy requirements were still minimal and supplied mainly by lipids.

During embryonic development, *P. ornatus* females also carry their eggs under their abdomen where the eggs turn from bright red when first extruded, to orange and subsequently to dark brown when close to hatching (Jones *et al*., 2006). Egg incubation period of *P. ornatus* was reported to vary up to 15 days at incubation temperatures between 24°C and 30°C (Sachlikidis *et al*., 2010). At incubation temperature of 27-28°C, *P. ornatus* eggs in this study hatched 26 days after oviposition, three earlier than the 29 days for hatching at a lower temperature of 26°C reported by Jones *et al*. (2006). The egg samples collected from the first female in this study were limited but they showed that significant increases in amylase and protease activity occurred during the later stage of egg development after heartbeat was visible, while significant increases in esterase activity began prior to the appearance of eyespots. These results were confirmed by analysis of the second set of eggs sampled from the second female that were successfully hatched.

The early increase in esterase activity during embryonic development agrees with the commonly accepted view that lipids are the main energetic reserves fuelling embryonic development of crustaceans (Herring, 1974; Holland, 1978; Amsler & George, 1984;

Wehrtmann & Graeve, 1998; Heras *et al*., 2000). Although protein is the more dominant component of marine invertebrate eggs (Holland, 1978), lipids are believed to play a central role in embryonic metabolism, providing at least 60% of the total energy expenditure of the developing crustacean embryo (Wehrtmann & Graeve, 1998). Eggs of decapod crustaceans are relatively high in lipids, which serve both as an energy source and as components of biological membranes and pigments of the compound eyes (Heras *et al*., 2000; Yao *et al*., 2006). Due to embryonic utilization, the lipid content of crustacean eggs has been found to decrease significantly during development (Clarke *et al.,* 1990; Petersen & Anger, 1997; Wehrtmann & Graeve, 1998; Wehrtmann & Kattner, 1988; Morais *et al*., 2002; Ates *et al*., 2012). A concurrent study at AIMS likewise found that during the embryonic development of *P. ornatus*, 61.28% of initial total lipids per egg was utilized while the percentage of total lipids (%DW) decreased by 54.96% (Wu, 2013).

The dramatic increase in amylase and protease activities which coincided with either decreasing or levelling of esterase activity towards the end of embryonic development in both species in this study may be related to a shift in sources of energy for the developing embryo. Rapid growth and tissue differentiation associated with higher metabolism that occur towards the end of embryonic development demands higher levels of energy (Heras *et al*., 2000; Kumlu *et al*., 2000). For example, energy consumption of developing embryos have been reported to increase substantially with increasing rate of heartbeat and movement of embryos within the egg membrane in order to break loose from the chorion (Pandian, 1970). A study on depletion rates of lipids, proteins and carbohydrates during embryonic development of the freshwater crayfish, *Cherax quadricarinatus*, reported that lipids were the first reserves to be metabolized at high rates in response to increased energy demands and that lipid consumption per day further intensified towards hatching (Garcia-Guerrero *et al*., 2003). However, the continous utilization of lipids during prolonged embryonic development

could cause lipid concentration to become too low to support all the energy demands during the last stages of embryonic development, hence protein may be increasingly utilized as an energy source (Garcia-Guerrero *et al*., 2003). While the fundamental role of proteins is to serve as structural components of tissues, under certain conditions, proteins could also be utilized as an important energy source (Lemos & Phan, 2001).

Carbohydrates are not the main energy source in aquatic eggs (Holland, 1978) but can be a useful source of energy with significant protein-sparing and lipid-sparing effects (Sanchez-Paz *et al.,* 2006). In the red claw, *C. quadricarinatus*, for example, it was observed that carbohydrates were consumed to provide energy when embryonic development was prolonged by low water temperature (Garcia-Guerrero *et al*., 2003). Longer embryogenesis requires extensive use of energy reserves stored as yolk (Susanto & Charmantier, 2001), and may force the embryos to use all available energy sources, including carbohydrates (Garcia-Guerrero *et al*., 2003). In addition to being used as an energy source in late embryonic development, carbohydrates are also essential for the synthesis of specific compounds, such as chitin during crustacean development (Garcia-Guerrero *et al*., 2003), which may explain the continued increases in amylase activity in *S. serrata* and *P. ornatus* during the latter part of embryonic development to hatching.

Higher energy consumption could be expected after hatching due to the more sustained movement of larvae (Garcia-Guerrero *et al.,* 2003). The energy requirement for respiration during post-embryonic development also increases because energy demand per individual is proportional to body mass (Mackeviciene, 1993). Newly hatched larvae in this study had to depend on remaining maternally-derived energy reserves to fuel metabolism because exogenous nutrition was not available to them. In the copepod, *Calanus finmarchicus,* a moderate decline in total protein content during the first 10 days of starvation was interpreted to suggest the utilization of endogenous reserves other than protein, while a

drastic reduction in protein content over the next 21 days suggested a shift towards utilization of protein as an energy source (Helland *et al*., 2003). In this study, protein was likely the major source of energy during the initial period of starvation of newly hatched larvae because protein levels significantly declined during the first 8 hours of starvation in *S. serrata* and during first 3 days in *P. ornatus*. Considering also the higher levels of protease compared to esterase and amylase activities in eggs prior to hatching, as well as in newly hatched larvae of both *S. serrata* and *P. ornatus*, it is more likely that protein continued to be the main energy source during the initial stage of starvation of these newly hatched larvae. The 2-3 orders of magnitude higher levels of protease compared to either amylase or esterase in larvae of both species suggest an over-riding importance of protein nutrition on the onset of feeding, in contrast to the dependence on lipids during the early stages of embryonic development.

In another spiny lobster, *J. edwardsii*, Ritar *et al*. (2003) reported that loss of lipid during starvation within Stages II, IV and VI phyllosoma was 41-81%, while the loss in dry weight due to lipid catabolism by Stage I phyllosoma during starvation up to day 8 after hatch was previously reported to be only 18% (Smith *et al*., 2003). Ritar *et al*. (2003) interprets this to suggest that lipid catabolism is a more important source of energy for the later stages of development, whereas protein catabolism may be more important in Stage I phyllosoma. Smith *et al*. (2003) also insinuated that protein, and to a lesser extent carbohydrate, would have been preferentially catabolised during starvation of *J. edwardsii* Stage I phyllosoma. In the western rock lobster *Panulirus cygnus*, lipid accounted for only 6.7% of the decrease in dry mass in starved Stage I phyllosoma, but this increased to 35.0% in Stage II larvae (Liddy *et al*., 2004), likewise indicating that lipid was not the major nutrient catabolised during starvation of the newly hatched larvae.

Carbohydrates likely played only a minor energetic role in the newly hatched larvae in this study because in most crustaceans they typically constitute less than 5% of body

mass and have a more rapid turnover (Anger, 1986). Free carbohydrates for energy metabolism are available in the form of glucose coming from the degradation of lipids and proteins to a lesser extent (Anger, 2001).

Enzyme levels in starved *S. serrata* expressed as *mU* mg^{-1} protein (specific activity) may have concealed the decreasing trend of the three enzymes during the starvation period due to the rapid decline in larval protein content. On the other hand, enzyme activities expressed as *mU* ind^{-1} (total activity) seem to have clearly reflected the effects of starvation. The significant drop in total activities of all the three major digestive enzymes in *S. serrata* at 4 h after hatch, plus decreasing activity levels with increasing starvation period, may suggest a lack of energetic reserves and a need to provide an exogenous nutrient source to larvae immediately. The lack of energy reserves may explain the observed poor survival of *S. serrata* larvae in hatcheries following a delay in initial feeding (personal observation). In the mud crab, *Scylla paramamosain*, it was also reported that even a short period of starvation after hatching likewise affected larval survival and development (Li *et al.*, 1999).

The drastic reduction in protease activity after 5 days of starvation in *P. ornatus* Stage I phyllosoma in the present study may indicate that the point-of-no-return (PNR) has been reached. PNR is the maximum period of starvation prior to first feeding which will still allow the larvae to recover and moult to the next stage (Mikami *et al.,* 1995). The elevated levels of esterase specific activity, followed by the drop in protease activity could possibly indicate degradation of structural lipids. It has been reported that lobster larvae have already passed their PNR when structural lipids are degraded and they cannot recover after re-feeding (Mikami *et al.*, 1995; Abrunhosa & Kittaka, 1997). The PNR for the newly hatched *J. edwardsii* and *J. verreauxi* phyllosoma was reported to be 4.2 and 6.5 days, respectively (Abrunhosa & Kittaka, 1997), while for newly hatched *P. ornatus* it was reported by researchers at AIMS to be 5.9 days (Smith *et al.*, 2010).

Anger (2001) described the pattern of energy utilization in decapod crustacean larvae. During short-term food deprivation, energy-rich lipid reserves are preferentially mobilized, but when much of the accessible lipid pool has been depleted, proteins are increasingly utilized as reflected in degradation of structures such as muscle and nervous tissue. A significant part of the lipid pool is bound in crucial cell structures such as membranes, and hence is normally unavailable for energy metabolism, however, in the final phase of starvation prior to death, structural lipids may also be degraded as a source of energy.

3.5 Summary and Conclusions

Enzyme activities during embryonic development and initial starvation of newly hatched larvae of *S. serrata* and *P. ornatus* reflected the pattern of energy utilization during ontogenetic development which is generally in agreement with the pattern of energy utilization reported for decapod crustaceans. Lipids were the first energy reserves utilized during the early periods of embryonic development as indicated by the early significant increase in amylase activities in contrast to the low and steady levels of amylase and protease activities. As readily available lipid reserves were depleted and became insufficient to meet the increasing energy demands towards the end of embryonic development, protein and to some extent carbohydrates, were increasingly utilized. This was demonstrated by the levelling of esterase activities, coinciding with significant increases in protease and esterase activities towards the final phase of embryonic development.

Starved newly hatched larvae of both species continued to utilize mostly proteins as shown by the relatively higher activities of protease compared to amylase and esterase, indicating the over-riding importance of protein nutrition on the onset of feeding. Persistent high protease activity in starved *S. serrata* larvae, suggests degradation of bodily or structural proteins, implying the need to immediately feed the larvae. While starved newly hatched phyllosoma of *P. ornatus* likewise initially utilised protein as an energy source as indicated by the highest levels of protease activities during the first three days, towards the final phase of starvation, close to larval death, lipids were degraded for energy as indicated by an increase in esterase activity. These results highlight the significance of lipid reserves in developing embryos and protein reserves in the newly hatched larvae of these species, as well as the importance of proper broodstock nutrition and management to ensure these reserves are accumulated sufficiently to produce high quality eggs and larvae.

CHAPTER 4

Digestive enzyme responses to intermittent food availability in first feeding larvae of *Scylla serrata* and *Panulirus ornatus*

4.1 Introduction

In their natural environment, newly hatched larvae of crustaceans may undergo periods of starvation due to intermittent food availability. This is particularly true for the larvae of mud crabs and tropical spiny lobsters since they are hatched and released in offshore marine environments where the density of food is several times less than in near-shore areas and estuaries, and much less to what is normally provided to them in aquaculture hatcheries (Anger & Dawirs, 1981). While prey options in natural environments are more diverse than the usually single or at most two live prey species provided in hatcheries, much of the potential prey organisms in the natural environment may not be available to newly hatched larvae because of unsuitable size and quality, faster swimming speed, and various defence mechanism or other characteristics preventing predation (Anger & Dawirs, 1981). Such limited and often highly variable food resources are likely to result in periods of starvation which may be critical for larval survival (Johnston *et al*., 2004) and inadequate food supply may also result in prolonged larval development (Anger & Dawirs, 1981; Dawirs, 1984).

Physiological adaptations to food deprivation have been studied in larvae of several species of crabs such as *Hyas araneus* (Anger & Dawirs, 1981; Anger 1986), *Carcinus menas* (Dawirs, 1984) and lobsters such as *Homarus americanus* (Anger *et al*., 1985; Abrunhosa & Kittaka, 1997), *Panulirus japonicus* (Mikami *et al*., 1995), *J. edwardsii* (Ritar *et al*., 2003; Smith *et al*., 2003; Johnston *et al*., 2004), and *J. verreauxi* (Abrunhosa &

Kittaka, 1997), but no similar research has been reported on *S. serrata* or *P. ornatus*, despite their status among the top commercially important tropical crustaceans. Clearly, understanding the digestive capacity and physiological adaptations of early larvae of *S. serrata* and *P. ornatus* to variable food availability and different situations of food deprivation may provide insights into which nutrients and energy reserves are metabolized or conserved under those conditions (Harms *et al*., 1991). Such information can help understand the nutritional requirements of these crustaceans and provide insights supporting improved feeding management when these species are reared in captivity.

Analysis of digestive enzyme activities in crustacean larvae has been shown to be an effective approach in understanding the crustacean digestive process and in determining the nutritional characteristics of natural diets (Lovett & Felder, 1990a; Fang & Li, 1992; Kamarudin *et al*., 1994; Jones *et al*., 1997; Hammer *et al*.,2000; Johnston, 2003; Johnston *et al*., 2004a).Variations in the activities of each major enzyme during periods of intermittent food availability may help identify the transitions in utilization of major nutrients employed by these crustaceans as a strategy to provide energy while at the same time attempting to maximize the chance of survival and growth.

The experiments reported in this Chapter examined changes in major enzyme activities of first feeding larvae of *S. serrata* and *P. ornatus* when subject to different conditions of food availability: (1) when food was immediately available (fed) vs when food was not immediately available (starved); (2) when food was initially available (fed) and then withdrawn; and (3) when initial feeding was delayed for different durations. The objective of the study was to generate information on how the larvae of *S. serrata* and *P. ornatus* adapt to various conditions of intermittent food availability in their natural environment through the analysis of the changes in major digestive enzymes. Considering that changes in digestive enzyme activity may indicate critical nutrient and energy reserves, as well as those

metabolised or conserved during food deprivation (Harms *et al*., 1991), information on the enzyme response of early larvae to various intermittent food availability situations can lead to a better understanding of the nutritional requirements of early larvae and help solve nutritional problems during larval rearing and serve as guide or basis for the development of their respective feeding protocols in the hatchery.

4.2 Materials and Methods

4.2.1 Experiments with Mud Crab Larvae.

All newly hatched mud crab, *S. serrata* larval samples used in the following experiments were sourced from broodstock caught from rivers and estuaries in Townsville, Queensland and maintained at the aquarium facilities of JCU following the methods described in *Section 2.1.1*.

The first experiment compared the enzyme activities of starved versus fed larvae. A berried crab was closely monitored for hatching of eggs which normally occurs in the early morning and then newly hatched larvae (Zoea 1) were collected as soon as possible after hatch and reared following the standard rearing protocol developed at JCU (Genodepa, 2003; Genodepa *et al*., 2004a, 2004b) described in *Section 2.1.1*. In this experiment, the larvae were stocked into 300-L tanks (20-22‰ salinity and 27-29°C water temperature) at a density of 150 larvae L^{-1} in the morning at 09:30 h, about 5 h after the larvae were hatched. Larvae were reared for three days in replicate tanks (4 per treatment) either without food or fed with rotifers (*Brachionus rotundiformis*) at a density of ~ 40 individuals mL^{-1}). Larval samples for enzyme assay were initially collected right after the larvae were stocked into the rearing tanks, just prior to feeding with rotifers, and then succeeding samples were collected every 24 h for three days in both fed and starved treatments.

The second experiment determined changes in digestive enzyme activities of newly hatched larvae that were initially fed for 36 h, and then subsequently starved to simulate the scenario, in a natural environment, where food was initially available but then became limiting. A berried crab was again closely monitored for hatching of eggs, and newly hatched larvae were likewise stocked and reared in tanks following the same protocol used in the first experiment, except for the feeding scheme. For this experiment, the larvae were stocked into 15-L plastic aquaria at a density of 150 larvae L^{-1} and were provided with rotifers at ~40 individuals mL^{-1} at 09:00 h (about 5 h after hatching) and allowed to feed for 36 h. After the larvae had fed for 36 h, the rotifers were removed by gently draining the contents of the larval tanks into a scoop net (300 μm mesh) held inside a basin of seawater to retain the larvae and allow the rotifers to pass through. In order to completely flush out all the rotifers, clean seawater was allowed to flow through the scoop net containing the larvae, taking extra precaution to prevent larval stress and physical damage during the process of separating them from the rotifers. After checking water samples using a microscope and ensuring that all rotifers had been removed, the larvae were stocked into new rearing tanks and kept without any food for another 36 h. Initial samples of larvae for enzyme assay were collected from four replicate aquaria just before larvae were fed with rotifers (right after the larvae were stocked), and then every 12 h thereafter. Aside from the initial sample (0 h), three samples of larvae were collected while food was available (i.e., 12 h, 24 h, and 36 h) and another three samples after food was removed (i.e., 48 h, 60 h and 72 h).

The third experiment compared the enzyme activities of normally fed larvae (fed based on the common practice in most mud crab hatcheries; *Section 2.1.1*; Fig. 2.1) with enzyme activities of larvae in treatments where feeding was delayed for different periods. To ensure accurate determination of the time of hatching, the berried crab was closely monitored for mass hatching of eggs, which occurs before sunrise, and is characterized by the vigorous

swimming activity of the berried crab. For this experiment, as soon as vigorous swimming activity and mass hatching of eggs was observed, the berried crab was transferred to a new hatching tank to release some of its larvae for 30 minutes (from 03:30 h to 04:00 h), before being removed and returned to its former tank to hatch and release the rest of its larvae.

The larvae that were synchronously hatched were collected and stocked into 300-L tanks at a density of 100 larvae L^{-1} and reared following the protocol used in first experiment under this section, except for the feeding schemes. In the treatment that was fed normally (control), feeding with rotifers (~40 individuals mL^{-1}) was done 6 h after the larvae were hatched, while in the delayed feeding treatments, feeding of larvae with rotifers was delayed for 12 h, 24 h or 36 h from the time that feeding in the control treatment commenced. In the control treatment, larval samples for enzyme assay were collected initially just before feeding, then after the larvae had fed for 12 h, 24 h, 36 h, and 48 h, while in the delayed feeding treatments, samples were collected immediately after the larvae had fed for 12 h.

All larval samples for enzyme assay were kept in 1.5 mL Eppendorf vials. The number of larvae in each sample was counted using a compound microscope, while the sample weights were measured using a Cahn C-33 micro-balance (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). The vials were then stored at -70ºC until assay.

4.2.2 Experiments with Lobster Larvae

All newly hatched (Stage I phyllosoma) *P. ornatus* larvae used for the following experiments were from broodstock held at the AIMS as described in *Section 2.1.2*.

In the first experiment, enzyme activities in starved versus fed phyllosoma were compared. Newly hatched phyllosoma from AIMS were transported to JCU and stocked into 300 L static rearing tanks (35‰ salinity; 28ºC temperature) at a density of 100 larvae L^{-1}.

They were reared for 5 days either without any food or fed with newly hatched *Artemia* (INVE GSL). Feeding with *Artemia* (~5 nauplii mL^{-1}) was begun 12 h after hatch. The exact time of hatching, which normally occurs around midnight between 22:00 h and 03:00 h (Smith *et al*., 2009) was determined with the aid of a hatching movement sensor placed in the hatching tank at AIMS. Larval samples for enzyme assay were initially collected just prior to feeding with *Artemia* at 14:00 h and succeeding samples were collected from both fed and starved treatments after 24 h, 72 h and 120 h from initial sampling.

The second experiment determined the changes in enzyme activities of newly hatched phyllosoma that were initially fed for 24 h and then food was removed to simulate the scenario in the natural environment where food was initially present followed by a period of starvation. Newly hatched phyllosoma were stocked and reared in 60 L conical static tanks at AIMS (35‰ salinity and 27-28ºC temperature) at the density of 100 larvae L^{-1}. The phyllosoma were fed with 5 *Artemia* (INVE GSL) nauplii mL^{-1} at 12:00 h (about 10 h after hatching) and were allowed to feed for 24 h when their food (*Artemia* nauplii) was removed by gently draining the contents of the larval tanks into a scoop net (500 μm mesh) held inside a basin of seawater to retain the larvae and allow the *Artemia* to pass through. In order to completely flush out all the *Artemia*, clean seawater was allowed to flow through the scoop net containing the larvae, taking extra precautions to prevent larval stress and physical damage during the process of separating the phyllosoma and the *Artemia*. After checking water samples in a beaker and ensuring that all *Artemia* had been removed, the phyllosoma were stocked into new rearing tanks and kept without any food for another 24 h. Initial samples of larvae for enzyme assay were collected just before the phyllosoma were fed with *Artemia* (right after the larvae were stocked), and then every 8 h thereafter. Aside from the initial sample (0 h), three samples of larvae were collected while food was available (i.e., 8 h, 16 h, and 24 h), and another three samples after food was removed (i.e., 32 h, 40 h and 48 h).

The third experiment compared the enzyme activities of newly hatched phyllosoma which were fed normally (control treatment), with enzyme activities of phyllosoma in treatments where feeding was delayed for different periods. A berried female was monitored for hatching of eggs with the aid of a hatching movement sensor, and the newly hatched phyllosoma were brought to JCU and reared in 20-L static rearing tanks (35‰ salinity, 28ºC temperature) at stocking density of 100 larvae L^{-1}. The phyllosoma in all rearing tanks were fed with *Artemia* (INVE GSL) at a density of 5 nauplii mL^{-1} but the feeding schedule varied according to the treatments. In the control treatment, *Artemia* nauplii were fed 12 h after the phyllosoma were hatched (based on the practice at AIMS), while in the other treatments, feeding with *Artemia* nauplii was delayed for either 24 h, 48 h, or 72 h from the time the control treatment was initially fed. Larval samples for enzyme assay from the control treatment were collected just before feeding (0 h), and then after the phyllosoma had fed for 24 h, 48 h, and 72 h. Larval samples for enzyme assay from the delayed feeding treatments were collected after the phyllosoma have fed for 24 h and a subsequent period of starvation according to the treatments.

All larval samples for enzyme assay were kept in 1.5 mL Eppendorf vials. The number of larvae in each sample were counted while the sample weights were measured using a Cahn C-33 micro-balance (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). The vials were then stored at -70ºC until assay.

4.2.3. Assay of Enzymes

The larval samples were assayed for α-amylase, trypsin-like protease and non-specific esterase activities following the methods described in *Section 2.2.1* and *Section 2.2.2*. Mud crab larval sample extracts for assay of amylase, protease and esterase were all diluted 1:50 (sample: buffer), while lobster larval sample extracts were all diluted 1:100. Enzyme activities were expressed both as specific activity (*mU* mg^{-1} protein) and as total activity (*mU* ind^{-1}).

4.2.4 Statistical Analyses

In experiments comparing larval enzymes in starved and fed treatments, the differences in activities of enzymes between hours and between starved and fed larvae were analysed using factorial experiment in completely randomized design. The data were tested for homogeneity of variances using the Levine's test and then Contrast was used for multiple comparisons. Data from the rest of the experiments were analysed using One-Way ANOVA to determine differences among treatments. Data were tested for homogeneity of variance using Levine's test and when necessary arcsine, square-root or logarithmic transformations were made to make the data normal and homogeneous before Duncan's Multiple Range Test (DMRT) was used for multiple comparisons. When normal distribution and/or homogeneity of the variance was not achieved, the data were subjected to the Kruskal-Wallis H nonparametric test followed by the Games-Howell nonparametric multiple comparison test. All statistical analyses were performed using SPSS for Windows Version12.0. Data is presented as mean, plus or minus (±) standard error of mean (SEM), and results were considered significantly different at $P \leq 0.05$.

4.3 Results

4.3.1 Mud Crab Experiments

4.3.1.1 Fed versus starved larvae

The specific and total activities of amylase, protease and esterase in starved, as well as in fed newly hatched larvae of *S. serrata* are presented in Fig. 4.1. Amylase activity in fed larvae clearly showed an increasing trend with longer feeding duration, but remained relatively unchanged in starved larvae (Fig 4.1A and B). In particular, specific activity of amylase in fed larvae began to increase significantly from the initial level after 24 h ($P<0.05$), then remained at about the same level at 48 h before increasing further at 72 h ($P<0.05$). In starved larvae there was also an initial significant increase in activity after 24 h, but then remained at about the same level up to the 72 h sample (Fig. 4.1 A). In the case of total amylase activity in fed larvae (Fig. 4.1 B), the trend was always increasing but increased significantly from the initial level after 48 h and showed another significant increase to 72 h ($P<0.05$). In contrast, total amylase activity in starved larvae did not differ significantly from the initial level regardless of the starvation period (Fig. 4.1 B).

Comparison of the amylase activity of starved versus fed Zoea 1 showed that specific activity was significantly higher in fed than in starved larvae at 72 h, while total activity was significantly higher in the fed larvae from 24 h onwards ($P<0.05$). After 72 h of culture, specific activity of amylase in fed larvae (380.2 ± 53.9 *mU* mg^{-1} protein) almost doubled that of the level in starved larvae (211.2 ± 15.5 *mU* mg^{-1} protein) while total activity more than tripled (358.7 ± 40.2 *mU* ind^{-1} versus 102.5 ± 18.8 *mU* ind^{-1}) (Fig. 4.1A and B).

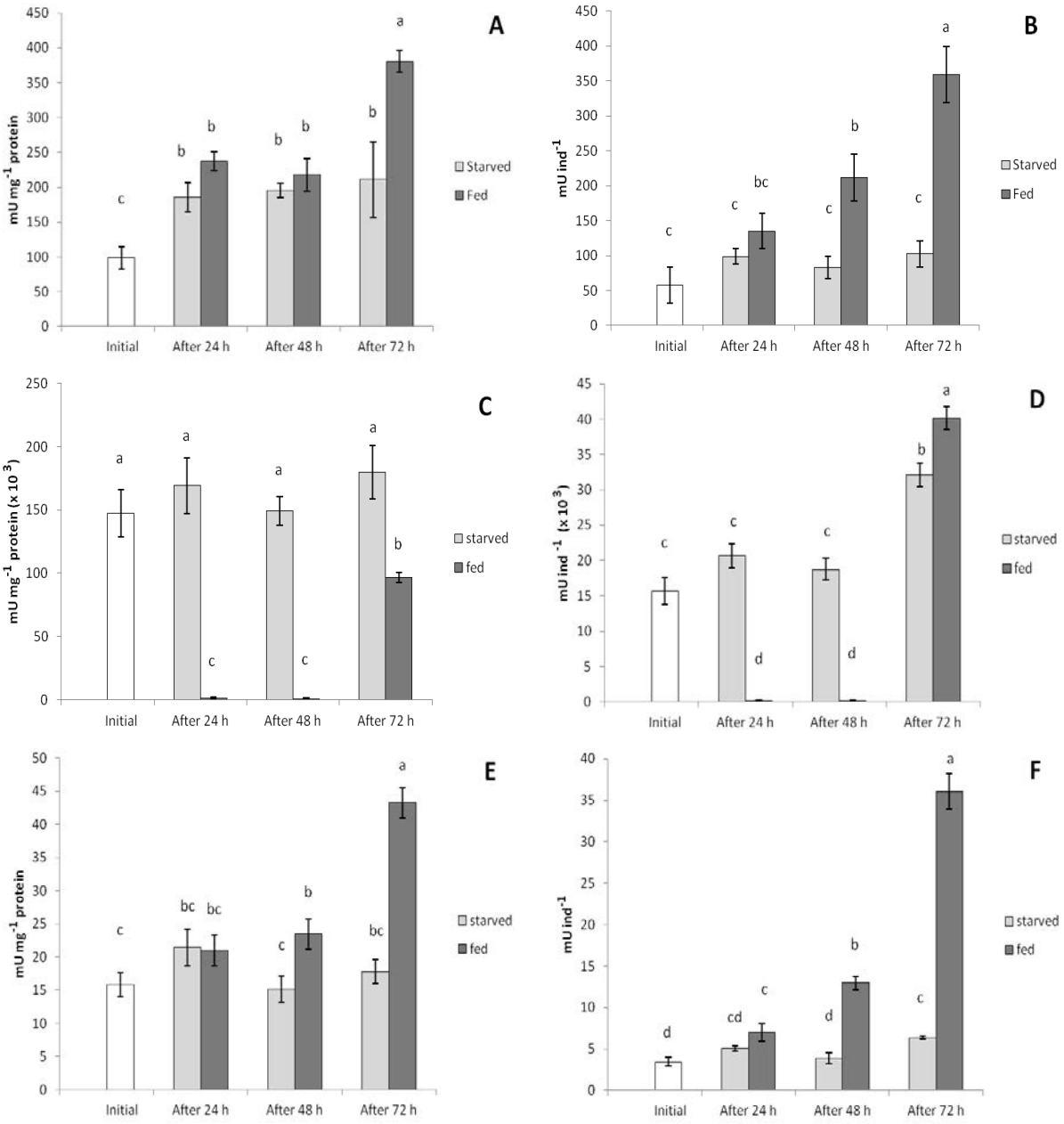

Fig.4.1. Mean (±SEM, n=4) specific (*mU* mg^{-1}protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D) and esterase (E & F) in starved and fed newly hatched (Zoea I) *S. serrata* larvae at various sampling periods from initial sampling (6 h post-hatch). Means with the same letters above bars are not significantly different (P>0.05).

In the case of protease activity, a very different pattern was shown. In starved larvae, while specific activity of protease did not differ with longer starvation (Fig. 4.1 C), total activity increased significantly ($P<0.05$) at 72 h (Fig. 4.1 D). Both specific and total protease activities in fed larvae dropped significantly to very low levels of near zero at 24 h and remained low until 48 h, before recovering with significant increases at 72 h ($P<0.05$; Fig. 4.1C and D). Despite the recovery in specific activity of fed larvae at 72 h, the activity level was lower than the initial level at the start of the experiment (Fig. 4.1C). In contrast, while total activity of fed larvae initially showed a trend that was similar to specific activity at 24 h and 48 h, total activity at 72 h (40154 ± 1609 *mU* ind^{-1}) recovered to a level that was significantly higher ($P<0.05$) and more than double the initial level (15702 ± 1877 *mU* ind^{-1}) (Fig. 4.1D).

Esterase specific activity in starved Zoea 1 did not change significantly throughout the duration of the experiment, however, total activity fluctuated but was significantly higher than the initial level only at 72 h ($P<0.05$) (Fig. 4.1 E and F). In fed larvae, esterase specific activity did not differ significantly from the initial level up to 48 h but then increased significantly at 72 h, while total activity increased significantly every 24 h from the initial level up to 72 h ($P<0.05$). Both specific and total activities of esterase were significantly higher in fed larvae compared to those in starved larvae at both 48 h and 72 h sampling periods, but the huge difference in esterase activities between fed and starved treatments occurred after 72 h ($P<0.05$; Fig. 4.1 E and F). Specific activity in fed larvae (43.25 ± 2.25 *mU* mg^{-1} protein) was more than twice that in starved larvae (17.8 ± 1.8 *mU* mg^{-1}protein) at 72 h, while total activity was almost six times higher in fed larvae: (36.0 ± 2.15 *mU* ind^{-1} versus 6.37 ± 0.18 *mU* ind^{-1}) (Fig. 4.1E and F).

4.3.1.2 Larvae fed initially then starved

Fig. 4.2 shows the time sequence of the changes in both specific and total amylase, protease and esterase activities of newly hatched *S. serrata* from the time of feeding, and from the time food was removed. Both specific and total activities of amylase gradually increased over time and appeared to be unaffected by the initial food availability and subsequent removal of food. Specific activity of amylase was significantly higher than the initial (0 h) level after 72 h ($P<0.05$) but did not change significantly while food was available (from 0 h to 36 h) and also when food was removed (from 36 h to 72 h) (Fig. 4.2 A). In contrast, total activity of amylase increased while food was available, and increased further when food was removed (Fig. 4.2 B). Total activity of amylase slightly fluctuated while food was available, but was significantly different from the initial (0 h) level only at 36 h where it was higher ($P<0.05$). Following removal of food, total activity of amylase monitored from 36 h to 72 h likewise fluctuated initially, and did not differ from the level at the time food was removed (at 36 h), except at 72 h where the level was significantly higher ($P<0.05$).

Specific and total activities of protease both dropped significantly ($P<0.05$) from a high level in the newly hatched larvae at 0 h to lowest levels after around 12 hours of feeding (from 172,298 *mU* mg^{-1} protein to 59,793 *mU* mg^{-1} protein and from 9,927 *mU* ind^{-1} to 3,586 *mU* ind^{-1}, respectively), and remained considerably low while food was available (Fig. 4.2 C and D). After food was removed, both specific and total protease activities remained consistently low and did not differ during the subsequent 30 h (from 36 h to 60 h) but interestingly, after 36 h without food, protease activities suddenly increased significantly ($P<0.05$) (Fig. 4.2 C and D).

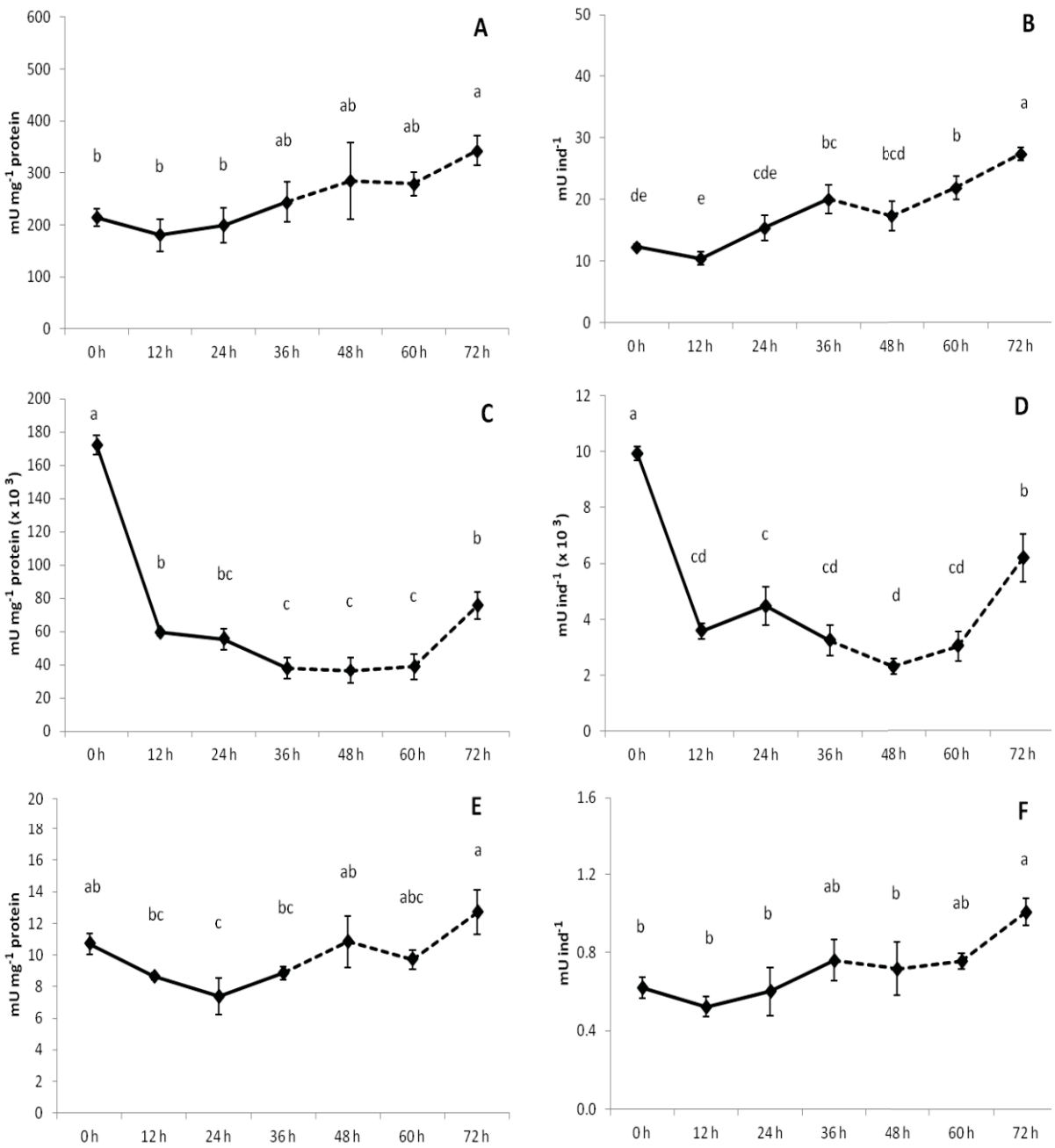

Fig. 4.2 Changes in mean (±SEM, n=4) specific (*mU* mg^{-1}protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D) and esterase (E & F) in newly hatched (Zoea 1) *S. serrata* larvae while food was available (from 0 h to 36 h) and after food was removed (from 36 h to 72 h). Means with the same letters above are not significantly different (P>0.05).

Specific and total activities of esterase appeared unaffected by initial food availability and the subsequent absence of food (Fig. 4.2 E and F). Esterase specific activity fluctuated while food was available for 36 hours, but the changes did not differ from the initial (0 h) level in the newly hatched larvae at the start of the experiment, except at 24 h where it was significantly lower ($P<0.01$). When food was removed, esterase specific activity increased slightly but did not differ from the level at the start of starvation (at 36 h), except at 72 h (after 36 hours starvation) where it was significantly higher ($P<0.05$). The specific activity of esterase when food was no longer available did not differ significantly from the initial (0 h) level in the newly hatched larvae at the start of the experiment. Total activity of esterase did not change significantly while food was available and also from the time when food was subsequently removed, however, total activity was significantly higher at the end of the experiment (at 72 h) compared to the initial (0 h) level in the newly hatched larvae.

4.3.1.3 Larvae subjected to delayed feeding

The enzyme activities in newly hatched (Zoea I) larvae under the various delayed feeding and normal feeding treatments are presented in Fig. 4.3. Overall, the pattern of amylase, protease and esterase activities of larvae which were fed normally for 12 h, 24 h, 36 h, and 48 h (F12/N, F24/N, F36/N and F48/N, respectively) were similar to the pattern observed in fed larvae in the previous experiments (Figs. 4.1 and 4.2).

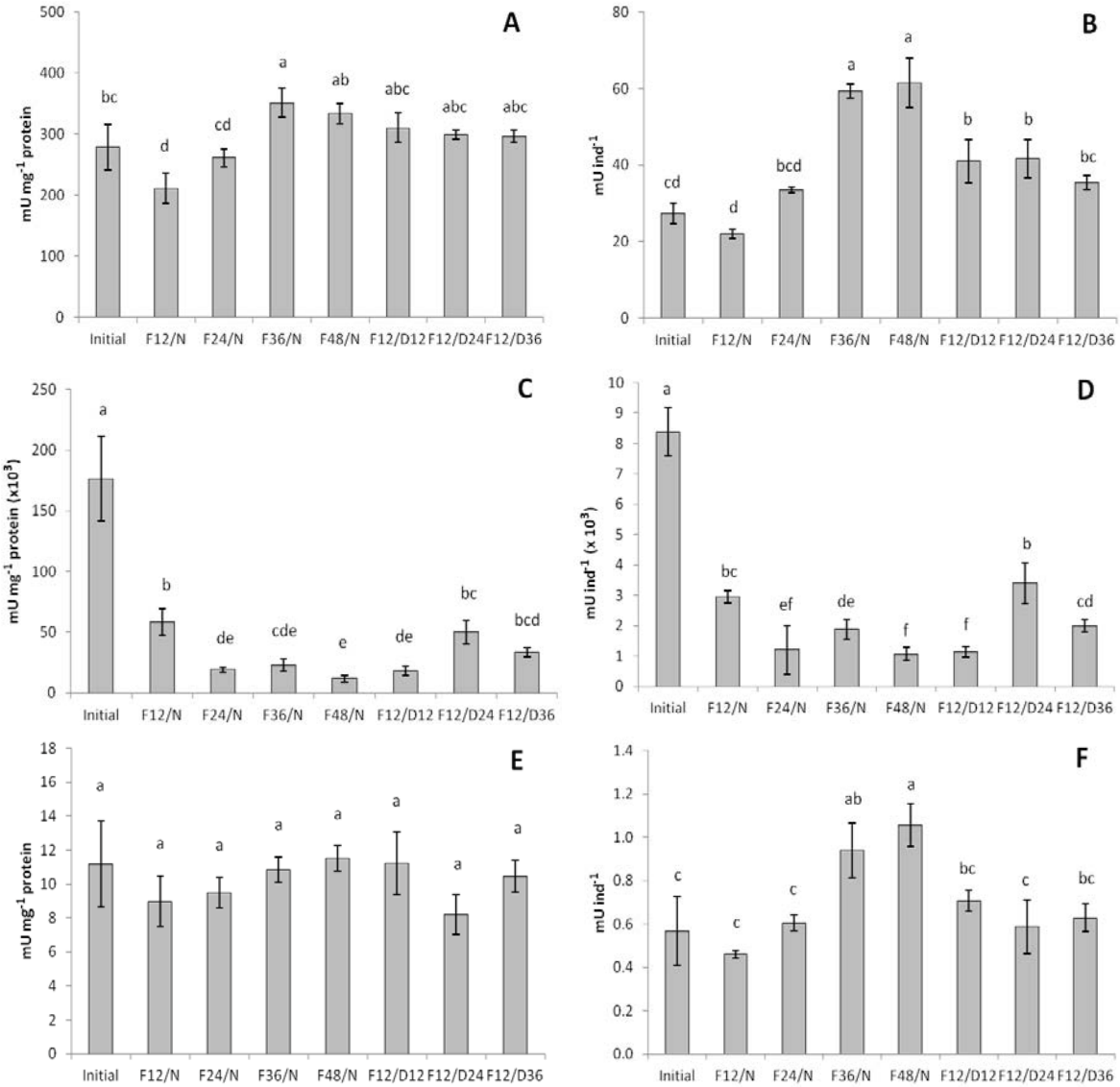

Fig. 4.3. Mean (±SEM, n=4) specific (*mU* mg^{-1}protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D), and esterase (E & F) in the newly hatched (Zoea 1) *S. serrata* larvae that were fed normally for 12 to 48 h or fed for 12 h but with the feeding delayed for 12 to 36 h. F12/N— fed normally for 12 h; F24/N—fed normally for 24 h; F36/N—fed normally for 36 h; F48/N—fed normally for 48 h; F12/D12— fed for 12 h after 12 h delay; F12/D24—fed for 12 h after 24 delay; F12/D36— fed for 12 h after 36 h delay. Means with the same letters above bars are not significantly different (P>0.05).

Newly hatched Zoea 1 showed the ability to compensate for short-term delay in food availability by increasing amylase activity. For example, both specific and total amylase activities of larvae fed for 12 h with the feeding delayed for 12 h, 24 h and 36 h (F12/D12, F12/D24, and F12/D36, respectively) were significantly higher ($P<0.05$) than in larvae that were fed normally for the same period of 12 h (F12/N) (Fig. 4.3 A and B). In fact, specific activities of amylase in the delayed feeding treatments did not differ from levels in larvae fed normally for longer periods of up to 48 h (i.e., F24/N, F36/N and F48/N) despite the shorter feeding period (Fig. 4.3 A). Total activities of amylase in the delayed feeding treatments likewise did not differ from levels in larvae fed normally 24 h (F24/N) but were significantly lower ($P<0.05$) than in larvae fed normally for longer periods (F36/N and F48/N) (Fig.4.3 B). Furthermore, amylase activity did not differ among the delayed feeding treatments regardless of the period of delay in feeding.

The interesting phenomenon of a general drop in protease activities soon after feeding was again shown in this experiment, confirming the results of the earlier experiments (*Sections 4.3.1.1* and *4.3.1.2*). Specific and total activities of protease in normally fed larvae dropped significantly ($P<0.05$) to the lowest level after feeding for 24 h (F24/N), and remained at more or less the same level after feeding for 36 h (F36/N) and 48 h (F48/N) (Fig. 4.3 C and D). The activities of protease in the delayed feeding treatments likewise dropped to very low levels but the drop was greater (often significant, $P<0.05$) when feeding was delayed for 12 h (F12/D12), compared to 24 h (F12/D24) and 36 h (F12/D36). The drop in protease activities of larvae where feeding was delayed for only 12 h (F12/D12) was comparable to the drop in activities of normally fed larvae (i.e., F24/N, F36/N and F48/N) as the levels did not differ, or were even significantly lower ($P<0.05$) in the delayed feeding treatment. In contrast, protease activities of larvae where feeding was delayed for 24 h

(F12/D24) and 36 h (F12/D36) did not drop to the lowest levels but were just about the same level as that of larvae fed normally for 12 h (F12/N).

The delay in feeding of newly hatched zoea up to 36 h showed no apparent changes in esterase activities. Specific activity of esterase in larvae from the delayed feeding treatments (F12/D12, F12/D24, and F12/D36) did not differ from the initial level in newly hatched zoea, as well as from the levels in larvae that were normally fed for various periods (F12/N, F24/N, F36/N and F48/N) (Fig 4.3 E and F). Total activity of esterase from the delayed feeding treatments did not differ from the initial level in the newly hatched zoea or from the levels in larvae that were fed normally for 12 h (F12/N) and 24 h (F24/N), but was significantly lower ($P<0.05$) than those fed for 48 h (F48/N).

4.3.2 Lobster Experiments

4.3.2.1 Fed versus starved phyllosoma

The amylase, protease, and esterase activities in starved versus fed newly hatched *P. ornatus* phyllosoma is presented in Fig. 4.4. The specific and total activities of amylase in starved phyllosoma remained low and did not differ from the initial level for the duration of the experiment (120 h). In contrast, specific and total activities of amylase in fed phyllosoma increased after 24 h, and remained significantly higher than the initial level until the end of the experiment despite significant fluctuations at various sampling periods ($P<0.05$). Amylase activity was significantly higher in fed than in starved phyllosoma ($P<0.01$); specific activity was at least four magnitudes higher in fed (347.3 ± 21.6 *mU* mg^{-1} protein) than in starved (78.7± 17.9 *mU* mg^{-1} protein) phyllosoma, while total activity was at least 16 times higher in fed than in starved phyllosoma (1351±87 *mU* ind^{-1} and 81.7±11 *mU* ind^{-1} respectively) (Fig. 4.4 A and B).

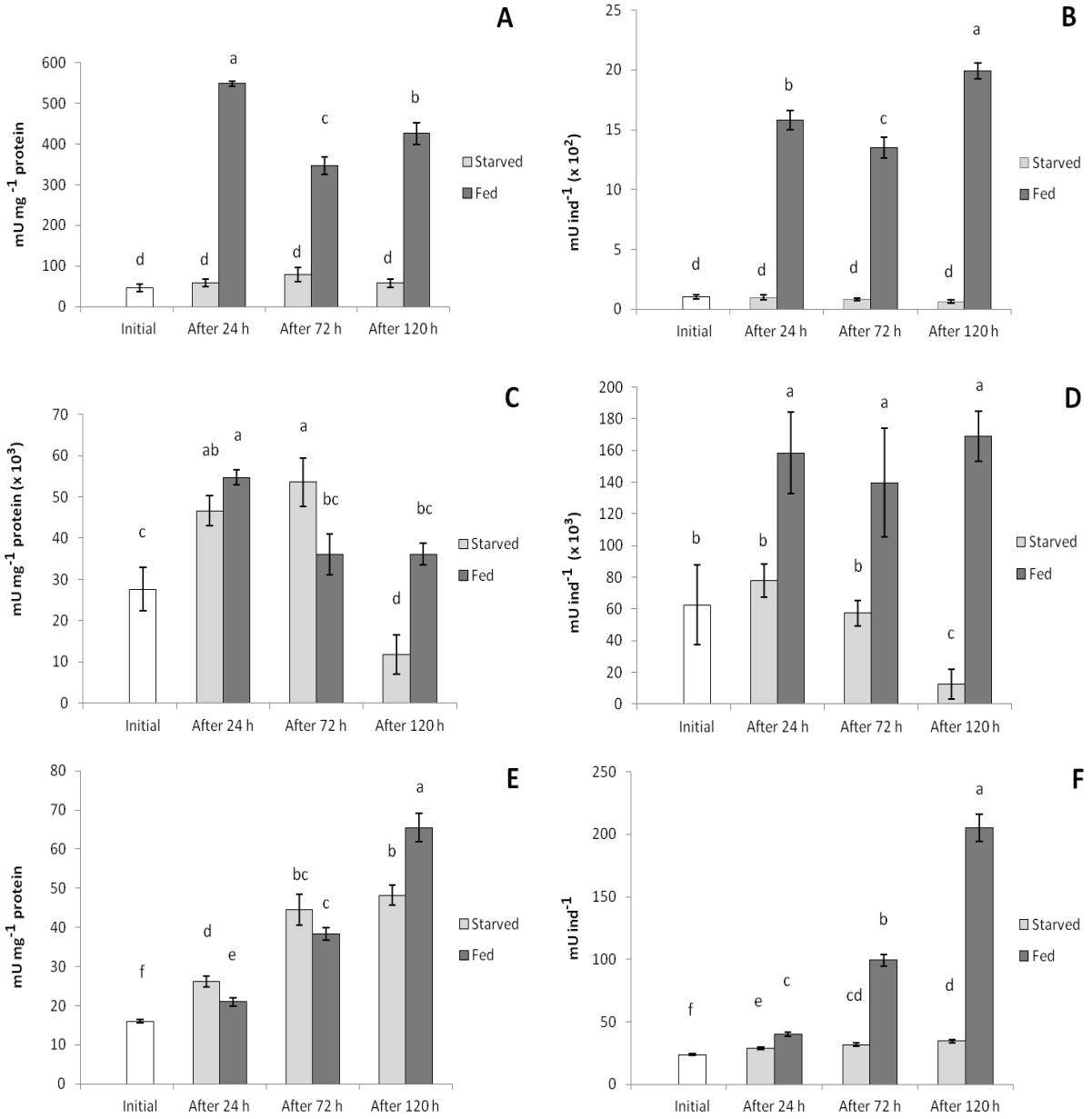

Fig. 4.4. Mean (±SEM, n=4) specific (*mU* mg^{-1}protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D), and esterase (E & F) in starved versus fed newly hatched (Stage 1) *P. ornatus* phyllosoma at various sampling periods from initial sampling (12 h post hatch). Means with the same letters above bars are not significantly different (P>0.05).

In starved phyllosoma, the specific activity of protease significantly increased after 24 h ($P<0.05$), and remained high until 72 h, before decreasing significantly to a level lower than the initial level after 120 h ($P<0.05$), while total activity remained at the same level as the initial activity for 72 h, then decreased significantly at 120 h ($P<0.05$). In fed phyllosoma, the specific activity of protease increased significantly after 24 h ($P<0.05$), but then decreased to a level that did not differ from the initial activity after 72 h of having food, and remained at the same level until the end of the experiment (120 h), while total activity significantly increased after 24 h ($P<0.05$) and maintained the same level until the end of the experiment. Comparison of protease activities in starved versus fed phyllosoma showed that specific activity was significantly higher in starved larvae at 72 h, but was higher in fed than in starved phyllosoma at 120 h, while total activity was always higher in fed compared to starved phyllosoma ($P<0.01$).

Both specific and total activities of esterase in starved phyllosoma increased significantly from the initial level up to 72 h but remained at the same level at the end of the experiment (120 h), while in fed phyllosoma, both specific and total activities of esterase increased significantly from the initial level until the end of the experiment ($P<0.05$). The comparison of esterase activity in starved versus fed phyllosoma showed that specific activity was significantly higher in starved phyllosoma at 24 h but it was the opposite at 120 h ($P<0.01$), while total activity was always signifcantly higher in fed phyllosoma ($P<0.01$).

4.3.2.2 Phyllosoma fed initially then starved

Changes in enzyme activities of newly hatched (Stage 1) phyllosoma after food was introduced, and then subsequently removed are shown in Fig. 4.5. Both specific and total amylase activities increased significantly 8 h after food was made available, and continued to increase up to 16 h ($P<0.05$); they remained at similar levels after 24 h, but when food was removed, both specific and total activity began to decrease significantly and was back to a low level, slightly above the initial (0 h) level at 48 h (after 24 h without food) (Fig. 4.5 A and B). The specific and total activities of protease both fluctuated, but did not differ significantly when food was made available or when food was subsequently removed (Fig. 4.5 C and D).

Changes in the specific activity of esterase showed an increasing trend, but did not differ while food was available (from 0 h to 24 h); when food was removed, specific activity continued increasing until the end of the experiment, but only started to be significant higher than the initial (0 h) level after 40 h, and significantly increased further at 48 h ($P<0.05$) (Fig 4.5 E). Total activity of esterase significantly increased from the initial (0 h) level while food was available, and continued to increase significantly from the time when food was removed (at 24 h) until the end of the experiment (at 48 h) (Fig 4.5 E).

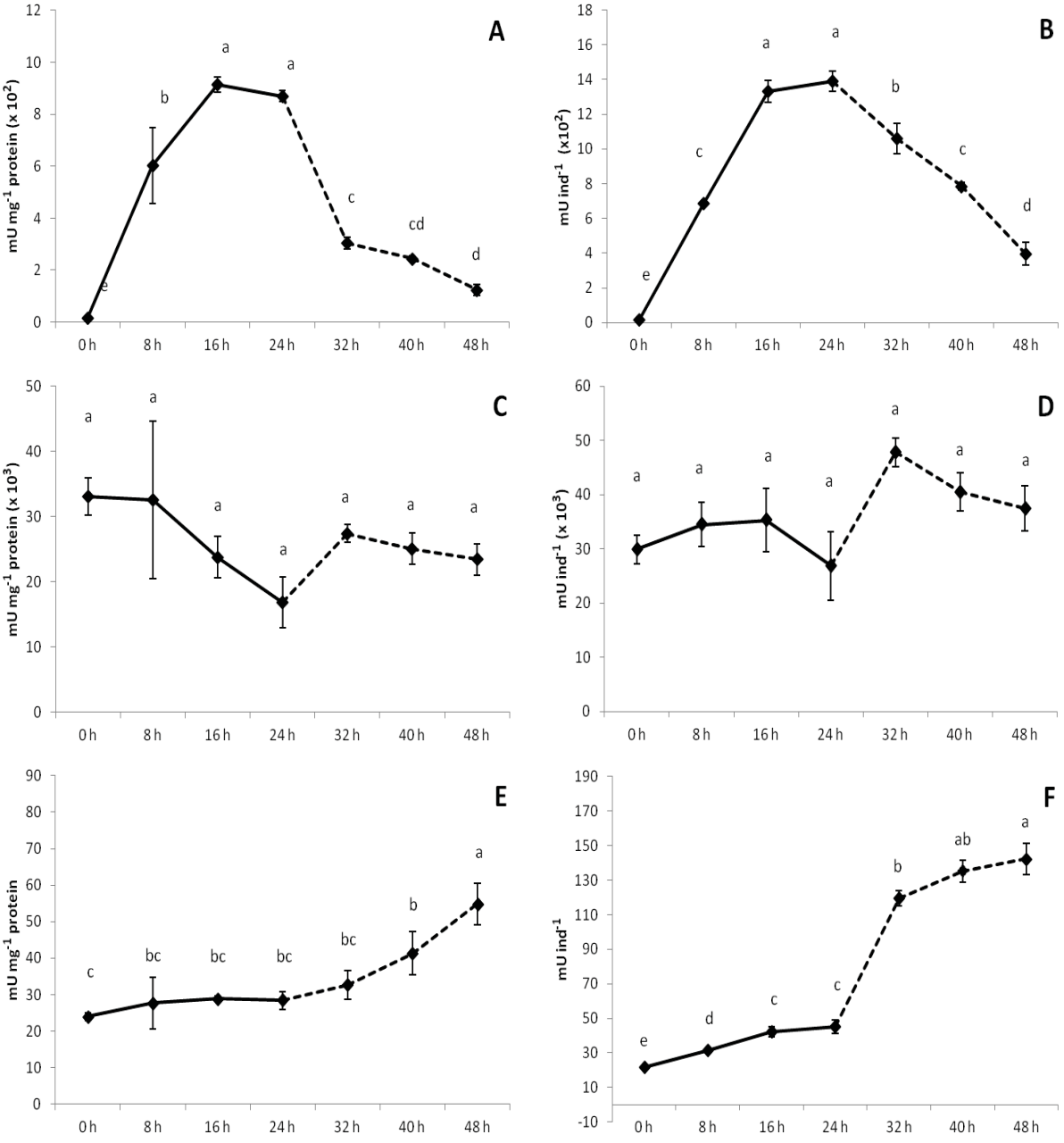

Fig. 4.5 Changes in mean (±SEM, n=4) specific (*mU* mg^{-1}protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D), and esterase (E & F) in newly hatched (Stage 1) *P. ornatus* phyllosoma while food was available food (from 0 h to 24 h) and after food was removed (from 24 h to 48 h). Means with the same letters above are not significantly different (P>0.05).

4.3.2.3 Phyllosoma subjected to delayed feeding

The enzyme activities of newly hatched (Stage 1) *P. ornatus* phyllosoma under various delayed feeding and normal feeding treatments are presented in Fig. 4.6. The general pattern of amylase, protease, and esterase activities of phyllosoma that were fed normally for 24 h, 48 h, 72 h, and 96 h (F24/N, F48/N, F72/N and F96/N, respectively) were similar to the pattern observed in fed, newly hatched phyllosoma in the previous experiments (*Section 4.3.2.1* and *Section 4.3.2.2*).

The specific and total amylase activities in phyllosoma under normal feeding and delayed feeding treatments are presented in Figs. 4.6 A and B. The specific and total activities of amylase in normally fed phyllosoma increased significantly ($P<0.05$) after 24 h (F24/N) and reached peak activity at 48 h (F48/N), before decreasing back to the 24 h level at 72 h (F72/N), and maintaining about the same level at the end of the experiment (after 96 h, F96/N). The delayed feeding of phyllosoma for 24 h (F24/D24) resulted in significantly higher ($P<0.05$) levels of specific and total activities of amylase than in larvae that were normally fed for the same period (F24/N), and even compared to larvae fed for 48 h (F48/N), which had the highest level of amylase activity among normally fed larvae. The delayed feeding of phyllosoma for 48 h (F24/D48) had significantly lower ($P<0.05$) amylase activity than in delayed feeding for only 24 h (F24/D24), but likewise did not differ in activity from that of larvae which were normally fed for 24 h (F24/N). Further delay in feeding from 48 h (F24/D48) to 72 h (F24/D72) resulted in further reduction of larval amylase activities, particularly the total activity which was significantly lower ($P<0.05$) than in larvae which were normally fed for 24 h (F24/N).

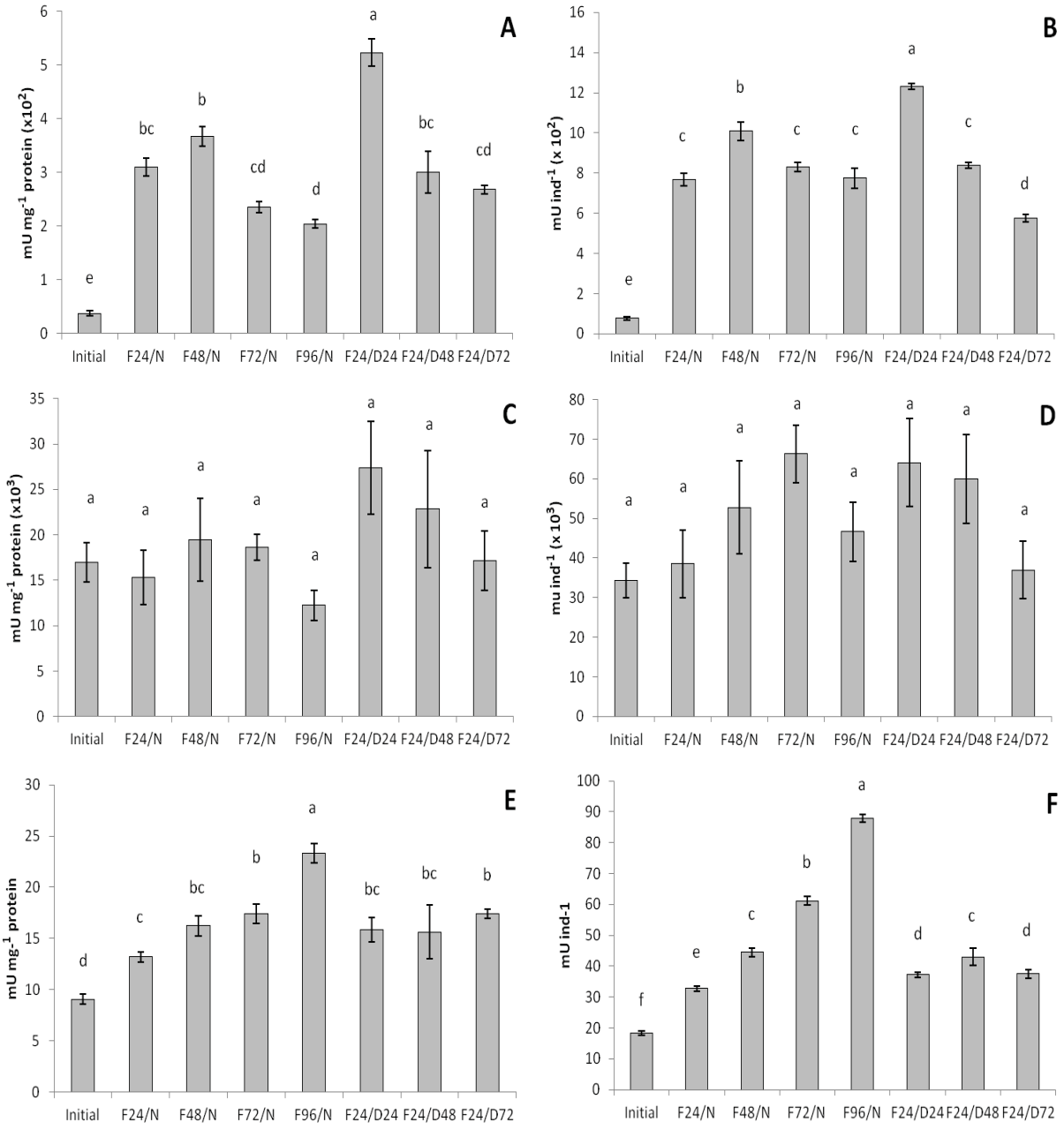

Fig. 4.6. Mean (±SE, n=4) specific (*mU* mg^{-1}protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D), and esterase (E & F) in the newly hatched *P. ornatus* phyllosoma that were fed normally for 24 to 96 h or fed for 24 h but with the feeding delayed for 24 to 72 h. F24/N— fed normally for 24 h; F48/N— fed normally for 48 h; F72/N— fed normally for 72 h; F96/N— fed normally for 96 h; F24/D24— fed for 24 h after 24 h delay; F24/D48—fed for 24 h after 48 h delay; F24/D72—fed for 24 h after 72 h delay. Means with the same letters above bars are not significantly different (P>0.05).

The specific activity and total activity of protease in normally fed phyllosoma (F24/N, F48/N, F72/N and F96/N) did not change significantly with longer feeding time, and did not differ at any point from the initial level at the start of the experiment; protease activities of phyllosoma subjected to delayed feeding for 24 h (F24/D24), 48 h (F24/D48), and 72 h (F24/D72) likewise did not differ from the initial activity and also from activities in normally fed larvae (Fig. 4.6 C and D).

The specific and total activities of esterase in phyllosoma under normally fed and delayed feeding treatments are presented in Figs. 4.6 E and F. Specific and total activities of esterase in normally fed phyllosoma increased significantly ($P<0.05$) from the initial level after 24 h (F24/N) and continued increasing (often significantly, $P<0.05$) until the end of the experiment. Phyllosoma subjected to delayed feeding for up to 72 hours had higher levels of esterase activity compared to phyllosoma that were normally fed for the same period of 24 hours. In particular, total activity turned out to be significantly higher ($P<0.05$) in all delayed feeding treatments (F24/D24, F24/D48 and F24/D72) than in larvae that were normally fed for 24 h (F24/N), while specific activity in the delayed feeding treatments were likewise higher, but did not differ from those fed normally for 24 h (F24/N), except for the 72 h (F24/D72) delayed feeding which turned out significantly higher ($P<0.05$). In fact, specific activity of esterase in the delayed feeding treatments did not differ from larvae that were fed normally for longer than 24 h (i.e., F48/N and F72/N).

4.4 Discussion

The enzyme activities in this study were expressed both as specific activity (*mU* mg^{-1} protein) and as total activity (*mU* ind^{-1}) of the major digestive enzymes measured, in order to provide better insights into larval enzyme dynamics allowing appropriate interpretation of results, especially when comparing enzyme activities between starved and fed larvae. For example, the increasing specific activity of major digestive enzymes in starved larvae may be primarily due to the decreasing larval protein content resulting from prolonged larval starvation, hence expressing larval enzymes as total activity should help confirm increasing or decreasing trends because it is not dependent on larval protein content.

The results of this study showed species-specific patterns of enzyme response of first feeding larvae of *S. serrata* and *P. ornatus* to different conditions of food availability. For instance, prominent differences in protease activity were detected between starved and fed Zoea 1 larvae of *S. serrata*, while in Stage I phyllosoma of *P. ornatus*, it was amylase activity that showed notable differences between starved and fed larvae. Such differences suggest that the strategies employed by these two tropical crustaceans to cope with food deprivation, to maximize their chances of survival and development to the next larval stage, are likely to be very different.

Enzymes catalyse chemical reactions that involve substrates (Tipton, 2002), among which are the various components of the diet (Le Vay *et al.*, 1994; Lemos & Rodriguez, 1998). Consumption of food by larvae is therefore likely to result in increased activities of certain enzymes due to the availability of substrates, which may explain the generally higher levels of certain enzymes in fed larvae compared to starved larvae in this study. While it is possible that the digestive enzymes in live prey such as rotifers or *Artemia* may have contributed to the enzyme activity measured in this study, these are unlikely to be substantial, based on available literature (Lovett & Felder, 1990a; 1990c; Kamarudin *et al.*,

1994; Jones *et al*., 1997a; Johnston *et al*., 2004a). For example, the contribution made by *Artemia* to the enzyme activity measured in *Penaeus setiferus* (Lovett & Felder, 1990a, 1990c) and *Macrobrachium rosenbergii* (Kamarudin *et al*., 1994) were found to be insignificant. Jones *et al*. (1997a) likewise reported similar findings on contributions made by live micro-algae and *Artemia* to enzyme activity at each larval stage of *Penaeus monodon* and *M. rosenbergii.* Furthermore, Johnston *et al*. (2004a) recorded significantly lower total enzyme activities in *Artemia* compared to the cultured phyllosoma of the lobster *Jasus edwardsii,* and they interpreted it as very unlikely that substantial proportions of the enzyme activities measured in the larvae originated from the ingested *Artemia.* Johnston *et al*. (2004a) explained that although phyllosoma of *J. edwardsii* can consume 15 to 25 *Artemia* nauplii per day, they are not ingested whole, but are torn apart and only small pieces of tissue are ingested (Cox & Bruce, 2002), hence, it is unlikely that enzymes contained in the gut of *Artemia* would remain in active form after ingestion. As typical crustaceans, the first stage larvae of *S. serrata* and *P. ornatus* also use their mouthparts to tear apart their prey before ingesting, hence, it is likewise unlikely that enzymes from either rotifers or *Artemia* made any significant contribution to the enzyme levels measured in the larvae in this study.

A common characteristic of crustacean larvae is their constant feeding activity, which requires continuous catabolism of nutrient reserves to provide the required energy (Cuzon *et al*., 2000). Due to rapidly decreasing nutrient reserves in newly hatched larvae, the availability of food was generally expected to result in increased or maintained levels of enzyme activities, however, this was not the case for protease activity of first feeding *S. serrata* larvae which decreased sharply to very low levels when food was introduced. It should be noted that such substantial decrease in protease activities was simultaneously coupled with a gradual increases in amylase and esterase activities in fed larvae, suggesting that fed larvae utilize carbohydrates and lipids more extensively while building-up protein

reserves. Although it appears that fed crab zoea initially relied heavily on carbohydrates and lipids, it eventually appeared to utilise protein extensively at the time closer to moulting. This was indicated by sharp increases in protease activities of fed larvae at 72 h, particularly total protease activity, which was more than twice the initial (0 h) level in newly hatched larvae. Such dynamic enzyme activity pattern was largely confirmed in the subsequent experiment wherein larvae were starved after initial 36 h of food intake.

In contrast, protease activity in starved larvae remained high throughout the 72 h sampling duration, and activity levels were relatively much higher (by 2-3 orders of magnitude) than that of amylase and esterase. Such enzyme activity pattern confirms the results presented in Chapter 3 indicating that protein is likely to be the major source of energy during the initial phase of starvation in newly hatched (Zoea l) larvae of *S. serrata*. While both proteins and lipids are degraded and utilized for energy during starvation in crustacean larvae, proteins are known to contribute relatively more energy than lipids because they are available in much greater quantities (Anger, 1986). Furthermore, as reported in Chapter 3, lipids are unlikely to be the main energy source of starved newly hatched larvae of *S. serrata,* because starting from the later phase of embryonic development (i.e. heartbeat stage), esterase activity had already dropped significantly from 59.8 to 25.7 $mU\ mg^{-1}$ protein, and remained low in starved Zoea l larvae. Such observations are confirmed in this experiment by the constantly low levels of esterase activity in starved Zoea 1 larvae (<20 $mU\ mg^{-1}$ protein). Comparison of protease activity patterns in starved and fed newly hatched Zoea 1 suggest that protein reserves were utilized when food is unavailable, but when food is available, carbohydrates and lipids from ingested prey were utilized more extensively, while protein was relatively spared initially. However, as larval development approaches the final phase of the moult cycle, stored proteins were also utilized. Together, these results highlight the significance of protein in the diet of mud crab larvae as well as the ability of the larvae to

respond to the different conditions of food availability by manipulating the relative activities of major digestive enzymes and selectively catabolise certain nutrients for energy, growth and development.

The results showing increased amylase activity in newly hatched Zoea in response to delayed food availability may be linked to the observations of Hofer (1982) and Harris *et al.* (1986) that the secretion of large amounts of enzymes may occur to maximize the use of a scarce component in the diet. Harms *et al.* (1991) also suggested that under conditions of high food availability, such as during larval culture, digestive enzyme levels are reduced as energy requirements are met without the need for highly efficient digestion, however, at lower levels of food availability or specific nutrient unavailability, but above the level of malnutrition, higher enzyme activity may be observed. It is possible that scarcity of certain dietary components may have occurred within the few hours of starvation in the newly hatched Zoea 1 in this experiment. In the mud crab, *S. paramamosain*, even a relatively short period of starvation after hatching reportedly could significantly affect larval survival and development (Li *et al.*, 1999), and the same is true with larvae of *S. serrata* (personal observation). The PNR_{50} (Point-of-No-Return) for Zoea l *S. paramamosain* was estimated to be about 1.3 days, while PRS_{50} (Point-of-Reserve Saturation) was around 2.3 days (Zeng & Li, 1992; Li *et al.*, 1999), which means that larvae should be fed soon after hatching. Immediate feeding of newly hatched larvae of the blue swimming crab, *Portunus pelagicus*, and the spanner crab, *Ranina ranina*, were also required for maximum survival (Mingawa, 1988; Mingawa & Morano, 1993).

The enzyme activities reported in this study also suggests that the capacity of newly hatched *S. serrata* larvae to produce digestive enzymes generally remained stable and did not drop significantly for a period of 36 h under the condition of food deprivation, which can be explained by the fact that starved, newly hatched larvae still needed enzymes to

catabolise existing nutritional reserves for maintenance, and to fuel the swimming activity in the hope of encountering food items during the limited time window of their relatively short moult cycle duration. It seems that the capacity of newly hatched Zoea 1 larvae to produce digestive enzymes is not the main reason related to the increased mortality and retarded development observed in crabs with delayed first feeding, but rather because of lack of energy reserves to fuel continued swimming activity. Once the larvae stops swimming and settles on the tank bottom, its chance of encountering food is great diminished.

The enzyme activity patterns in Stage I phyllosoma of *P. ornatus* showed that while they appeared to utilize carbohydrates as well as proteins and lipids, catabolism of carbohydrates from prey was prioritized, as shown by the notable and immediate increase in amylase activity which remained high in the subsequent days for fed phyllosoma, but was not observed in starved larvae. Based on field observations of common ecological associations of wild phyllosoma as well as various indirect studies on their natural diets, Jeffs (2007) deduced that the natural diets of phyllosoma consist of mainly gelatinous zooplankton and small crustaceans. Given the large sizes of some gelatinous zooplankton and the body form of phyllosomes, Jeffs (2007) suggested that in the field, phyllosoma larvae often attach to living gelatinous zooplankton and feed on them for some time.

Pelagic zooplankton generally have high protein and lipid but low carbohydrate content (Le Vay *et al*., 2001), which is typical of the *Artemia* nauplii used in this study. Hence, the finding of immediate and notable increase in amylase activity in fed *P. ornatus* phyllosoma was somewhat unexpected. However, it may suggest that compared to proteins and lipids, carbohydrates from the prey represent a relatively less important energy source that larvae choose to prioritize to provide energy for maintenance, to maximize their survival in oligotrophic environments and, in order to effectively utilize the relatively limited carbohydrates, amylase activity was increased significantly. Jones *et al*. (1997a) likewise

reported a similar mechanism of adaptation in some crustacean larvae which exhibited high protease and trypsin activities when they consumed low protein diets in order to maximize assimilation efficiency of a relatively limited substrate.

The immediate and continuous drop in amylase activity after food was removed following initial 36 h feeding supports the above hypothesis suggesting that phyllosoma utilized mainly the carbohydrates of ingested prey, but ceased doing so when the supply became unavailable. Furthermore, this suggests that carbohydrates from prey were utilized quickly and not conserved when larvae were fed. This result also agrees with previous reports that carbohydrates generally do not make an important contribution to crustacean energy during starvation (Holland, 1978; Regnault, 1981; Dawirs, 1987). Carbohydrates are known to have a more rapid turnover and constitute less than 5% of the body mass of crustaceans in general, which have limited capability for carbohydrate storage (Anger, 1986).

The enzyme activity pattern of starved Stage I phyllosoma agrees with the results presented in Chapter 3, confirming that protein is initially the main energy source during starvation, however, as protein reserves were exhausted with prolonged starvation, lipids became increasingly utilised. This was shown by sharp drops for both specific and total protease activities in starved phyllosoma after 120 h which coincided with significant increases in esterase activities. The continuous increases in esterase activity in both starved and fed phyllosoma suggest increasing importance of lipids as energy source regardless of their feeding conditions. While protease activity initially increased and then levelled in fed phyllosoma, esterase activity increased continuously, suggesting increased utilization of lipids, and possibly the storage of proteins. Storage of lipids and carbohydrates are generally more limited in marine crustaceans (Dall & Smith, 1986) compared to proteins. Proteins have been observed to be the principal substrates used to meet the energy requirements of starved decapods, but more proteins were also accumulated compared to other components upon

feeding (Anger, 2001). Compared to the initial activity levels measured in newly hatched phyllosoma, the relatively lower increase of protease activities (maximum of 2 fold) in fed phyllosoma compared to amylase activities (> 7 fold) may suggest that a large proportion of ingested proteins from the high protein prey (*Artemia* nauplii) were conserved, while carbohydrates which, though available in limited amounts, were prioritized.

The results of the delayed feeding experiment showed that Stage I phyllosoma elevated both specific and total amylase activities to significantly higher levels when feeding was delayed for 24 h, likely to compensate for delayed food intake. The elevated activity of amylase in response to delayed feeding by *P. ornatus* phyllosoma may again highlight its ability to utilise carbohydrates as discussed earlier. However, such response was no longer observed when feeding was further delayed for more than 24 h, suggesting that such ability diminishes as the period of starvation extends. In contrast, the activities of protease and esterase remained stable and did not diminish even when feeding was delayed up to 72 h, which is in agreement with the results of the earlier experiment on enzyme activities of newly hatched *P. ornatus* phyllosoma in starved and fed treatments. As discussed in Chapter 3, protein and lipid reserves are utilised by starved *P. ornatus* Stage I phyllosoma as sources of energy for maintenance and for fuelling swimming activity, which explains the elevated protease and esterase activities in this study despite delayed feeding. Furthermore, protease and esterase activities in this study remained stable and did not diminish within 72 h, probably because the maximum period of starvation prior to first feeding which will still allow the larvae to recover and moult to the next stage (PNR) was not reached. Phyllosomae of palinurid lobsters can survive longer periods of starvation and the PNR for the newly hatched phyllosoma were reported to be around 4.2 to 6.5 days (Mikami *et al.*; 1995, Abrunhosa & Kittaka, 1997; Johnston *et al.*, 2004).

4.5 Summary and Conclussions

The patterns of enzyme activity in first feeding larvae of *S. serrata* and *P. ornatus* subject to different conditions of food availability provide insights into how these larvae cope with such conditions in their natural environment characterized by intermittent food availability. The enzyme response of first feeding zoea of *S. serrata* suggests that protein reserves were prioritized while no food was available, but when food was available, larvae utilized carbohydrates and lipids more extensively while building-up protein reserves and eventually utilise protein extensively at the time closer to moulting. The enzyme reponse of first feeding *P. ornatus* phyllosoma suggests an ability to utilise carbohydrates, protein and lipids, but highlights the ability to prioritise the use of carbohydrates when food is available. In the absence of food or even during short-term starvation, the phyllosoma immediately cease to utilise carbohydrates and shift to protein and lipid utilisation. During short-term delayed feeding, both species showed elevated levels of amylase activity which suggests increased capability to utilize carbohydrates, but such ability is lost as starvation resulting from delayed feeding is prolonged.While *S. serrata* and *P. ornatus* larvae showed distinct adaptations to different food availability conditions, the orders of manitude higher levels of protease in both crustaceans highlight the significant role of protein nutrition in first feeding larvae of these species.

CHAPTER 5

Digestive enzyme responses of *Scylla serrata* and *Panulirus ornatus* larvae to quantity and quality of feeds: the effects of food density and food type

5.1 Introduction

One of the least understood aspects crucial to hatchery culture of *S. serrata* and *P. ornatus* is the digestive physiology of their larvae. Different aspects of the digestive physiology of larval crustaceans can be studied through various means, such as observations of the behaviour of prey organisms and corresponding larval feeding behaviour, structures of larval feeding apparatus (e.g. feeding appendages and mouthparts), and investigations into gut morphology and digestive enzyme profiles (Jeffs, 2007). In Chapter 4, the enzyme responses of first feeding *S. serrata* and *P. ornatus* larvae to various food availability conditions were examined, including the comparison of enzyme activities of starved versus fed newly hatched larvae, changes in enzyme activities under the conditions where food was initially introduced but subsequently removed, and enzyme activities of newly hatched larvae subject to different periods of delayed feeding. The result provided new insights into nutrient utilization strategies and physiological adaptations of these larvae to cope with the different conditions of food availability in their natural environments, and highlight the importance of timing when providing food to first feeding larvae reared in captivity. The previous experiments focused on extreme situations of food availability or unavailability that newly hatched larvae sometimes encounter in the wild, however, food is generally readily available for the larvae in aquaculture hatcheries following initial feeding, although their quantity and

quality could vary substantially. Analysis and comparison of larval enzyme responses to both quantity and quality of foods commonly used for hatchery culture of *S. serrata* and *P. ornatus* is therefore the next important and logical step to better understand the digestive physiology of the larvae of these economically important crustaceans.

In hatcheries, it is a common practice to ensure that food is always available and the density of larval food is usually, and intentionally, several times greater than would occur naturally in order to improve the chances of larvae encountering the prey. In many cases, survival and growth of larvae are used as indicators of appropriate ration and nutritional quality of foods, however, the close relationships between food availability and the activities of certain digestive enzymes reported in Chapters 3 and 4 indicates that the enzyme responses of larvae may be a useful indicator of whether the appropriate quantity and quality of food is supplied in hatcheries. Furthermore, considering that it is commonly assumed that live prey are generally superior to formulated diets because enzymes from live prey are utilized by the larvae for digestion of food (Kurmaly *et al*. 1990; Jones *et al*. 1993), comparative studies on larval enzyme responses to feeding on different live prey as well as formulated diets may help evaluate the suitability of these foods for a particular larvae stage and may highlight potential deficiencies in these foods.

The enzyme responses of *S. serrata* and *P. ornatus* larvae to both quantity and quality of foods commonly used in aquaculture hatcheries has not been reported yet despite the high aquaculture potential of these species. This Chapter therefore aimed to determine and compare the enzymic responses of first feeding *S. serrata* and *P. ornatus* larvae to different densities of rotifers and *Artemia,* respectively. Moreover, the enzyme responses of *S. serrata* Zoea II fed with either rotifers or Artemia nauplii and megalopae fed with either Artemia or a formulated diet were also determined and compared in this Chapter.

5.2 Materials and Methods

5.2.1. Food Quantity Experiments

Larval enzyme responses to live prey provided at different densities were determined for Zoea I larvae of *S. serrata* and Stage I phyllosoma of *P. ornatus*.

Mud crab larvae used for the experiments were sourced from broodstock kept at the aquarium facilities of JCU and reared following the standard protocol developed at JCU (Genodepa, 2003; Genodepa *et al*., 2004a, 2004b) described in *Section 2.1.1*. For this experiment, newly hatched Zoea I were reared in sixteen 15-L static culture plastic aquaria (20-22‰ salinity and 27-29°C temperature) at a stocking density of approximately 150 L^{-1} and were fed with rotifers, *B. rotundiformis* at densities of 10, 20, 40 or 80 mL^{-1}, replicated in four aquaria per treatment. Larval samples were collected from each of the four replicates for each treatment for enzyme analyses every 6 h for 24 h from the time larvae were first fed with rotifers.

Phyllosoma larvae of *P. ornatus* were sourced from broodstock kept at AIMS as described in *Section 2.1.2*. Newly hatched phyllosoma were reared at AIMS at a density of 100 L^{-1} in twelve 60-L static, conical rearing tanks (35‰ salinity, 28ºC temperature) and were fed newly hatched *Artemia* (INVE GSL) at densities of 0.5, 2.5, and 5.0 *Artemia* nauplii mL^{-1}, with four replicate tanks per treatment. Phyllosoma samples were collected from each of the four replicates for each treatment every 8 h for 24 h from the time the phyllosoma were first fed *Artemia.*

The number of larvae in each sample were counted using a compound microscope while the sample weights were measured using a Cahn C-33 micro-balance (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). All larval samples for enzyme assay were kept in 1.5 mL Eppendorf vials that were stored at -70ºC until assay.

5.2.2. Food Quality Experiments

The larval enzyme responses to different types of food were determined for Zoea II and Megalopa stages of *S. serrata*. Mud crab larvae used for these experiments were sourced from broodstock kept at the aquarium facilities of JCU and reared to Megalopa stage following the standard protocol developed at JCU (Genodepa, 2003; Genodepa *et al*., 2004a, 2004b) as described in detail in *Section 2.1.1*.

For the experiment with Zoea II larvae, newly hatched larvae reared in 300-L tanks following the method described in *Section 5.2.1* were closely monitored for moulting to Zoea II, which usually occurs after 3 to 4 days of rearing Zoea I. Larvae that moulted simultaneously to Zoea II were collected from one of the 300-L rearing tanks in the morning of the day of moulting (Day 0) and randomly stocked into twenty-four 500-mL plastic aquaria at a density of approximately 100 larvae L^{-1}. Larvae in half of these aquaria (12) were fed rotifers at a density of ~40 mL^{-1} while the other half were fed *Artemia* nauplii at a density of ~5 mL^{-1}. Larval samples for enzyme analyses were collected daily for three days, randomly selecting four replicate aquaria from each treatment fed either rotifers or *Artemia* nauplii. Considering that normal larval duration of Zoea II is from 3 to 4 days, all larval samples were checked carefully to make sure that all of them were at Zoea II stage and had not moulted to Zoea III.

The megalopal experiment was conducted with megalopae that have synchronously moulted within a few hours in 300-L larval rearing tanks at JCU. The 300-L rearing tanks with Zoea V were closely monitored and the first few megalopae were removed but those that moulted synchronously within 6 h were collected and stocked individually in 700 mL plastic culture vessels filled with filtered seawater (28‰ salinity) to prevent cannibalism that commonly starts at this stage in mud crabs (Heaseman *et al.,* 1985; Genodepa *et al*., 2004b). The plastic culture vessels were placed in a water bath to maintain a culture temperature of 27-28ºC during the experiment. Half of the megalopae (200 individuals) were fed *Artemia* nauplii at a density of ~5 mL^{-1} while the other half were fed *ad libitum* with a formulated diet. The formulated diet (Table 5.1) was a microbound diet (MBD) based on a formulation developed over many years of research for crab larvae at JCU (Genodepa *et al*., 2004b; Holme *et al*., 2006a ; Mat Noordin, 2010). To maintain water quality, the megalopae were transferred to new culture vessels daily before new feeds were provided. Larval samples for enzyme analyses (4 replicates with 2 megalopae each) were randomly collected from each treatment after feeding on *Artemia* or MBD for 1, 3, 5, and 7 days, respectively. An additional part of the experiment determined survival and moulting of megalopae to the first crab stage (C1) for both treatments with 50 replicates per treatment to assess the performance of the diets. Because of the absence of space in the water bath, the plastic culture vessels were kept at room temperature (~26 ºC). The megalopae were likewise transferred to new culture vessels daily before new feeds were given, and at the same time, mortalities and successful moulting to the C1 were monitored.

The number of larvae in each sample were counted using a compound microscope while the sample weights were determined using a Cahn C-33 micro-balance (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). All larval samples for enzyme assay were kept in 1.5 mL Eppendorf vials that were stored at -70ºC until assay.

Table 5.1. Composition of microbound diet (MBD) used to compare the enzyme response and moulting to first crab instar (C1) of mud crab, *S. serrata* megalopae fed MBD or *Artemia* nauplii (from Mat Noordin, 2010).

Ingredient	Source	% Dry weight
Defatted fish meal	A	50
Total lipids (2 fish oil : 1 corn oil)	B	6
Cholesterol	B	1
Lecithin	B	6
Vitamin mix[a]	C	4
Mineral mix[b]	C	4
Choline chloride	B	1
Dibasic calcium phosphate (DCP)	B	0.6
Agar	B	12
Starch	B	8
Cellulose	B	7.4
TOTAL		100

Sources of Ingredients: (A) Skretting, Tasmania, (B) Sigma-Aldrich Pty. Ltd, (C) Rabar Pty. Ltd., [(a)] ZZ600 DPI, each 1 kilogram contains: Retinol (vit.A), 2 mIU; Cholecaliferol (vit.D3), 0.8 mIU; Tocopherol (vit. E), 40 g; Phytomenadione (vit. K), 2.02 g; inositol (vit Bh), 50 g; Niacin (vit. B3), 30.4 g; Pantothenic Acid (vit. B5), 9.18 g; Folic Acid (vit. B9) 2.56 g; Riboflavin (vit. B2) 4.48 g; Cyanobalamin (vit. B12) 0.004 g; Biotin (vit H) 0.1g; Pyridoxine (vit. B6) 4 g; Thiamine (vit. B1) 3.4 g; Ascorbic Acid (vit. C) 44.4 g; para amino benzoic acid 20 g; tixosil™(anticoagulant) 5g and antioxidant 30 g. [(b)] ZZ603 DO 067 DPI, each 1 kilogram contains: copper 1 g; cobalt 100 mg; magnesium 59.4 mg, manganese 5 g; iodine 800 mg; selenium 20 mg; iron 8 mg; zinc 20 g; aluminium 100 mg; chromium 100 mg.

5.2.3. Assay of Enzymes

Larval samples were assayed for α-amylase, trypsin-like protease and non-specific esterase activities following the methods described in *Section 2.2.1* and *Section 2.2.2*. Mud crab Zoea I sample extracts for assay of amylase, protease, and esterase were all diluted 1:50 (sample: buffer). Zoea II sample extracts for assay of amylase and protease were diluted 1:100 while for assay of esterase it was diluted 1:50. Megalopae sample extracts for assay of amylase was diluted 1:200 while those for the assay of protease and esterase were diluted 1:100. Lobster Stage I phyllosoma sample extracts for assay of amylase, protease and esterase were all diluted 1:100. Enzyme activities were expressed as specific activity (*mU* mg^{-1} protein) and as total activity (*mU* ind^{-1}).

5.2.4 Statistical Analyses

Data from the various experiments were analysed using factorial experiment in completely randomized design to determine the differences in activities of enzymes and interactions between food density or food type and feeding duration. Data were tested for homogeneity of variances using the Levine's test and then Contrast was used for multiple comparisons. Statistical analyses were performed using SPSS for Windows Version12.0. Data is presented as mean, plus or minus (±) standard error (SE), and results were considered significantly different at $P \leq 0.05$.

5.3 Results

5.3.1 Larval Enzyme Responses to Food Quantity

5.3.1.1 Zoea 1 fed different densities of rotifers

The enzyme activities of *S. serrata* Zoea I fed with rotifers provided at different densities for different feeding durations are presented in Figure 5.1. Specific amylase activity did not differ significantly among food density treatments of 10, 20, 40 or 80 rotifers mL^{-1} at any of the feeding durations examined ($P>0.05$) (Fig.5.1A). For total amylase activity, while no significant differences were detected among treatments fed 10, 20 and 40 rotifers mL^{-1} at all feeding durations, significant differences were detected between the highest (80 rotifers mL^{-1}) and the lowest (10 rotifers mL^{-1}) food density treatments at 24 h feeding duration ($P<0.05$) (Fig. 5.1B). In the case of amylase activity, variation related to feeding duration within the same food density treatment, no significant differences were detected over the 24 h feeding duration for the treatments fed 10, 20 and 40 rotifers mL^{-1}, however, total amylase activity of the treatment fed 80 rotifers mL^{-1} significantly increased to reach the highest level at 24 h of feeding ($P<0.05$) (Fig. 5.1A & B).

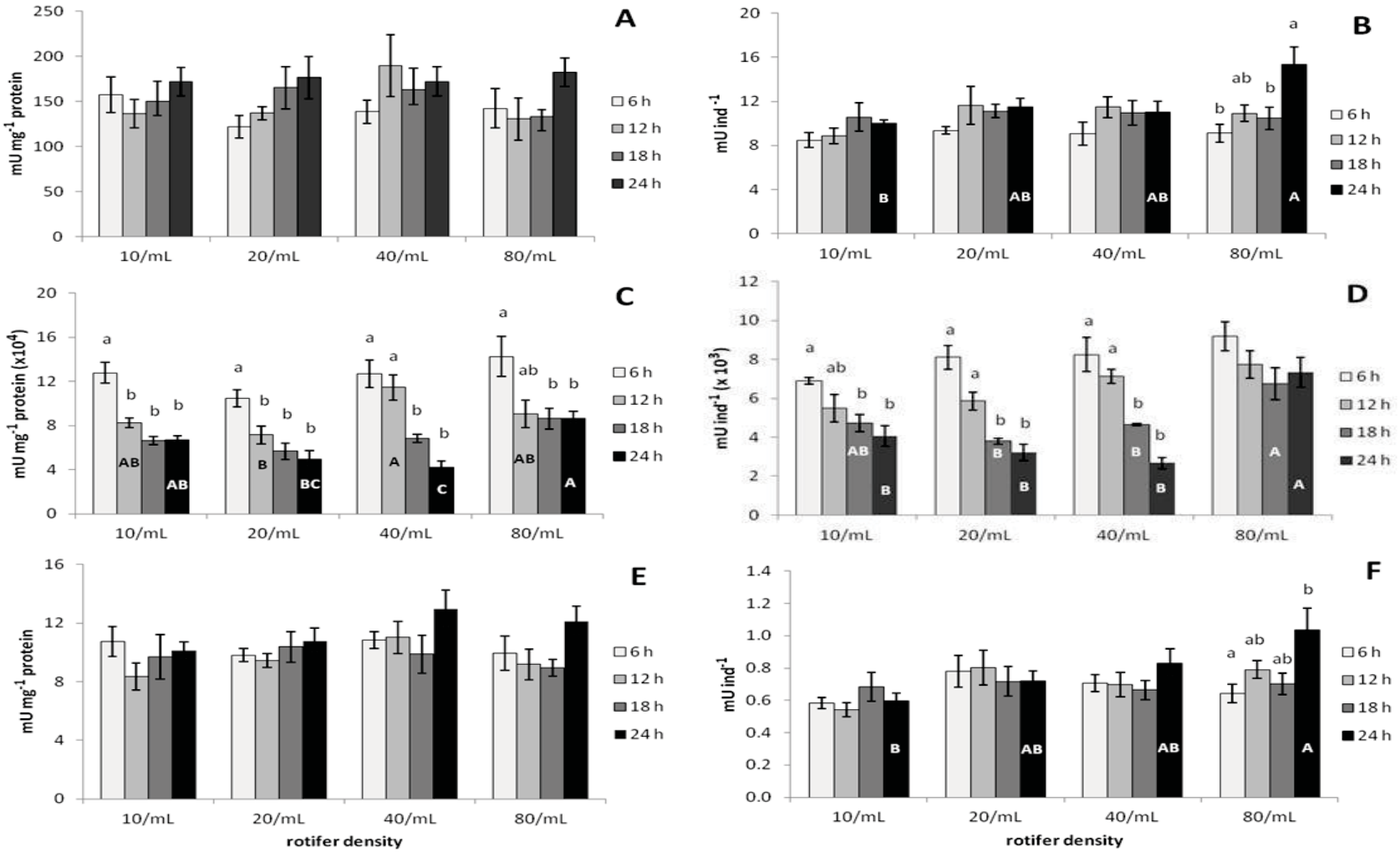

Fig. 5.1 Mean (±SEM, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D) and esterase (E & F) in *S. serrata* Zoea I fed different densities of rotifers for different durations. Means within same rotifer density treatment having no labels or with the same lower case letters labelled on top bars are not significantly different (P>0.05). Means within the same feeding duration having no labels or with the same capital letters labelled within bars are not significantly different (P>0.05).

In the case of protease activity, the treatment fed 80 rotifers mL^{-1} had generally higher specific and total activity than those at low densities, particularly after 24 h of feeding, where both specific and total activity of the 80 rotifers mL^{-1} treatment were significantly higher than those fed at lower food densities (Fig. 5.1C & D). For the protease activities within the same food density treatment, a general trend of decreasing activity with longer feeding duration was observed in all treatments. In particular, both specific and total protease activities of larvae from treatments fed 10, 20 and 40 rotifers mL^{-1} dropped significantly with increased feeding duration ($P<0.05$). However, in the treatment fed 80 rotifers mL^{-1}, while specific protease activity likewise dropped significantly with longer feeding duration ($P<0.05$), total protease activity did not differ significantly despite the decreasing trend.

Esterase activity showed a trend similar to that of amylase activity. Specific esterase activity did not differ significantly among the different rotifer density treatments at all feeding durations examined ($P<0.05$) (Fig. 5.1E). The total esterase activity also did not differ significantly among 10, 20 and 40 rotifers mL^{-1} treatments at all feeding durations, however, for the treatment fed 80 rotifers mL^{-1}, activity increased at 24 h of feeding to become significantly higher than at 6 h of feeding ($P<0.05$) (Fig. 5.1E). In the case of esterase activity variation related to feeding duration within the same food density treatment, no significantly differences were detected over the 24 h feeding duration for the treatments fed 10, 20 and 40 rotifers mL^{-1}, however, total esterase activity in the treatment fed 80 rotifers mL^{-1} increased and was significantly higher after 24 h of feeding than after 6 h of feeding ($P<0.05$) (Fig. 5.1A & B).

5.3.1.2 Stage I phyllosoma fed different densities of Artemia

The enzyme activities of Stage I phyllosoma of *P. ornatus* fed with *Artemia* nauplii provided at different densities for different feeding durations are presented in Figure 5.2. Amylase activity monitored every 8 h for 24 h showed that significant differences in both specific and total activity among the food density treatments occurred only after 24 h feeding with the treatment fed 2.5 *Artemia* mL^{-1} having the highest amylase activity, which was significantly higher ($P<0.05$) than that of the treatment fed 0.5 *Artemia* mL^{-1} but did not differ from the treatment fed 5.0 *Artemia* mL^{-1} (Fig. 5.2A & B). For changes in amylase activity related to feeding duration within the same food density treatment, the two higher food density treatments (i.e., 2.5 and 5.0 *Artemia* mL^{-1}) showed clear trends of increasing specific and total amylase activities with longer feeding duration, which were significantly higher after 24 h of feeding than after 8 h of feeding ($P<0.05$). However, in the treatment where newly hatched phyllosoma were fed at a low density of only 0.5 *Artemia* mL^{-1}, no significant differences related to feeding duration were detected for both specific and total amylase activity ($P>0.05$) (Fig. 5.2A & B).

In contrast to the results with Zoea I mud crab (Fig. 5.1 C & D), no significant difference were detected for both specific and total protease activities of Stage I phyllosoma of *P. ornatus* fed *Artemia* at densities of 0.5, 2.5 and 5.0 nauplii for different feeding durations ($P<0.05$)(Fig. 5.2 C & D).

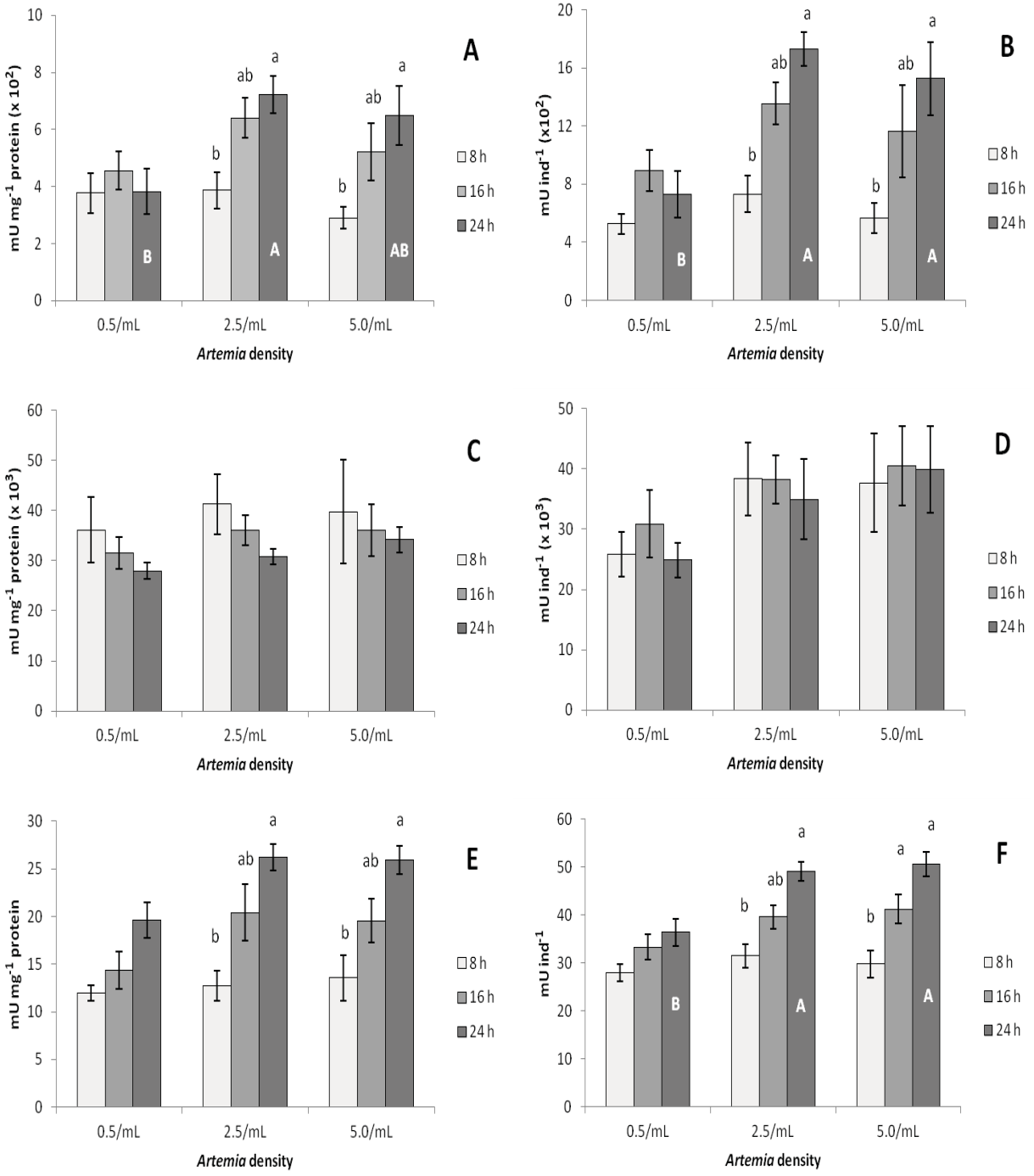

Fig. 5.2 Mean (±SEM, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D) and esterase (E & F) in Stage I phyllosoma of *P. ornatus* fed different densities of *Artemia* for different durations. Means within the same *Artemia* density treatment having no labels or with the same lower case letters labelled on top bars are not significantly different (P>0.05). Means within the same feeding duration having no labels or with the same capital letters labelled within bars are not significantly different (P>0.05).

Both specific and total esterase activities of phyllosoma sampled at 8 h and 16 h of feeding showed no significant differences among the different *Artemia* density treatments ($P>0.05$) (Fig. 5.2 E & F). However after 24 h of feeding, total activities of larvae in the treatments fed 2.5 and 5.0 *Artemia* mL^{-1} were significantly higher than that of larvae fed 0.5 *Artemia* mL^{-1} only, while specific activities showed no significant differences among the treatments ($P>0.05$) (Fig. 5.2 E & F). Changes in esterase activities related to feeding duration within the same food density were similar to the pattern shown for amylase activity. Both specific and total esterase activities increased with longer feeding duration for the treatments fed 2.5 and 5.0 *Artemia* mL^{-1} and were significantly higher after 24 h of feeding than after 8 h of feeding ($P<0.05$); however, no significant differences were detected at different feeding durations for the low food density treatment fed 0.5 *Artemia* mL^{-1} ($P>0.05$) (Fig. 5.2 E & F).

5.3.2 Enzymes Response of Mud Crab Larvae to Food Types.

5.3.2.1 Zoea II fed either rotifers or Artemia

The enzyme activities of Zoea II *S. serrata* fed different prey consisting of rotifers or *Artemia* nauplii are presented in Figure 5.3. The specific and total amylase activities did not differ significantly between treatments fed either rotifers or *Artemia* nauplii at different feeding periods of 1-3 days ($P>0.05$) (Fig. 5.3A & B). The changes in both specific and total amylase activities within the culture period (3 days) also did not differ in the treatment fed rotifers, as well as in the treatment fed *Artemia* nauplii ($P>0.05$) (Fig. 5.3A and B).

In contrast to amylase activity, protease activity of Zoea II fed the two different types of prey showed differences in pattern. This time, both specific and total protease activities of larvae fed *Artemia* nauplii were significantly higher than that of larvae fed rotifers at all feeding periods/sampling times (day 1 to 3) ($P<0.05$), except on day 1, where specific activity did not differ between the two treatments ($P>0.05$; Fig. 5.3C and D). Regarding changes in protease activity within the feeding/culture period, specific and total protease activities of larvae fed rotifers did not differ significantly despite a trend of decreasing levels with longer feeding time/culture period. Similarly, for the larvae fed *Artemia* nauplii, both specific and total protease activities did not differ significantly with longer feeding time/culture period ($P>0.05$) (Fig. 5.3 C & D).

Specific and total esterase activities of larvae fed *Artemia* nauplii were significantly higher than those fed rotifers on day 2 ($P<0.05$), but not on days 1 and 3 (Fig.5.3 E and F). Regarding the changes in esterase activities within the feeding/culture period, neither specific and total esterase activities of larvae fed *Artemia* nauplii varied significantly, while for larvae fed rotifers, total activity increased significantly from day 2 to day 3 ($P<0.05$), although neither differed significantly from the level on day 1 ($P>0.05$) (Fig. 5.3 E & F).

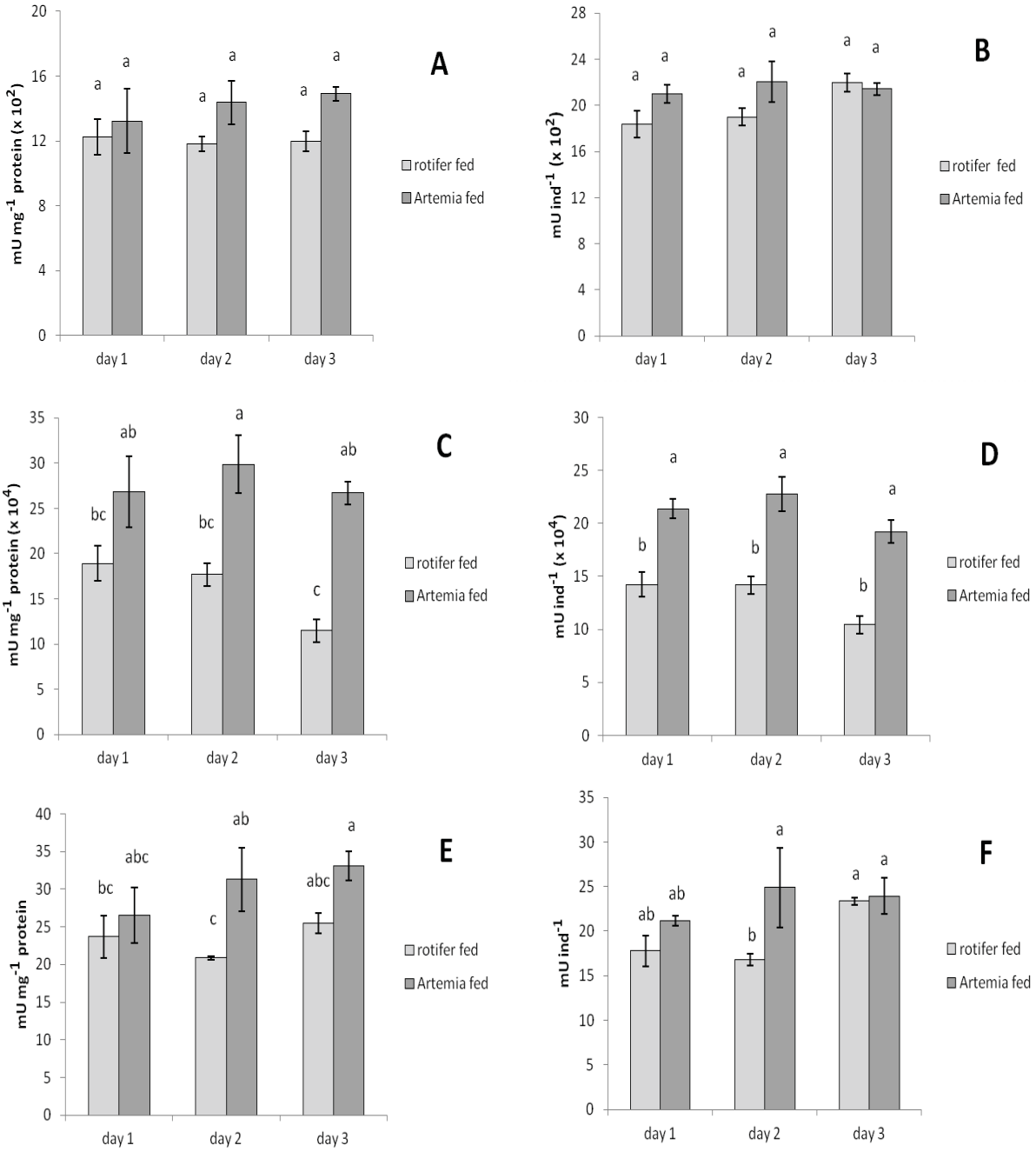

Fig. 5.3 Mean (±SEM, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (graphs A and B), protease (graphs C and D) and esterase (graphs E and F) in Zoea II *S. serrata* fed either rotifers or *Artemia* nauplii. Means with the same letters on top bars are not significantly different (P>0.05).

5.3.2.2 Megalopae fed either Artemia or MBD

The enzyme activities of *S. serrata* megalopae fed either microbound diet (MBD) or *Artemia* are presented in Figure 5.4. Both specific and total amylase activities were many times higher in megalopae fed with *Artemia* than in those fed with MBD, particularly in the middle of megalopal development (i.e. day 3 and 5) ($P<0.01$). In terms of changes in amylase activity within the culture period, amylase activities of megalopae fed with MBD showed a decreasing trend with significantly lower levels at day 5 and day 7 compared to day 1, while those fed *Artemia* showed a bell curve with an increase in activity from day 1 to day 5 before a substantial decline on day 7 ($P<0.05$) (Fig. 5.4 A & B).

Overall, the specific activity of protease in megalopae fed with MBD was consistently higher than those fed with *Artemia*, and the differences between the two treatments were always significant ($P<0.05$) except on day 3. On the other hand, total protease activity of megalopae fed with MBD was initially significantly lower than that of larvae fed *Artemia* on day 3 and day 5 ($P<0.05$), but increased continuously as culture duration increased, and eventually became significantly higher than those fed with *Artemia* on day 7 ($P<0.05$) (Fig. 5.4 C & D). The patterns of protease activity changes with megalopal development were also very different between larvae fed with MBD and those fed with *Artemia*. Both specific and total protease activities of megalopae fed with MBD showed a clear trend of increasing activity with longer culture duration, resulting in significantly higher levels on day 7 compared to days 1, 3, and 5. However, those fed with *Artemia* showed a bell curve, where protease activity initially increased but then decreased towards the late phase of larval development, resulting in highest activity levels on day 5, which did not differ in specific activity, but was significantly higher than both at day 1 and day 7 for the total activity ($P<0.01$) (Fig. 5.4 C & D).

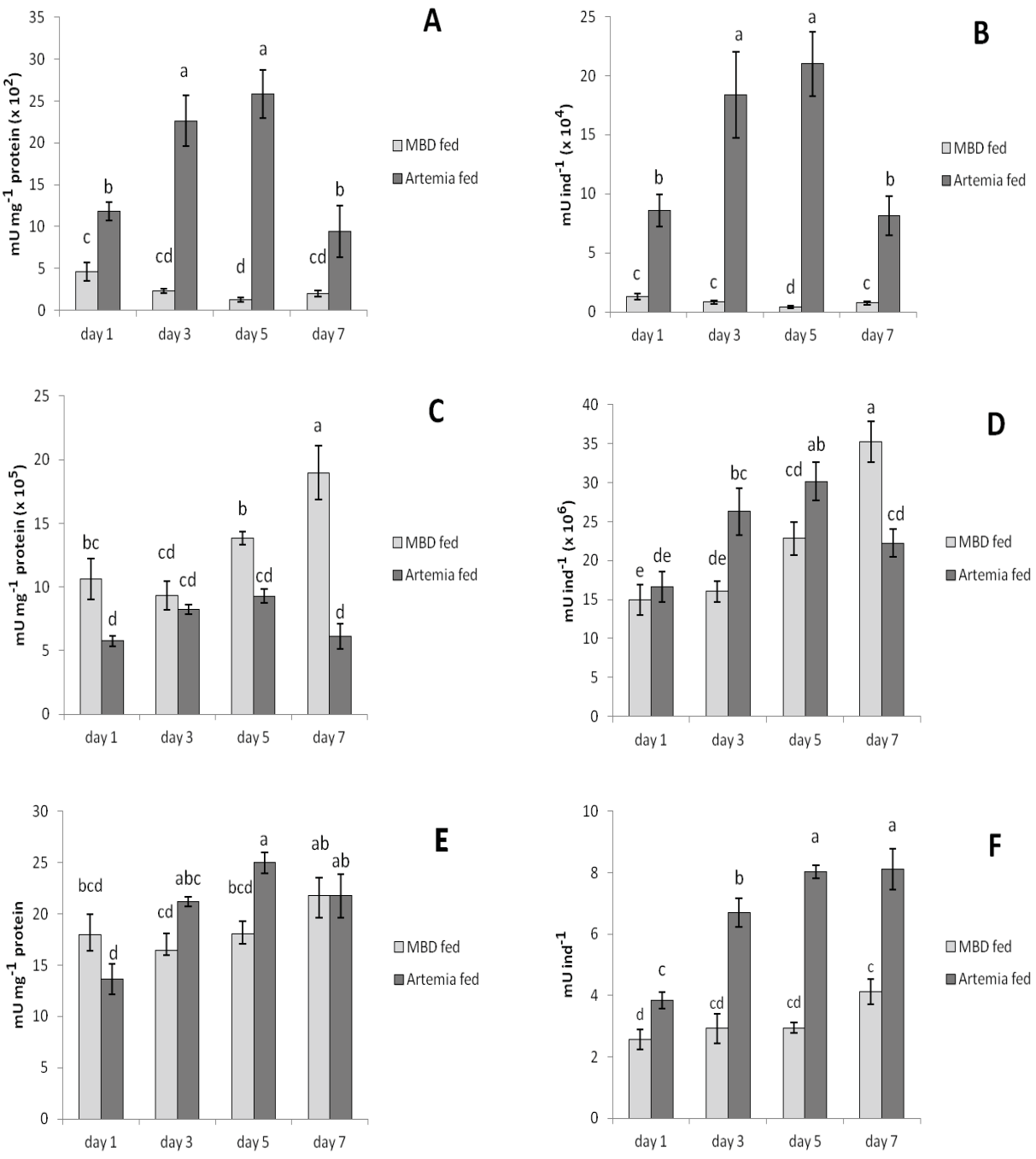

Fig. 5.4. Mean (±SEM, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (A & B), protease (C & D) and esterase (E & F) in *S. serrata* megalopae fed either microbound diet (MBD) or *Artemia* nauplii. Means with the same letters on top bars are not significantly different (P>0.05).

Specific esterase activity of megalopae fed either *Artemia* or MBD generally did not differ significantly except on day 5 when activity was significantly higher in those fed with *Artemia* ($P<0.05$) (Fig.5.4 C). However, in terms of total activity, it was always significantly higher in megalopae fed with *Artemia* than in those fed with MBD ($P<0.05$), and except on day 1, total activity of *Artemia*-fed megalopae was more than double that of those fed with MBD (Fig. 5.4 E & F). Changes in patterns of esterase activity with megalopal development generally showed an increasing trend, in both megalopae fed with *Artemia* and in those fed with MBD, although these increases were more substantial in the former (Fig. 5.4 E & F).

As expected, the relatively low rearing temperature (~26°C) in the concurrent culture experiment, where individual megalopae were reared to monitor their survival and moulting to C1 stage resulted in an extended inter-moult duration, with still some surviving megalopae in both *Artemia*-fed and MBD-fed treatments (9 and 1, respectively) when the experiment was terminated after 20 days. Over a 20-day period, megalopae fed *Artemia* obtained showed 80.8% survival with 78.8% success in moulting to C1, while those fed MBD had lower overall survival (63.5%) and lower moulting success (46.2%) (Fig. 5.5). Mortalities were observed to occur only during the first 2 days in *Artemia* fed megalopae, while it was during the first 5 days in those fed MBD. After initial mortalities, all the remaining megalopae survived up to the end of the experiment (20-day culture period) and when calculated in terms of percentage of all surviving megalopae for each treatment, the successful moulting rate to C1 was 97.6% for those fed with *Artemia* but only 72.8% for those fed the MBD (Fig. 5.5).

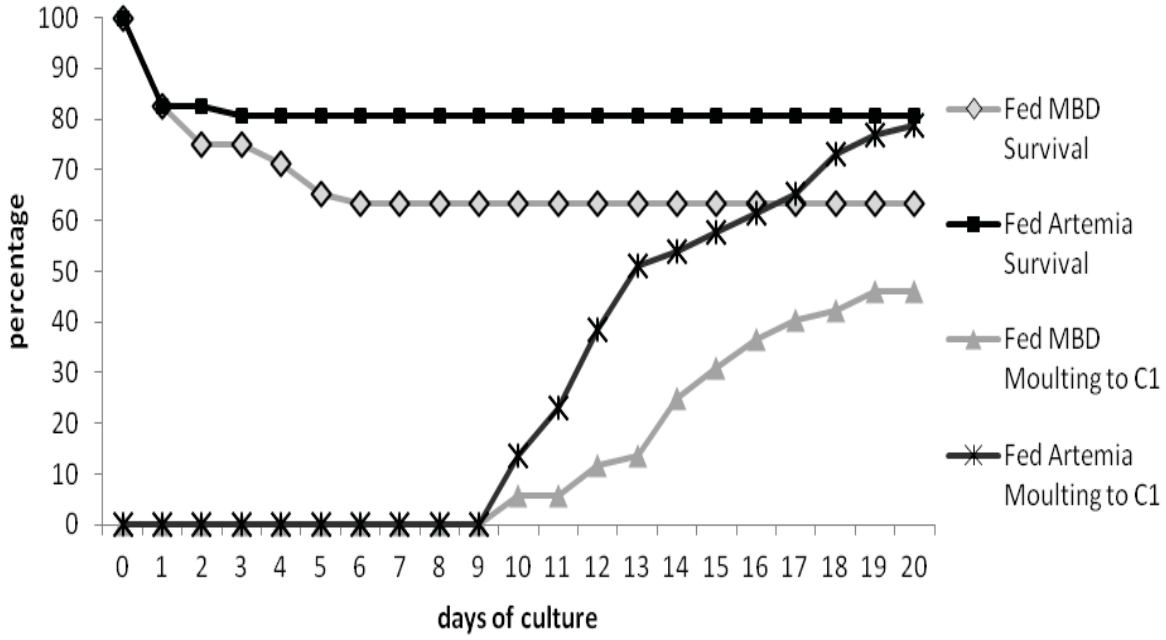

Fig. 5.5. Survival and moulting to first cab instar (C1) of *S. serrata* megalopae fed either *Artemia* or a microbound diet (MBD) for 20 days.

5.4 Discussion

Digestive enzymes play a crucial role in the digestion and assimilation of food by catalysing the chemical breakdown of food particles into forms that can be assimilated by the animal (Gilbault, 1976). For this reason, the presence, as well as the concentration of digestive enzymes, have been used to indicate the relative importance of each component in the diet (Lee *et al*., 1984; Rodriguez *et al*., 1994; Johnston, 2003). Increases in amylase, protease, and lipase activities, for example, are believed to be indicative of enhanced carbohydrate, protein and lipid catabolism respectively (Kamarudin *et al*., 1994; Rodriguez *et al*., 1994; Johnston, 2003). The abundance of substrates, however, may not always result in increased enzyme activity, as some crustaceans have the ability to elevate their enzyme activities as an adaptive mechanism to scarcity of substrates (Harris *et al*., 1986). For example, during ontogenetic development of penaeid shrimp larvae, high proteolytic activities were detected during the herbivorous feeding phase of zoeal stages, while lower levels were measured during the carnivorous feeding phase of late mysis stages (Jones *et al.,* 1993; Le Vay *et al.,* 1993; Rodriguez *et al.,* 1994; Kumlu & Jones, 1995b). Similarly, high proteolytic activity was also detected in herbivorous copepods such as the *Temora* and *Centropages* (Harris *et al.,* 1986).

As demonstrated in Chapter 4, the first feeding Zoea 1 of *S. serrata* were observed to decrease their protease activity as their initial response to the availability of food, but their protease activity eventually increased, most likely as a scheme for building-up high levels of protein reserves. In the present experiment in which Zoea I were provided with rotifers at different densities, they responded similarly to food availability by decreasing protease activity in all treatments within the first 24 h of feeding. However, the drop in protease activity, particularly the total activity, was much milder in individuals within the highest rotifer density treatment (80 rotifers mL^{-1}) compared to other treatments with lower

rotifer densities (10 to 40 rotifers mL^{-1}) suggesting that the build-up of higher protein reserves was probably accomplished more quickly within the high food density treatment.

In contrast to reduced protease activity upon initial food intake, the amylase and esterase activities in this experiment generally increased with feeding, which is again consistent with the results of the earlier research in this study reported in Chapter 4. As it was further shown that total activities of both enzymes also increased significantly after 24 h of feeding when the rotifer density was increased to 80 rotifers mL^{-1}, the increasing enzyme activities in response to increased food density suggest that the zoeal *S. serrata* are likely capable of modulating their enzyme activity levels to maximize the utilization of available ingested food.

The reported density of rotifers used for mud crab larval rearing varies from 10 to 60 mL^{-1}, but there are considerable differences of opinion as to optimal feeding rates (Fielder & Heasman, 1999). Since first feeding mud crab larvae do not actively pursue their prey but capture food randomly by pleon lashing (Zeng & Li, 1999; Genodepa *et al*., 2004a), in principle, a greater density of rotifers should increase the number of encounters between larvae and prey, resulting in increased food consumption and consequently enhancing survival and development. The ability of mud crab larvae to maximize food utilization by increasing their enzyme activities as shown in the results of this study, supports the findings of prior studies that better survival can be attained by using higher rotifer densities. However, there should be a density threshold at which such an effect is maximized, and beyond which, water quality problems may occur that could adversely affect larval performance (Zeng & Li, 1999).

In rearing spiny lobster phyllosoma, *Artemia* and shellfish have been used as foods since they are readily available at lower costs and relatively easy to produce and dispense for phyllosoma culture (Kittaka, 1999; Ritar *et al.*, 2003). *Artemia* is a convenient

food, and the nauplii may be on-grown to a larger size for the growing older larvae, and enriched to improve their nutritional profile (Ritar *et al*., 2002). Kittaka (1997) observed that the availability of *Artemia* as food source for lobster phyllosoma was dependent on their concentration in the culture water and that the optimal density of *Artemia* nauplii for first-instar phyllosoma of *P. japonicus* was 4 individuals mL^{-1}, as this provided maximal ingestion. In rearing *P. ornatus*, some earlier reports have used food densities as low as 0.5 individual mL^{-1} (Bourne *et al*., 2004, 2005) but others have also used 4 individuals mL^{-1} (Johnston *et al*., 2007; Smith *et al*., 2009a), which was adopted from the results of Kittaka (1997). In this study, after 24 h of feeding, both amylase and esterase activities of newly hatched phyllosoma increased significantly when *Artemia* were provided at higher densities (2.5 mL^{-1} and 5.0 mL^{-1}) compared to the lower density of only 0.5 mL^{-1}, suggesting that food intake and digestion of food were enhanced at higher densities. Such suggestion is backed up by the results of amylase and esterase activity changes over the 24 h feeding period, which showed no significant changes at a low density of 0.5 *Artemia* mL^{-1}, but significantly increases at higher densities (2.5 and 5.0 *Artemia* mL^{-1}).

The capability of modulating enzyme activity in response to diet quality has been reported only in late larvae of crustaceans in the past, such as in the later mysis stages of some penaeids (Jones *et al*., 1993; Le Vay *et al*., 1994; Rodriguez *et al*., 1994) and in megalopae of crabs as well as later stage larvae of caridean shrimps (Harms *et al*., 1991; Kumlu & Jones, 1995b). In this study, marked differences in enzyme activities were detected in mud crab larvae as early as Zoea II stage when fed different types of live prey. In particular the protease activities of Zoea II fed with *Artemia* nauplii were almost twice as high as those fed with rotifers, implying overall superior nutritional value of *Artemia.* Such enzyme responses of Zoea II to different types of prey can probably be explained best by the differences in proximate compositions of rotifers and *Artemia* as shown in Table 5.2. The

notably higher protease activity of larvae fed with *Artemia* can be attributed to the substantially higher protein content of *Artemia* compared to rotifers, while less prominent differences in amylase and esterase activities may be attributed to similar levels of carbohydrates and lipids in these two commonly used hatchery prey. Zeng & Li (1999) reported that in another mud crab species, *S. paramanosain*, survival of Zoea II fed rotifers and *Artemia* were very similar, as were the C, H, and N contents of larvae (expressed as percentages of dry weight), suggesting that both prey were able to meet the basic nutritional requirements for the survival of Zoea II. However, both the inter-moult duration of Zoea II and the dry weight of the newly moulted Zoea III from the Zoea II fed with rotifers were significantly inferior to those fed with *Artemia* (mean dry weight of newly moulted Zoea III: 34.7 µg/larva for the rotifer fed larvae vs. 53.9 µg/larva for the *Artemia* fed larvae). These data provide supporting evidence that the higher protein content of *Artemia* resulted in improved larval nutritional status, as reflected by significantly enhanced development and weight gain.

Table 5.2. Average nutritional composition (% dry matter ± standard deviation) of newly hatched *Artemia* nauplii and *Brachionus plicatilis* fed with *Nannochloropsis* sp. (data compiled from Ledger *et al*., 1987 and Jeeja *et al.,* 2011).

	Artemia nauplii	*Brachionus plicatilis*
Protein	52.2 ± 8.8	32.10 ± 0.83
Lipid	18.9 ± 4.5	19.85 ± 0.19
Carbohydrate	14.8 ± 4.8	14.62 ± 0.10
Ash	9.7 ± 4.6	5.20 ± 0.06

The significantly higher specific activity of trypsin-like protease in *S. serrata* megalopae fed with MBD, compared to those fed with *Artemia* in this study, was similar to observations reported for late stage larvae of *M. rosenbergii* (Kumlu & Jones, 1995b) and penaeid mysis larvae (Jones *et al*. 1993; Le Vay *et al*., 1994, Kumlu & Jones, 1995a), where larvae fed with artificial diets had higher trypsin activities compared to those fed with *Artemia*. Due to the highly digestible nature of *Artemia* nauplii fed as live prey compared to dry fishmeal powder as the main protein source for formulated diets, the increase in trypsin activity when larvae were fed formulated diets was interpreted by Harris *et al*. (1986) as a response to lower digestibility of the protein source. Although MBD appear to be suitable for larval crustaceans as they are relatively easier to digest compared to other forms of formulated diets (Genodepa *et al*., 2004b; Holme *et al*., 2006a), because of the nature of its main protein source and the formulation process, it is clearly not as digestible as live *Artemia*, as pointed out by Harris *et al*. (1986).

It is worth noting that there appeared to be problems with the acceptability of MBD to megalopae during the first few days of rearing, as survival of megalopae fed with MBD showed a bigger drop compared to those fed with *Artemia*. This indicates that the megalopae in the treatment fed with MBD went through a weaning period during the early phase of the experiment, as the larvae had been with *Artemia* only since the late Zoea II. This weaning period also probably contributed to a delay in moulting to first stage crabs of megalopae fed with MBD compared to those fed *Artemia*. Decreases in enzyme activities of *Artemia* fed megalopae on day 7, which was not found in MBD fed megalopae, could be explained by the fact that megalopae fed with *Artemia* developed faster, hence, were closer to metamorphosis. The sharp decline in food consumption is common in crab megalopae close to metamorphosis as they reduce or even stop eating once they have accumulated the required nutrients for metamorphosis (Anger, 2001).

The consistently lower amylase and esterase activities in MBD fed megalopae compared to those fed with *Artemia*, may also have contributed to the lower survival and delayed moulting found in the former. The magnitude of difference in amylase activities of MBD fed and *Artemia* fed megalopae was surprising; it suggests that carbohydrates may play a significant role in supporting megalopal survival and development. Considering that research on mud crab feed development has so far focused on protein and lipid requirements (Catacutan, 2002; Sheen & Wu, 1999; Holme *et al*., 2006b; Holme *et al*., 2007a, 2007b), these results showing huge differences in amylase activity between MBD fed and *Artemia* fed megalopae warrants further investigation, particularly on the roles played by carbohydrates in larval mud crab nutrition, as well as the optimal dietary carbohydrate requirements.

5.5. Summary and Conclusions

The results of this study clearly showed that both prey quantity and quality can significantly influence larval digestive enzyme activities. Considering that the presence and concentration of digestive enzymes are known to indicate the digestive potential of an organism (Hammer *et al*., 2000; Lemos *et al*., 2000; Furne *et al*., 2005), the ability of both early *S. serrata* and *P. ornatus* larvae to increase their digestive enzyme activities in response to increasing food density reflects their ability to maximize the utilization of available food. A comparison of the levels of enzyme activity in Zoea I *S. serrata* and in Stage I phyllosoma of *P. ornatus* fed different densities of rotifers and *Artemia*, respectively, revealed that generally, the food density range that resulted in maximal activities of digestive enzymes fall within those currently used in larval rearing of both species. This may suggest that digestive enzyme activities can serve as indicators for suitable food densities used for larval rearing.

The digestive enzyme response of Zoea II and Megalopae of *S. serrata* to different types of feeds also helped identify the value of these feeds to the larvae. In Zoea II,

the digestive enzyme response to feeding on rotifers or *Artemia* largely reflected the nutritional values of these two most commonly used hatchery prey in terms of their relative proximate contents. The enzyme activities of *S. serrata* megalopae fed with either MBD or *Artemia* also indicated the differences in digestibility and nutritional quality of live prey and formulated diets. Previous research has reported that formulated diets were not as digestible as live *Artemia*, and this led to higher trypsin-like protease activities in larvae fed the formulated diet (Harris *et al*., 1986), which were likewise observed with enzyme activities of MBD fed megalopae in this study. Finally, the huge differences in levels of amylase activity detected between megalopae fed with MBD and *Artemia* indicate the possible significant role of carbohydrates in larval nutrition, as well as the apparent deficiency of carbohydrates in the MBD used. Clearly, these results points to the need for substantial investigation in this area, considering the fact that, compared to protein and lipid, dietary carbohydrate requirements of crustacean larvae have so far received very little attention.

CHAPTER 6

Changes in digestive enzyme activities related to the moulting cycle and ontogeny of *Scylla serrata* and *Panulirus ornatus*

6.1 Introduction

Decapod crustaceans are characterized by having hard and rigid exoskeletons that cover and protect their bodies. In order to grow, they must go through a critical process known as moulting or ecdysis, which is a recurring episode that leads to complete shedding of the old exoskeleton and its replacement with a new exoskeleton (Anger, 2001). The crustacean moulting cycle is known to be affected by multiple factors including environmental conditions such as water temperature, light (Bermudes & Ritar, 2008; Bermudes *et al*., 2008), salinity (Romano & Zeng, 2006) and feeding treatments (Mingawa & Murano, 1993), and it results in significant physiological, biochemical and behavioural changes (Chang, 1995; Anger, 2001; Chang & Mykles, 2011; Sugumar *et al*., 2013).

The period during and immediately after moulting is perhaps the most critical and challenging time for crustaceans. The increased demand for energy during moulting (Cuzon *et al*., 1980; Cuzon *et al*., 2000; Sugumar *et al*., 2013) adds to the complexity of the process, hence it is important to know the main sources of energy utilized during the moulting cycle, particularly under conditions of starvation, as these could provide new insights to facilitate improved food formulation and management of feeding. Considering the current low and inconsistent survival in hatchery production of *S. serrata* and *P. ornatus*, such insights may hold special relevance to the larvae of these two species.

In the earlier chapters of this study, while the activities of the major digestive enzymes were measured to identify the energy sources of the newly hatched larvae of *S. serrata* and *P. ornatus* subject to starvation (Chapters 3) as well as under conditions of intermittent food availability (Chapter 4), these results did not provide adequate information on the changes of enzymes activities related to larval moulting cycle. Better knowledge of fluctuations in the major digestive enzymes during larval moulting cycle should facilitate more efficient larval feeding management and better formulation of larval feeds (Lee *et al*., 1984; Rodriguez *et al*., 1994; Johnston, 2003). To date, while there are some reports on digestive enzyme activity changes during the moulting cycle of crustaceans such as the swimming crab, *P. pelagicus* (Sugumar *et al*. 2013), the spiny lobster, *P. argus* (Travis, 1955, 1957; Perera *et al*., 2008), the Norway lobster, *Nephrops norvegicus* (Alvarez-Fernandez *et al.,* 2005), and penaeids, such as *Penaeus esculentus* (Barclay *et al.,* 1983), *Penaeus notialis* (Fernandez *et al*.,1997), *Litopenaeus vannamei* (Muhlia-Almazan & Garcia-Carreño 2002) and *Masupenaeus japonicus* (Zilli *et al*., 2003), these studies were done with juveniles and adults. So far, nothing has been reported on the changes in digestive enzyme activities during the moulting cycle of *S. serrata* and *P. ornatus*, particularly on the larval stages of these species.

Both the qualitative and quantitative profiles of digestive enzymes were found to differ relative to the stages of development in crustaceans (Van Wormhoudt, 1973; Van Wormhoudt *et al*., 1980; Galgani & Benyamin, 1985); on this basis, the changes in digestive enzyme activities were used by several authors to infer differences in digestive potential among different life stages of an organism (Hammer *et al*., 2000; Lemos *et al*., 2000; Furne *et al*., 2005). For example, in penaeid larvae, changes in enzyme activities have been suggested to reflect their modification in feeding strategy when making a transition in trophic level during development (Jones *et al.*, 1997b; Le Vay *et al*., 2001). The changes in enzyme

activity were also linked to morphological changes in the larval digestive system during ontogeny (Abubakr & Jones, 1992). Despite recent studies that have examined ontogenetic changes in amylase, trypsin, chymotrypsin and carboxypeptidases A & B for *S. serrata* (Serrano, 2012, 2013), more studies are required to shed new lights into larval digestive capabilities of *S. serrata*. In the case of *P. ornatus*, ontogenetic changes in enzyme activities were monitored only for Stages I - IV phyllosoma due to lack of samples of older larvae in a previous study (Johnston, 2006).

Better knowledge of the qualitative and quantitative changes in digestive enzymes during larval development, particularly over critical transitional periods, may hold the key to understanding the reasons for the occurrence of high mortalities during larval rearing of both *S. serrata* and *P. ornatus* (*e.g.*, Zoea V to Megalopa stage for *S. serrata* and Stage V to Stage VI phyllosoma for *P. ornatus*). The present study therefore examined changes in the activities of major digestive enzymes related to the moulting cycle in selected larval stages of both *S. serrata* and *P. ornatus*. Furthermore, ontogenetic changes in digestive enzyme activities throughout larval development (from Zoea 1 to Crab Instar I) of *S. serrata* and (from Stage I to V phyllosoma) of *P. ornatus* were also examined.

6.2 Materials and Methods

6.2.1 Mud Crab Experiments

All larval samples of *S. serrata,* used in the following experiments came from broodstock caught from estuaries in Townsville, Queensland and maintained at the aquarium facilities of JCU following the methods described in *Section 2.1.1*. Larvae were reared following the standard rearing protocol developed at JCU as described in *Section 2.1.1* (Genodepa, 2003; Genodepa *et al*., 2004a, 2004b).

6.2.1.1 Changes in enzyme levels related to the moulting cycle

Changes in enzyme levels during the moulting cycle were first investigated for the Zoea I. Newly hatched larvae from a berried crab were stocked at a density of ~100 larvae L^{-1} in 300-L rearing tanks (20-22‰ salinity and 27-29°C temperature) in the early morning, about 5 h after hatch and were fed rotifers (*B. rotundiformis*) at a density of ~ 40 mL^{-1} until moulting to Zoea II stage. Larval samples for enzyme assay were initially collected right after the newly hatched Zoea I were stocked into the rearing tanks, and subsequently every 24 h throughout the duration of the Zoea I moulting cycle, up to day 1 of Zoea II. In order to ensure accurate sampling of the larval stage and their corresponding phase within the moulting cycle, larval cultures were closely monitored for any Zoea II. Samples of day 0 Zoea II were carefully checked and picked individually from the mixture of Zoea I and Zoea II taken from the mass rearing tanks on day 3 where only a few newly moulted Zoea II samples were available, and again on day 4 where the majority of the larvae had moulted to the Zoea II stage. A portion of the day 0 Zoea II collected from the mass rearing tanks were reared in a separate tank with identical conditions as the mass rearing tanks and were used on the next day as samples of day 1 Zoea II.

Changes in enzyme levels during moulting cycle during the megalopal stage up to the newly metamorphosed Crab Instar I juveniles (C1) were also assessed. The megalopae used for this experiment were from the same batch of larvae used in the Zoea I experiment except that they were reared further to reach the megalopal stage following the larval rearing protocol developed at JCU (*Section 2.1.1*). Considering the fact that moulting or metamorphosis of Zoea V to Megalopa was normally less synchronized, newly moulted megalopae (day 0) were collected daily from the mass rearing tanks and reared in separate containers in order to accurately determine their development, which was based on the number of days from the date they have metamorphosed from Zoea V. To achieve this, the 300-L larval rearing tanks containing Zoea V which were about to moult to megalopae were closely monitored for the appearance of any megalopae every 6 h. All new megalopae were collected and placed individually in 500-mL plastic containers which were labelled to indicate the date and time of collection, and then the plastic rearing containers were placed in a water bath to maintain a water temperature of ~27ºC. Rearing megalopae in individual containers is necessary to prevent cannibalism which commonly occurs at this stage (Heaseman *et al*., 1985). Megalopae were fed *Artemia* at a density of ~5 nauplii mL^{-1} daily, but prior to feeding, a 100% water exchange was carried out by transferring the megalopae individually into new rearing containers filled with clean seawater (28‰ salinity). Collection of megalopal samples (2 megalopa $replicate^{-1}$; 4 $replicates\ treatment^{-1}$) for enzyme assay was carried out each day during the moulting cycle of megalopae while samples of C1 (1 crab $replicate^{-1}$; 4 $replicates\ treatment^{-1}$) were collected on the day of moulting from megalopae to C1.

The number of larvae in each sample were counted using a compound microscope while the sample weights were measured using a Cahn C-33 micro-balance (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). All larval samples for enzyme assay were kept in 1.5 mL Eppendorf vials that were stored at -70ºC until assay.

6.2.1.2 Ontogenetic changes in enzyme levels through larval development

The larvae used in this experiment were from the same batch of larvae in the above experiments described in *Section 6.2.1.1*. Larvae from each developmental stage were collected from the 300-L mass rearing tanks. To be sure about the day of larval development for a particular stage, the mass rearing tanks were monitored closely, and every day, the newly moulted day 0 larvae of each stage were collected and transferred into a 20-L aquarium for further rearing under conditions identical to that of the mass rearing tanks. Except for the megalopae and C1 which were reared individually as described in *Section 6.2.1.1* to avoid cannibalism, day 0 larvae of the other larval stages were reared communally in 20-L aquaria until the designated day that samples were taken for enzyme essay. To ensure that the larvae sampled were mostly at the inter-moult phase of the moulting cycle of each stage, Zoea I to III samples were collected on day 2 after the larvae moulted to the stage; for Zoea IV to V and Megalopae, samples were taken on day 3, while C1 samples were collected on day 2. Processing and storage of all the larval samples prior to enzyme essay were the same as described in *Section 6.2.1.1*.

6.2.2 Lobster Experiments

All larvae of *P. ornatus* used in the following experiments were hatched from wild-caught broodstock reared at AIMS following the protocol described in *Section 2.1.2*.

6.2.2.1 Changes in enzyme levels related to the moulting cycle in early phyllosoma

Changes in enzyme levels related to the moulting cycle of *P. ornatus* were investigated in early phyllosoma from Stage I to Stage III. Newly hatched phyllosoma were reared to Stage III at AIMS using 100-L upwelling culture vessels designed specifically for rearing lobster larvae. A flow-through system with water turnover rate of 100% h^{-1} was operated. Phyllosoma were reared following the standard procedures developed by AIMS (*Section 2.1.2*). Newly hatched phyllosoma were stocked at a density of 30 larvae L^{-1} and were fed newly hatched *Artemia* every morning at a density of 1.5 nauplii mL^{-1}. Prior to daily feeding, any left-over *Artemia* from the previous feeding were flushed-out for 2 h by allowing the water to flow through a mesh screen (500 μm) that retained the phyllosoma but allowed *Artemia* to pass through. Larval samples at various phases of the moulting cycle from Stages I to III were collected for enzyme assay: Stage I— day 0, 1, 3, 5; Stage II— day 0, 2, 4; and Stage III— day 0 and 2, respectively. To ensure the accuracy of the days of development of the larvae sampled within each stage, phyllosoma in the 100-L upwelling tanks were closely monitored daily and any larvae that had newly moulted to the next stage within the same day were transferred into separate rearing containers (10-L capacity) and were reared further under the same feeding regime and physical condition as in the 100-L upwelling tanks until they were sampled for enzyme assay.

All phyllosoma samples for enzyme assay were kept in 1.5 mL Eppendorf vials. The number of phyllosoma in each sample were noted and their corresponding sample weights were measured using a Cahn C-33 micro-balance (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) before the vials were stored at -70 ºC until assay.

6.2.2.2 Ontogenetic changes in enzyme levels during early phyllosoma development

Ontogenetic changes in enzyme levels were investigated for early phyllosoma up to Stage V. The newly hatched phyllosoma were cultured in 100-L upwelling tanks designed for larval rearing of lobsters (*Section 6.2.1*) following the standard rearing procedures developed at AIMS (*Section 2.1.2*). The initial stocking density was 40 larvae L^{-1} but the density was gradually reduced as larvae for enzyme assay were collected and transferred into separate 10- L upwelling tanks for further rearing. The Stage I to III phyllosoma were fed *Artemia* at 1.5 nauplii mL^{-1} daily, while Stages IV and V phyllosoma were fed a combination of *Artemia* nauplii (1.5 nauplii mL^{-1}) and chopped mussel, *M. galloprovincialis* gonad (20 mg L^{-1} day^{-1}). The phyllosoma sampled for enzyme assay were taken at the inter-moult phase of their moulting cycle; 4 days after hatching or moulting to the stage for Stages I to III phyllosoma, but 5 days after moulting to the stage for Stages IV and V phyllosoma. In order to determine accurately the number of days since moulting, the newly moulted (day 0) phyllosoma of Stages II, III, IV and V were collected from the 100-L rearing tanks on their respective moulting day and transferred into smaller culture vessels for further culture until the designated day of development when they were sampled for enzyme assay. The phyllosoma developmental stages were identified according to Smith *et al* (2009b). The processing and storage of larval samples prior to enzyme essay were the same as described in *Section 6.2.2.1*.

6.2.3 *Assay of Enzymes*

Larval samples were assayed for α-amylase, trypsin-like protease and non-specific esterase activities following the methods described in *Section 2.2.1* and *Section 2.2.2*. Sample extracts for assay of amylase, protease, and esterase during the Zoea I moulting cycle

were all diluted 1:100 (sample: buffer). During the moulting cycle of Megalopa, sample extracts for assay of amylase was diluted 1:200 while those for protease and esterase were diluted 1:100. In the lobster phyllosoma moulting cycle from Stage I to Stage III, sample extracts for the assay of amylase and esterase were diluted 1:150 while those for the assay of protease were diluted 1:100. Sample extracts of the developmental stages of mud crab from Zoea I to C1 crabs were all diluted 1:200 for the assay of all the three enzymes, while the sample extracts of the developmental stages of lobster from Stage I to Stage V phyllosoma were all diluted 1:100.

6.2.3 Statistical Analysis

The results of all experiments were analysed using One-Way ANOVA to determine differences among treatments. Data were tested for homogeneity of variance using the Levine's test, and when necessary, arcsine, square-root or logarithmic transformations were made to make the data normal and homogeneous before Duncan's Multiple Range Test (DMRT) was used for multiple comparisons. When normal distribution and/or homogeneity of the variance was not achieved, data were subjected to the Kruskal-Wallis H nonparametric test followed by the Games-Howell nonparametric multiple comparison test. Statistical analyses were performed using SPSS for Windows Version12.0. Data are presented as mean, plus or minus (±) standard error (SE), and results were considered significantly different at $P \leq 0.05$.

6.3 Results

6.3.1. Mud crab larvae

6.3.1.1 Changes in enzyme levels related to the moulting cycle

The changes in enzyme activities related to the moulting cycle of Zoea I stage of the mud crab *S. serrata* are presented in Fig. 6.1 with enzyme activities of the day 0 and day 1 Zoea II also included. Overall, amylase activities increased throughout the Zoea I moulting cycle, peaked on the day the larvae moulted to Zoea II, then showed a major drop the next day. In particular, specific amylase activity increased every day from 436±87 *mU* mg^{-1} protein in the newly hatched Zoea I at day 0 (Z1-D0) to 2477±136 *mU* mg^{-1} protein on day 4 (Z1-D4) and to 2666±101 *mU* mg^{-1} protein in the newly moulted Zoea II (Z2-D0), before a significant drop to 1607± 133 *mU* mg^{-1} protein the next day (Z2-D1). The daily increases in specific activities during the Zoea I moulting cycle were significant ($P<0.05$) except at the beginning between day 0 to day 1. The difference between the activities of 4 day old Zoea I (Z1-D4) and newly moulted Zoea II (Z2-D0) was also not significant (Fig. 6.1A). In the case of total amylase activity, the same trend was shown although the daily increases in total activity from the newly hatched Zoea I (Z1-D0) to newly hatched Zoea II (Z2-D0) were constantly significant ($P<0.05$), while the decrease from day 0 to day 1 for Zoea II was not significant ($P>0.05$) (Fig. 6.1B).

Protease activity through the Zoea I moulting cycle showed more fluctuations compared to amylase activity. Both specific and total protease activities dipped significantly on day 1 (Z1-D1) from the initial level at day 0 (Z1-D0), then established a sustained trend of significant daily increases until day 4 of Zoea I (Z1-D4) ($P<0.05$). A further sharp increase in protease activities was found in the newly moulted Zoea II (Z2-D0), but this was followed by a steep drop the next day (Z2-D1) ($P<0.01$) (Fig. 6.1C and D).

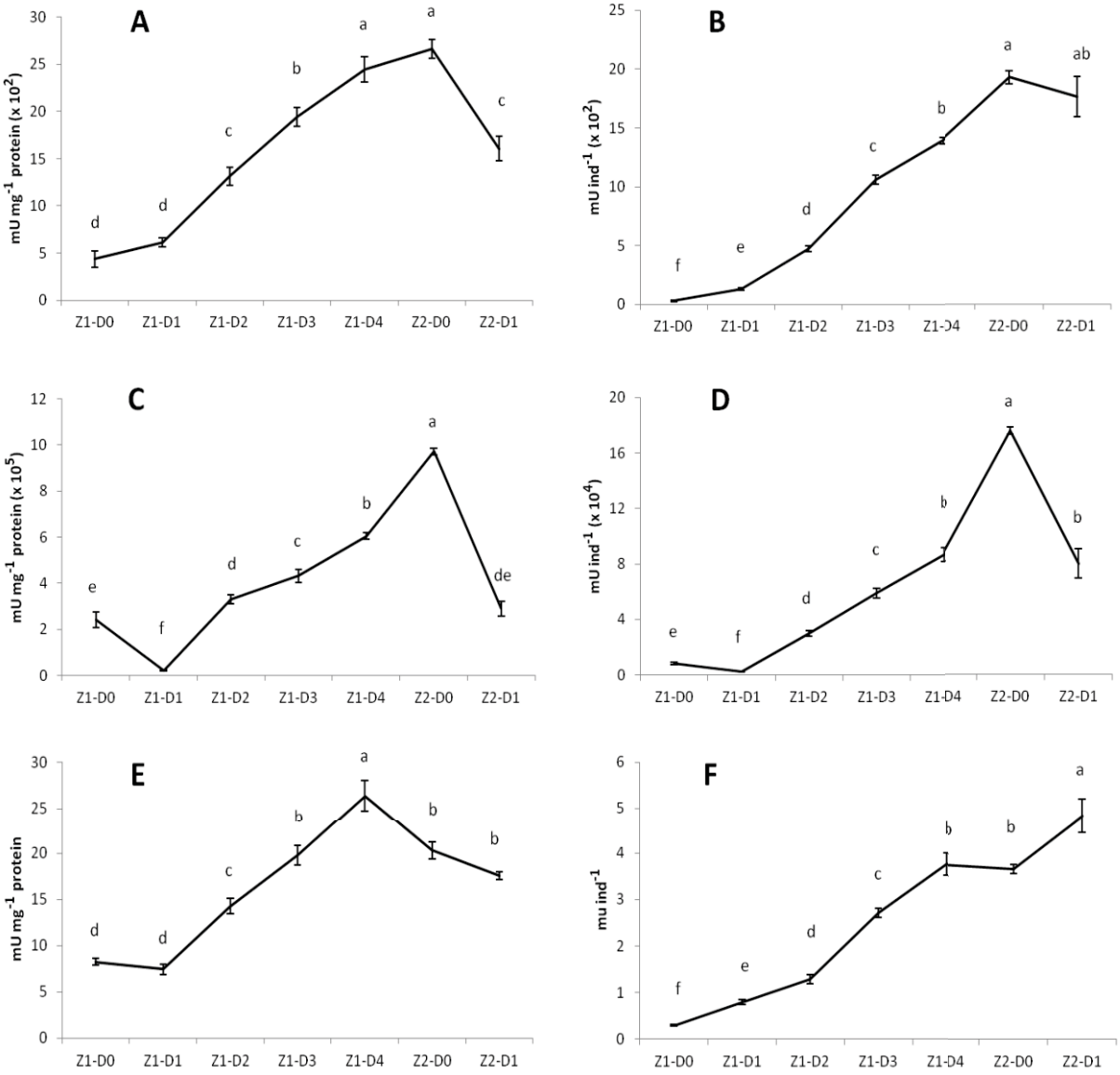

Fig. 6.1 Changes in mean (±SE, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (A and B), protease (C and D) and esterase (E and F) in *S. serrata* during the Zoea I moulting cycle and in the early Zoea II larvae. Z1-D0— newly hatched, day 0 Zoea I; Z1-D1— day 1 or 1 day old Zoea I; Z1-D2— day 2 Zoea I; Z1-D3—day 3 Zoea I; Z1-D4— day 4 Zoea I; Z2-D0— newly moulted, day 0 Zoea II; and Z2-D1— day 1 Zoea II. Means with same letters above are not significantly different (P>0.05).

Esterase activities of Zoea I larvae similarly showed an overall increasing trend towards the end of the moulting cycle. However, unlike amylase and protease, esterase activities did not further increase upon moulting to Zoea II and the differences between specific and total activities were more substantial (Fig. 6.1E and F). Specific esterase activity showed a slight insignificant dip as the newly hatched (day 0) larvae developed to day 1 (Z1-D1) before significant daily increases established peak activity on day 4. However, there was a significant drop in specific activity upon moulting to Zoea II (Z2-D0) ($P<0.01$), and this decreased further the next day (Z2-D1) but was not significant (Fig. 6.1E). In contrast, total esterase activity significantly increased daily during the Zoea I development from day 0 (Z1-D0) to day 4 (Z1-D4), then remained at a similar level upon moulting to Zoea II (Z2-D0), and increased significantly again on the following day as Zoea II (Z2-D1) ($P<0.05$) (Fig. 6.1F).

Changes in enzyme activities related to the moulting cycle of the megalopal stage of *S. serrata* are presented in Fig. 6.2. Both specific and total amylase activities of megalopae showed a sharp increase from the initial level at day 0 (M-D0) to the peak level the day after metamorphosis (M-D1) ($P<0.01$) and remained high at similar levels for four days. However, both specific and total activities then dropped significantly on day 5 ($P<0.05$), largely back to the initial levels of day 0 and remained low until the end of the moulting cycle and also in the newly moulted first stage crabs (C1-D0) (Fig. 6.2A and B).

Protease activities of megalopae generally showed a similar trend as amylase activities which had an initial increase (although not significant) on day 1 and then remained at about the same levels on the following days until a significantly drop occurred on day 5 ($P<0.05$). The activities remained at low levels from day 5 onwards until the megalopae moulted to become C1 (Fig. 6.2 C and D).

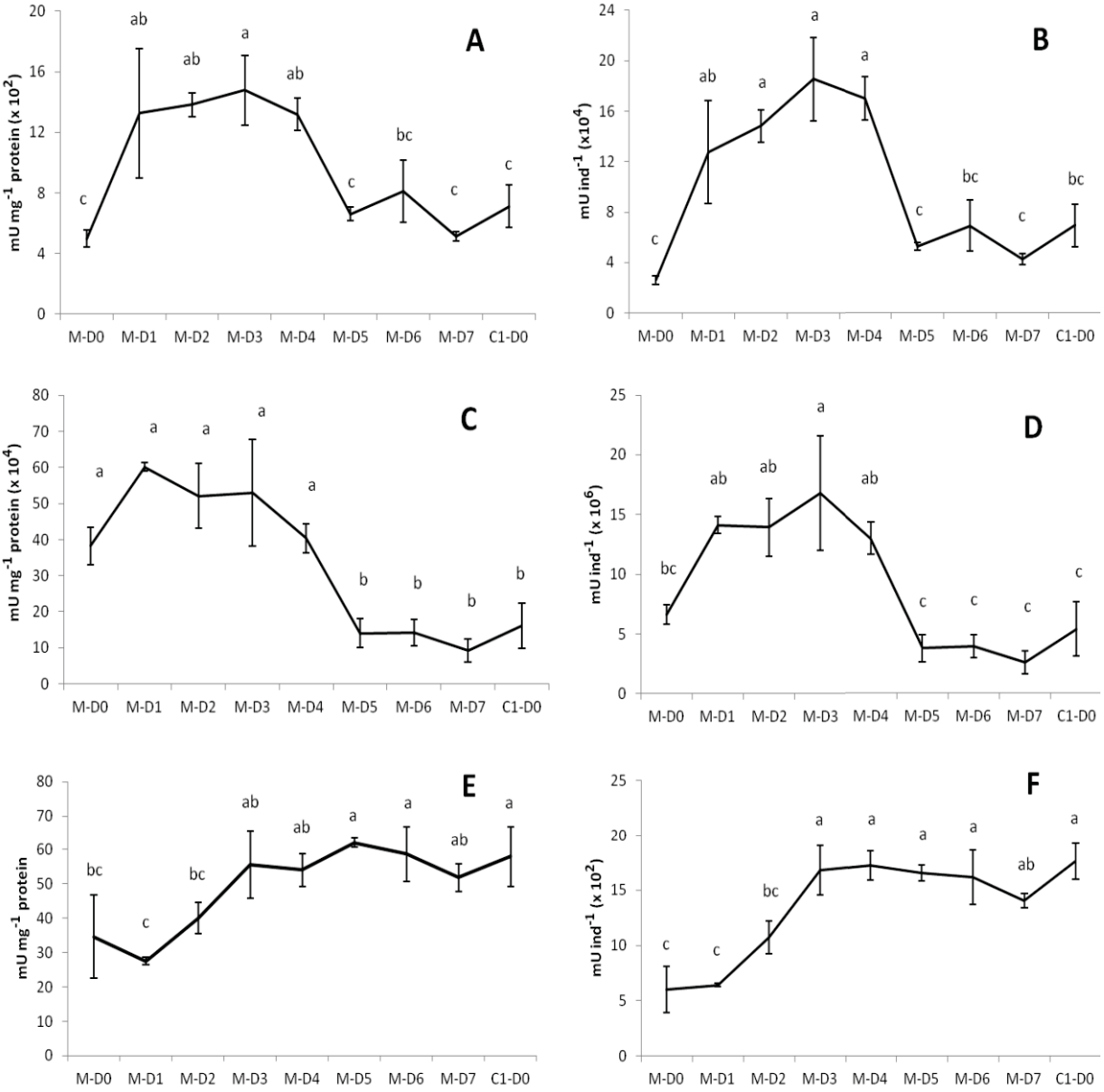

Fig. 6.2 Changes in mean (±SE, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (A and B), protease (C and D) and esterase (E and F) in *S. serrata* during the Megalopa moult cycle and in the early Crab instar. M-D0— newly moulted, day 0 megalopae; M-D1— day 1 or 1 day old megalopae; M-D2— day 2 megalopae; M-D3—day 3 megalopae; M-D4— day 4 megalopae; M-D5— day 5 megalopae; M-D6—day 6 megalopae; M-D7—day 7 megalopae; and C1-D0— newly moulted, day 0 Crab instar. Means with same letters above are not significantly different (P>0.05).

The general pattern of esterase activities during the megalopal moult cycle was somewhat opposite to that of amylase and protease activities. Both specific and total esterase activities showed a gradually increasing trend which peaked at day 3 and then remained at elevated levels until moulting to C1 (Fig. 6.2 E and F). Hence, esterase activities were at elevated levels when the megalopae were about to moult, in contrast to amylase and protease activities that were both at reduced levels at this stage (Fig. 6.2).

6.3.1.2 Ontogenetic changes in enzyme levels through larval development

Enzyme levels during the various stages of larval development of *S. serrata* are presented in Fig. 6.3. While both specific and total amylase activities generally showed an increasing trend through larval development, distinct patterns were demonstrated (Fig. 6.3A and B). From Zoea1 to Zoea II, specific activity increased sharply by more than 5-fold (from 457 ± 46 to 2356 ± 209 *mU* mg^{-1} protein) ($P<0.01$), but then remained at similar levels until Zoea IV, before another significant increase to a peak level occurred at the Megalopa stage ($P<0.05$). Following metamorphosis from megalopae to first stage crab (CI), the specific activity level dropped slightly but did not differ significantly from the other larval stages except Zoea I ($P>0.05$) (Fig. 6.3A). In contrast, total activity showed significant and steady increases at each stage as the larvae developed from Zoea I to Megalopa ($P<0.05$). A further increase was shown as the megalopae developed to CI, but the difference was not significant ($P>0.05$) (Fig. 6.3B).

The general pattern of protease activities during larval development was similar to amylase (Figs. 6.3C and D). Specific protease activities increased significantly by almost 3-fold when larvae developed from Zoea I to Zoea II (from 2.7 x 10^5 to 6.7 x 10^5 *mU* mg^{-1} protein) ($P<0.01$), then remained at more or less similar levels with no significant differences until Zoea V. At the megalopal stage, activity increased further to a peak level which was significantly higher than at Zoea IV stage ($P<0.05$). Following metamorphosis to C1, the activity level dropped but did not differ significantly from that of megalopal and zoeal stages except Zoea I ($P>0.05$) (Fig. 6.3C). In contrast, total protease activities increased significantly at each stage throughout larval development from Zoea I to Megalopa stage ($P<0.05$). However, as the megalopae developed to CI, the total activity also dropped but was likewise not significant ($P>0.05$) (Fig. 6.3D).

The general pattern of esterase activities somewhat differed from that of amylase and protease activities (Figs. 6.3E and F). While specific esterase activity also showed an initial significant increase from Zoea I to Zoea II ($P<0.05$), the increase was not as dramatic as that of amylase and protease. After showing no significant increase in activity from Zoea II and Zoea III stages, the activity increased substantially at Zoea IV stage ($P<0.05$) and increased further to reached a peak at the Zoea V stage before dropping back at the Megalopa stage. However, the specific activities of Zoea IV, Zoea V and Megalopa stages were not significantly different ($P>0.05$). As the megalopae developed to C1, the specific activity dropped further and the decrease was significant ($P<0.05$) (Fig. 6.3E). In contrast, total esterase activity showed a clear trend of significant increases at each stage throughout larval development from Zoea I to the Megalopa stage. However, as megalopae developed to C1, a significant decrease in specific activity was detected ($P<0.05$) (Fig. 6.3E).

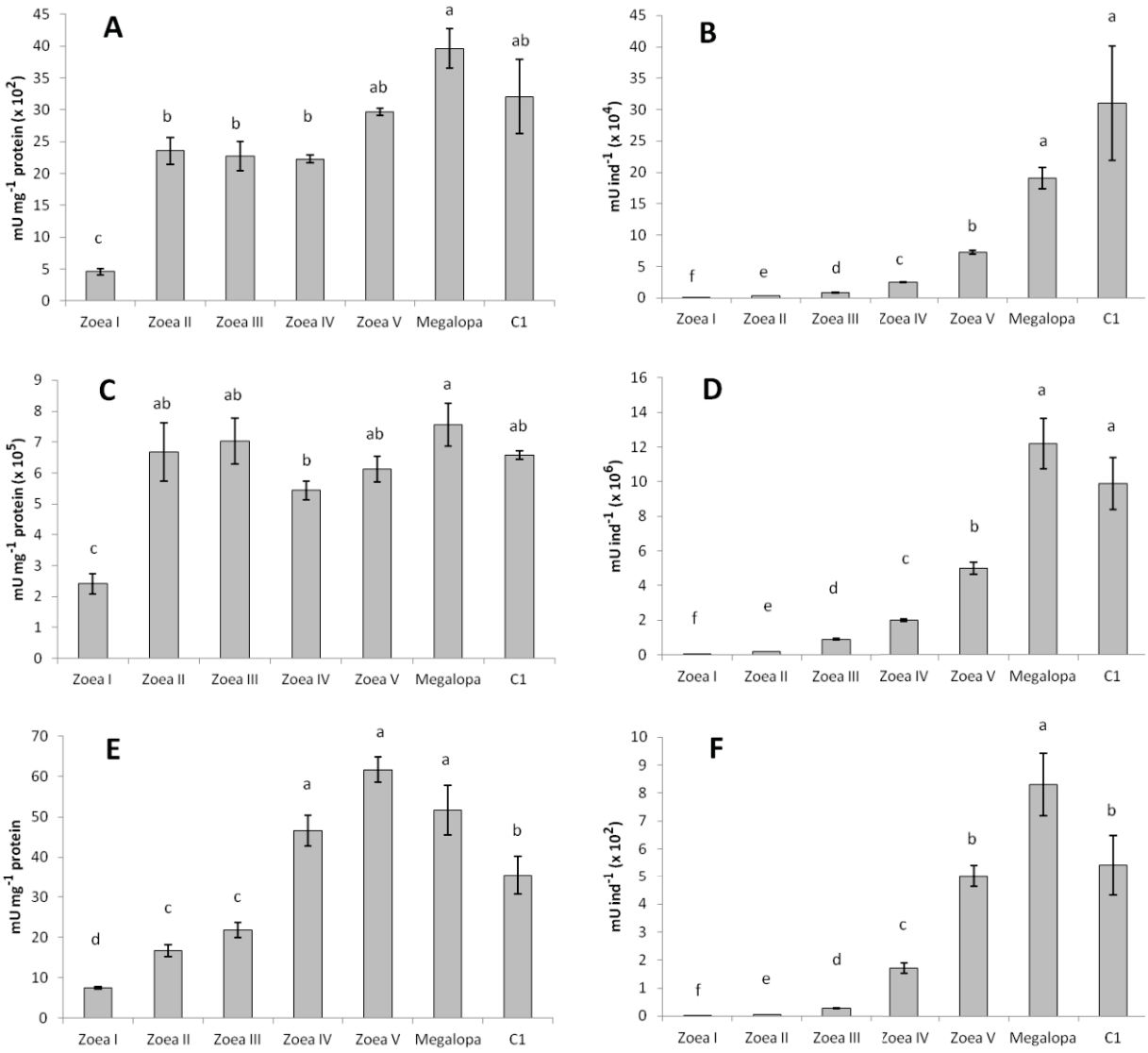

Fig 6.3. Changes in mean (±SE, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (A and B), protease (C and D), and esterase (E and F) in *S. serrata* at various larval developmental stages from Zoea I to Megalopa and as the settled first stage crabs (C1). Means with the same letters on the top of bars are not significantly different (P>0.05).

6.3.2 Lobster Larvae

6.3.2.1 Changes in enzyme levels related to the moulting cycle of early phyllosoma stages

The changes in enzyme activities related to the moulting cycle of *P. ornatus* phyllosoma were investigated during the early stages, from Stage I to Stage III (Fig. 6.4). In Stage I phyllosoma, both specific and total amylase activities increased rapidly from the initial low levels on day 0 to peak levels on day 1 ($P<0.01$). However, for specific activity, it subsequently decreased significantly on day 3 ($P<0.05$) and then remained at similar level until day 5 before a significant drop was detected in the newly moulted Stage II phyllosoma ($P<0.05$) (Fig 6.4A). In contrast, total activity remained more or less stable from day 2 to day 5 before a similar significant decrease occurred upon moulting to Stage II ($P<0.01$) (Fig. 6.4B). For Stage II phyllosoma, both specific and total activities had similar sharp increases from the low levels in newly moulted larvae to peak levels on day 2 ($P<0.01$) followed by a significant decrease on day 4. It is worth noting that the peak activities achieved on day 2 of Stage II phyllosoma were significantly higher than the peak activities detected for Stage I phyllosoma. As for the newly moulted Stage III phyllosoma, both specific and total activities dropped to significantly lower levels compared to the levels at day 4 of Stage II phyllosoma, which was a day prior to moulting ($P<0.01$). On day 2 of Stage III phyllosoma, both specific and total activities did not increase but remained at low level similar to that of day 0 ($P>0.05$); this is a different pattern to that observed for Stage I and II phyllosoma on day 2 (Fig. 6.4A and B).

The pattern of protease activities related to the moulting cycle was generally similar to that of amylase activity, except that peak protease activities during the moulting cycle were higher in Stage I compared to Stage 2 phyllosoma, while it was the reverse in the case of peak amylase activities (Fig. 6.4A and B vs. Fig. 6.4C and D). In Stage I phyllosoma, the specific activity of protease increased significantly from day 0 to day 2 ($P<0.05$) and continued to increase to reach the peak at day 3 before decreasing at day 5; however, changes in levels did not differ from that of day 2. On day 0 of moulting to Stage II, the activity decreased further to a level similar to that of day 0 Stage I phyllosoma but was not significantly different to the level at day 5 of Stage I. Changes in specific activities of Stage II phyllosoma were relatively mild and did not vary significantly; activity increased slightly on day 2 and decreased back on day 4. The specific activity of the newly moulted (day 0) Stage III phyllosoma decreased further from the level prior to moulting (day 4 of Stage II) and continued to decrease on day 2 but these levels were not significantly different ($P>0.05$). Unlike in Stages I and II, the activity in Stage III did not pick up on day 2, a trend which was similarly observed for amylase activity (Fig. 6.4C). In the case of total protease activity, significant increases occurred at each sampling period from day 0 to day 3 for the Stage I phyllosoma ($P<0.01$) and then remained at about the same level until day 5. In newly moulted Stage II phyllosoma, the total activity on day 0 dropped sharply to a level similar to that of day 0 Stage I phyllosoma ($P<0.01$) before a steep increase in activity occurred on day 2 ($P<0.01$), followed by a decrease on day 4, albeit not significant. Similar to specific activity, the total activity significantly decreased on day 0 of moulting to Stage III ($P<0.01$) and remained at a low level at day 2 (Fig. 6.4D).

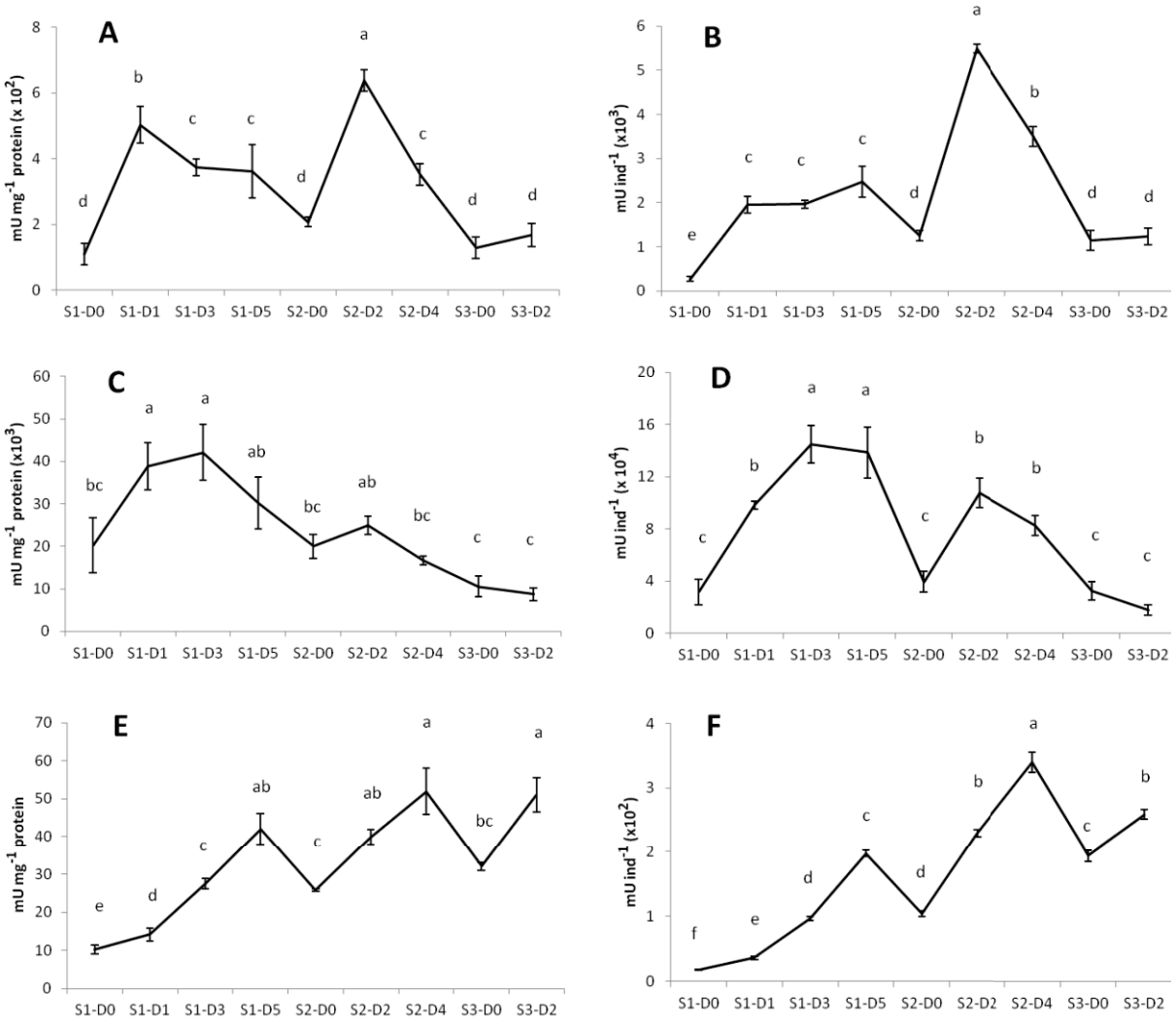

Fig. 6.4 Changes in mean (±SE, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (graphs A and B), protease (graphs C and D), and esterase (graphs E and F) at various stages of the moult cycle of Stages I to III *P. ornatus* phyllosoma. S1-D0 — newly hatched or day 0 Stage I; S1-D1 — day 1 Stage I ; S1-D3 — day 3 Stage I; S1-D5 — day 5 Stage I; S2-D0 — day 0 Stage II; S2-D2 — day 2 Stage II; S2-D4 — day 4 Stage II; S3-D0 — day 0 Stage III; and S3-D2 — day 2 Stage 3. Means with the same letters above are not significantly different (P>0.05).

In contrast to amylase and protease activities, esterase showed a consistent continuously increasing activity pattern within each moulting cycle of the early phyllosoma stages but with significant drops in activities on day 0 of each moult (Fig. 6.4E and F). In particular, both specific and total esterase activities of Stage I phyllosoma increased significantly at each sampling day from day 0 until activities peaked on day 5. The activities dropped significantly upon moulting to Stage II, then increased again from day 0 of Stage II to day 2 and continued to increase until peaks were achieved on day 4. Upon moulting to Stage III phyllosoma, activities dropped significantly again but then increased significantly from day 0 to day 2 ($P<0.05$) (Fig. 6.4E and F).

6.3.2.2 Ontogenetic changes in enzyme levels during early phyllosoma development

Changes in enzyme activities during the early larval development from Stages I to V of *P. ornatus* phyllosoma are presented in Fig. 6.5. Specific and total amylase activity showed distinct patterns during early larval development. Total activity steadily and significantly increased at each developmental stage ($P<0.05$) (Fig. 6.5B). In contrast, specific activity initially decreased significantly as the larvae developed from Stage I to II ($P<0.05$), then remained at similar level at Stage III, before significantly increasing at Stage IV to a level to similar to Stage I ($P<0.05$); they again decreasing significantly at Stage V ($P<0.05$) (Fig. 6.5A).

Specific protease activity showed a pattern similar to that of amylase activity; it likewise decreased significantly at Stage II ($P<0.05$), then remained at a similar level at Stage III, before significantly increasing at Stage IV and decreasing back at Stage V to about its former level ($P<0.05$) (Fig. 6.5C). On the other hand, total protease activity showed steady increases at each stage of larval development, however, these increases were not significantly different from those at Stage II to III and from IV to V ($P>0.05$) (Fig. 6.5D).

The pattern of specific esterase activity differed from that of amylase and protease activities. It remained low and did not differ between Stages I and II, but started to increase significantly at Stage III ($P<0.05$), followed by another sharp increase at Stage IV, leading to a more than doubling of activity from 31.0 ± 1.61 to 72.4 ± 3.6 $mU\ mg^{-1}$ protein respectively ($P<0.01$). It then decreased significantly at Stage V ($P<0.01$) (Fig. 6.5E). On the other hand, the total esterase activity was similar to total amylase activity, showing steady and highly significant increase at each stage of the early larval development ($P<0.01$) (Fig. 6.5F).

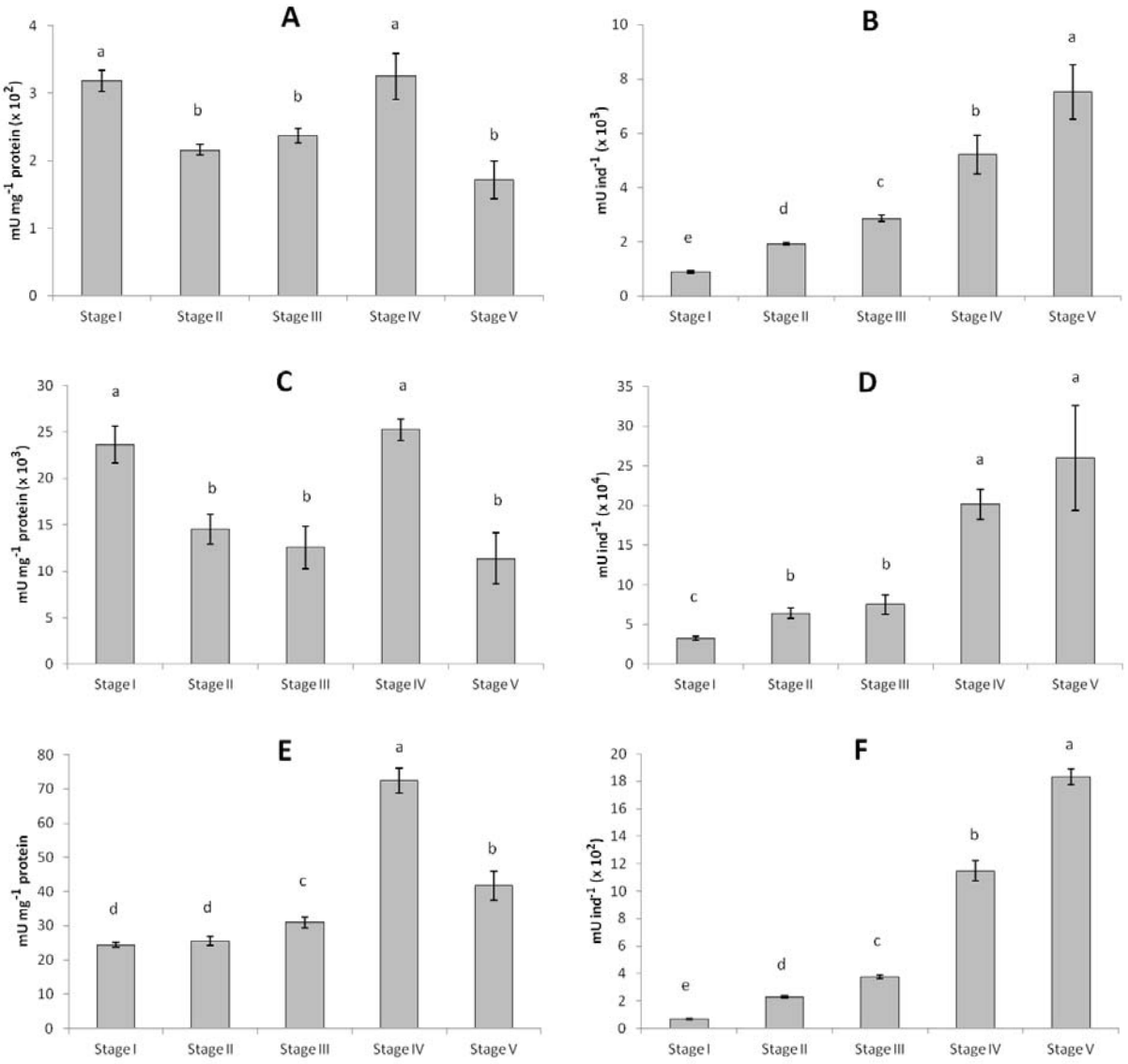

Fig 6.5. Changes in mean (±SE, n=4) specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activities of amylase (graphs A and B), protease (graphs C and D), and esterase (graphs E and F) at various stages of development in phyllosoma larvae of *P. ornatus*. Means with the same letters above bars are not significantly different (P>0.05).

6.4 Discussion

Crustacean growth happens in sequential steps that is directly related to moulting, a critical recurring process throughout their life cycle (Chang & Mykles, 2011). During each moulting cycle, chitinous components of the mouthparts and other feeding appendages of crustaceans are shed off; associated with this is a profound change in feeding activity that results in alternate episodes of feeding and fasting during development (Sanches-Paz *et al*., 2006). Active feeding in crustaceans generally happen during the late post-moult stage when new exoskeleton hardens and continues throughout the whole inter-moult period; it declines as the cycle approaches the next moult (pre-moult stage) and stops completely during ecdysis and immediately after a moult, when the new exoskeleton is still soft (Phlippen *et al*., 2000).

The differences in patterns of enzyme activities during the moulting cycle of the different larval stages of the mud crab, *S. serrata* and the spiny lobster, *P. ornatus* in this study suggested differences in the main energy sources utilised for metabolism during the episodes of feeding and fasting within moulting cycles. During the Zoea I moulting cycle of *S. serrata*, an initial significant drop in protease activities on day 1 (Fig. 6.1C and D) was also observed in a previous experiment (Chapter 4) and this was interpreted as an initial sparing of proteins by the larvae and reliance primarily on carbohydrates and lipids for metabolism. However, as the moulting cycle advanced, all the three major nutrients appeared to be increasingly utilized, as indicated by the significant increases in amylase, protease and esterase activities over subsequent days in Zoea I. As it was likely that the larvae would reduce food intake at pre-moult stage and cease feeding altogether during moulting, the continued increase in amylase and protease activities even in the newly moulted Zoea II was unexpected. Remarkably, amylase and protease activities continued to increase in newly moulted (day 0) Zoea II from the previous levels in larvae close to moulting (day 4 Zoea I), while specific esterase activity dropped significantly and total esterase activity levelled off;

this suggests that the newly moulted Zoea II probably relied more on carbohydrates and proteins as an energy source and reduced their utilisation of lipids. However, as the moulting cycle progressed, lipid reserves appeared to be increasingly utilized, as indicated by the significant drop in amylase and protease activities on day 1 of Zoea II that coincided with the significant increase in total esterase activity and largely maintained levels of specific esterase activity.

The moulting cycle of *S. serrata* megalopae was substantially longer than the zoeal stages, which enabled more sampling points for the major digestive enzymes. Amylase and protease activities in newly moulted (day 0) megalopae increased sharply by day 1, but specific esterase activity decreased slightly while total esterase activity was maintained at a similar level; this may suggest that early megalopae probably utilized more carbohydrates and proteins while sparing lipids. However, all three major nutrients were utilised as the moulting cycle advanced into inter-moult stage, as indicated by elevated levels of all three enzymes during the subsequent few days. However, during late inter-moult stage and pre-moult stages, it appeared that lipids were preferentially utilised as indicated by the continued elevated esterase activities which contrasted to the significantly reduced amylase and protease activities.

Changes in enzyme activities during moulting cycle of *P. ornatus* Stage I and II phyllosoma suggest that early Stage I phyllosoma initially utilised carbohydrates and proteins to a greater extent compared to lipids, since there were sharp increases in amylase and protease activities from day 0 to day 1 but relatively limited increase in esterase activities (Fig. 6.4). However, as the moulting cycle progressed into inter-moult and pre-moult stages, there appeared to be a gradual shift from metabolizing carbohydrates and proteins to lipids, as indicated by the overall levelling-off or decreasing amylase and protease activities, which were accompanied by steady increases in esterase activities. Earlier research h in this study

on the digestive enzyme activities of newly hatched *P. ornatus* phyllosoma that were first fed for 24 h then starved for the next 24 h, also showed that after food was removed, amylase activity dropped significantly, but protease activities remained at similar levels while esterase activities increased; this was interpreted to suggest that proteins and lipids were increasingly utilized when food became unavailable (Chapter 4). In the current experiment with Stage I phyllosoma, while all three nutrients were probably utilized during reduced feeding prior to moulting and total fasting during moulting, it was likely that proteins served as the major nutrient utilized. While both proteins and lipids are degraded during starvation, proteins contribute conspicuously more energy than the lipids because they are available in much larger quantities. In contrast, carbohydrates likely played a lesser role as they constitute less than 5% of body mass of crustaceans and have a more rapid turnover (Anger, 1986, 1987). In the spiny lobster *J. edwardsii,* Smith *et al*. (2003) reported that protein and to a lesser extent carbohydrate, were preferentially catabolised during starvation of Stage I phyllosoma. In the western rock lobster, *P. cygnus*, lipids accounted for only 6.7% of the decreased dry mass in starved Stage I phyllosoma, likewise indicating that lipids were not the major nutrients catabolised during starvation of newly hatched larvae (Liddy *et al*., 2003, 2004).

The enzyme activity during the moult cycle of Stage II phyllosoma showed a pattern similarly observed for Stage I phyllosoma. Comparably greater increases in amylase activity (more than 3-fold) during the post-moult period (2 days after moulting) may suggest that upon recovery from brief fasting due to moulting, utilization of carbohydrates from prey were prioritized over proteins and lipids probably because of their more rapid turnover (Anger, 1986). As the moulting cycle advanced further, there was again a shift in priority of nutrient utilization as implied by the sharp drop in amylase activities from day 2 to day 4, which coincided with the levelling-off in protease activities and significant increases in esterase activities. Considering that the larvae reduced feeding immediately prior to moulting

and completely ceased feeding during, and soon after moulting, the significant drop in activities of all the three major enzymes in newly moulted (day 0) Stage III phyllosoma may suggest that lesser levels of the three major nutrients were utilized due to the fasting period or starvation related to moulting. However, as starvation extended in the Stage III phyllosoma, the larvae may have relied primarily on lipids as indicated by the levelling of amylase and protease activities which coincided with significant increases in esterase activities during Stage III.

Changes in enzyme levels in this study were expressed as both specific (*mU* mg^{-1} protein) and total (*mU* ind^{-1}) activity. While these two different ways of expressing enzyme activity generally showed more or less similar trends regarding changes in enzyme levels related the moult cycle, they showed very different patterns when ontogenetic changes in enzyme activities during larval development are concerned. For both *S. serrata* and *P. ornatus* larvae, specific activities of three major digestive enzymes fluctuated during larval development; however, total activities showed consistent increasing trends. Since specific enzymes activities are expressed as per unit larval protein content (*mU* mg^{-1} protein), the values are affected by differences in larval biomass, which could be influenced by various factors, but especially by larval developmental stage because larval biomass increased substantially with each moult (Lovett & Felder, 1990a; Anger, 2001).

Another major influencing factor on larval biomass is the stages within a moulting cycle, however, since all larval samples of different larval stages for enzyme assay were collected during the inter-moult period, this factor less likely played a major role. It has been suggested that only the protein content of a major digestive organ (usually the hepatopancreas) rather than the whole animal should be used for calculating specific enzyme activity (Anger 2001), unfortunately, this is impractical for larvae due to their small size. On

this basis, total activity appears to be a better indicator of larval enzyme activities when considering enzyme level changes during larval ontogeny.

For *S. serrata* larvae, the significant increases in total activities of the major digestive enzymes at each stage of larval development from Zoea I to Megalopa clearly suggested continuous increases in digestive capacity with larval ontogeny, which helps explain the increasing larval ability in utilizing microbound diets (MBD) with larval development (Genodepa, 2003; Genodepa *et al*., 2006; Holme *et al*., 2006a). It is interesting to note that the steep absolute increases in total activities of all the three major enzymes occurred at late larval stages, i.e., Zoea IV, Zoea V and megalopae in this study coincided with periods of substantially increased food intakes of MBD reported in prior research with this species where ^{14}C-labelling was used as a tool to trace intake of MBD (Genodepa, 2003; Genodepa *et al*., 2006). This may suggests that substantial improvement in the digestive capacity occurred precisely during these late larval stages in preparation for the critical metamorphoses and long migration back to the coastal nursery grounds during the megalopal stage (Hill, 1994; Hamasaki, 2002).

In contrast, a significant drop in total esterase activity, coupled with a lack of significant changes in both total amylase and protease activities as the megalopae settle (as benthic first stage crabs) may be related to the shift from active planktonic larval stages to a more sedate benthic life as juvenile crabs and development of the digestive gland, particularly the hepatopancreas. In fact, in the freshwater crayfish, *Procambarus clarkii*, Hammer *et al*. (2000) reported that digestive enzyme activities increased steadily through the 42nd day from the on-set of feeding, however from that point onwards, enzyme activities remained largely stable and did not differ significantly; it was suggested that such a phenomenon was related to the full development of the hepatopancreas.

Similarly, during early development up to Stage V phyllosoma of *P. ornatus*, total activities of all the three major digestive enzymes also showed clear increasing trends with ontogenetic development, suggesting continuously improving digestive capacity. Such improvements appeared to be more prominent at Stage IV and Stage V where increases in levels of protease and esterase total activities more than doubled from those of the previous stage. Such a result implies a better chance for introducing formulated diets at these more advanced stages of phyllosoma. Research into ontogenetic changes in structure and function of mouthparts and foregut of *P. ornatus* phyllosoma by Johnston (2006) also revealed that multiple key morphological changes in both the mouthparts and foregut occurred at Stage IV, including an increase in size of mouth aperture, lateral setae and main brushes and the presence of a well-formed filter press with eight ampullary channels (Johnston 2006; Johnston *et al*., 2007). These changes coincide well with the significant increases in digestive enzymes of Stage IV phyllosoma revealed in this study, suggesting a substantially increased ingestion and digestive capacity. In fact, a previous study that compared the ability of *P. ornatus* phyllosoma to capture, feed on, and process diets of different textures and particle sizes reported that Stage I phyllosoma only fed on live newly hatched *Artemia*, while Stage III phyllosoma could also consume mussel gonad and mussel foot but not an extruded microbound formulated diet (Smith *et al*. 2009a). In contrast, Stage V phyllosoma readily consumed all the diets tested, including the formulated diet, and had the maximum size-discriminatory ability in the proventriculus, which also agrees with the results of this study. Another study on the development and function of the filter-press of a different spiny lobster species, *Sagmariasus verreauxi,* showed that although the filter-press of phyllosoma was fully functional at Stage III, it only became fully developed at Stage IV (Simon *et al*., 2012), which again agrees with the results of this study. Collectively, the results of the above research, including those of the current study, confirm that digestive enzymes can indeed be

used as an indicator of the digestive potential of an organism (Hammer *et al*. 2000; Lemos *et al*. 2000; Furne *et al*. 2005).

6.5 Summary and Conclusions:

Changes in digestive enzyme activity related to larval moulting cycles of *S. serrata* and *P. ornatus* helped identify the major sources of nutrients for metabolism during the recurring episodes of feeding and fasting associated with larval moulting cycles. For example, during the moult cycle of *S. serrata* Zoea I, newly hatched larvae appeared to initially spare protein and rely more on carbohydrates and lipids, but as the moult cycle advanced further, all three major nutrients, carbohydrates, proteins and lipids, were utilized for metabolism. During the brief fasting immediately prior to, during, and immediately after moulting, the larvae appeared to prioritise catabolism of proteins and carbohydrates initially, but relied more on lipids later on. During the moulting cycle of postlarval megalopae, there were both similarities and differences to Zoea I; newly moulted megalopae initially appeared to rely more on carbohydrates and proteins while sparing lipids, but as the moult cycle progressed, all three major nutrients were utilized. However, during the second half of the moult cycle, megalopae utilised lipids to a greater extent.

Over the moult cycle of the *P. ornatus* Stage I phyllosoma, the newly hatched phyllosoma appeared initially utilise carbohydrates and proteins to a greater extent; however, there was a gradual shift from utilizing carbohydrates and proteins to lipids as the moult cycle advanced towards ecdysis. Immediately following the moult to Stage II phyllosoma, the metabolic rate of newly moulted larvae was low as all the three enzymes showed a low level of activity, which was likely related to fasting during moulting. The larvae then increased their metabolism substantially and utilized all three nutrients but relied on proteins to a greater extent. As the moulting cycle advanced towards ecdysis, lipids had become the most

utilized nutrient for catabolism, as esterase activity remained at about the same level in early Stage III phyllosoma.

Compared to specific activity, total activity appeared to be a more appropriate way of expressing in enzyme activity when comparing ontogenetic changes during larval development of both *S. serrata* and *P. ornatus*. Changes in total activities of the major digestive enzymes during larval development clearly demonstrated continuous improvement in digestive capacity of both *S. serrata* and *P. ornatus* throughout larval ontogeny. For *S. serrata*, total activities of the major digestive enzymes increased significantly at each stage during larval development, but more dramatic increases occurred during late larval stages of Zoea IV, Zoea V and Megalopa. Enzyme activities did not further increase at first stage crabs, which may be related to the shift from active swimming of megalopae to the more sedate benthic habit of young crabs.

In *P. ornatus*, the activities of all three major digestive enzymes also increased with phyllosoma development to Stage V, especially at Stage IV and V when the increases in protease and esterase activities were more than doubled that of the previous stage. These changes indicated significantly improved ingestion and digestive capacity and suggest a better chance of success if formulated foods are introduced at Stage V or later.

CHAPTER 7

General Discussions and Conclusions

7.1 Significance of the Study

The mud crab *Scylla serrata* and the spiny lobster, or tropical ornate rock lobster, *Panulirus ornatus,* are emerging aquaculture species in tropical and sub-tropical areas of the Indo-Pacific region. Despite huge economic potential, further development of aquaculture industries for these species is constrained by dependence on wild seeds for stocking of grow-out farms (Keenan, 1999; Shelley, 2008; Johnston, 2006). On this basis, hatchery production of both species has received significant attention over recent years and improvements to hatchery practices have been made, however, further research is required to achieve consistent, routine, large-scale production that is required for commercial viability of these industries. Among the most important yet poorly understood aspects of hatchery production of these tropical crustaceans is nutrition of their larvae, particularly the specific nutrient requirements of different larval stages and ontogenetic development in digestive capacity. These issues have considerable importance as a basis for developing hatchery feeding protocols that support required hatchery production. Considering that the larvae of aquatic animals rely primarily on chemical digestion aided by enzymes, determination of digestive enzyme activities have been an important tool used in past studies to gauge larval capability to absorb and utilize nutrients from ingested foods (Le Vay *et al*., 1994; Le Moullac *et al*., 1997; Lemos & Rodriguez, 1998). Changes in digestive enzyme activities have also been used to indicate the relative importance of major nutrients in the diets of larvae (Lee *et al*., 1984; Rodriguez *et al*., 1994; Johnston, 2003). On such basis, this study comprehensively assayed the major digestive enzymes at various larval stages of both *S. serrata* and *P.*

ornatus, under various feeding scenarios, with the expectation that information generated would lead to a better understanding of larval nutrition and provide vital clues for devising optimal feeding strategies and appropriate feeds for their larvae; both are crucial for the improvement of hatchery production of these species.

7.2 Major Findings from this Study

7.2.1. Chapter 3 - Changes in digestive enzyme activities and nutrient utilization during embryonic development and starvation of the newly hatched larvae of *Scylla serrata* and *Panulirus ornatus*

Examination of the changes in digestive enzymes during embryonic development of *S. serrata* and *P. ornatus* revealed that lipids were the energy reserve most heavily utilized during the early phase of embryonic development of both species, however, as lipid reserves were low and insufficient to meet the increasing demand for energy towards the end of embryonic development, proteins, and to some extent carbohydrates, were increasingly utilized. These results agree with earlier reports that lipids are the main energy reserve fuelling embryonic development of crustaceans (Herring, 1974; Holland, 1978; Amsler & George, 1984; Wehrtmann & Graeve, 1998; Heras *et al*., 2000). Although proteins are the main component of marine invertebrate eggs (Holland, 1978), lipids were reported to provide more than 60% of the total energy expenditure of developing crustacean embryos (Wehrtmann & Graeve, 1998), which is generally in agreement with the transitions in levels of enzyme activities in this study. The higher levels of protease compared to esterase and amylase in starved newly hatched larvae of both species indicated that proteins continued to be the main energy source during the initial phase of starvation of newly hatched larvae. These results largely agree with the proposition of Anger (1986) that while both proteins and

lipids are generally degraded during starvation of crustacean larvae, proteins contribute conspicuously more energy because they are available in much larger quantities.

7.2.2 Chapter 4 - Digestive enzyme responses to intermittent food availability in first feeding larvae of *Scylla serrata* and *Panulirus ornatus*

This study examined changes in major digestive enzyme activities of first feeding larvae of *S. serrata* and *P. ornatus* subject to different conditions of food availability: (1) fed vs. starved; (2) fed initially, but then all food withdrawn; and (3) initial feeding delayed for varying durations. This study was designed to gain insights into how the larvae of *S. serrata* and *P. ornatus* adapt to various conditions food availability, which should provide clues for the development of formulated larval diets and for devising hatchery protocols. The results of experiments with first feeding zoea of *S. serrata* suggested that protein reserves were the main sources of energy utilized when no food was available. In starved *S. serrata* larvae, protease activity remained high throughout the 72 h sampling duration and protease activity levels were much higher than those of amylase and esterase. On the other hand, where food was available, first feeding larvae could have utilized carbohydrates and lipids more extensively while sparing proteins, as protease activity initially decreased sharply to very low levels although it eventually increased as the larvae approached moulting. This drop in protease activities when fed, contrasted with a gradual increases in amylase and esterase activities, suggesting that fed first feeding larvae were possibly building-up protein reserves.

The enzyme reponse of first feeding *P. ornatus* phyllosoma suggests an ability to utilize all three major nutrients, i.e., carbohydrates, proteins and lipids, but highlights a capacity to prioritise the use of carbohydrates when food was available. This was illustrated by the immediate and notable increase in amylase activity in fed phyllosoma, which remained high during subsequent days; however, such a phenomenon was not observed in starved

larvae. When food was removed after the phyllosoma were fed for a period of 24h, amylase activity decreased to low levels, suggesting that the phyllosoma quickly responsed to the unavailability of food by substantially reducing their utilization of carbohydrates and shifting to greater utilization of proteins and lipids.

When initial feeding was delayed for varying durations, both newly hatched zoea of *S. serrata* and phyllosoma of *P. ornatus* showed an ability to compensate for delayed food availability by increasing amylase activity. Both specific and total amylase activities of newly hatched zoea fed for 12 h, but with the feeding delayed from 12 to 36 h after hatching were significantly higher than those of larvae fed immediately after hatch for a same period of 12 h. In newly hatched phyllosoma fed for 24 h, but with feeding delayed for 24 h, both specific and total amylase activities were also significantly higher than those of larvae fed immediately after hatching for a same period of 24 h; however, such a response was no longer observed when initial feeding was further delayed beyond 24 h, suggesting that the ability of phyllosoma to compensate for delayed food intake diminished as the starvation period extended. Such response to delayed food availability may be related to the findings of Hofer (1982) and Harris *et al.* (1986) who reported that secretion of high levels of enzymes may occur as a way to maximize the use of scarce nutrients, although in this particular study, scarcity of dietary components was induced by the delayed feeding. Harms *et al.* (1991) suggested that at lower levels of food availability or relatively unavailability of a specific nutrient, secretion of higher enzyme levels has a compensatory function.

7.2.3 Chapter 5 - Digestive enzyme responses of *Scylla serrata* and *Panulirus ornatus* larvae to quantity and quality of feeds: the effects of food density and food type

The results of this chapter clearly showed that prey quantity and quality influenced larval digestive enzyme activities. Both *S. serrata* and *P. ornatus* larvae showed the ability to maximize utilization of available food by increasing their digestive enzyme activities in response to increasing prey density. Comparison of enzyme activities of Zoea I larvae of *S. serrata* fed different densities of rotifers showed that the rotifer densities resulting in maximal digestive enzyme activity fell within the range considered optimal in larval rearing of this species. A similar result was obtained for Stage I phyllosoma of *P. ornatus* fed different densities of *Artemia* nauplii. These results together suggest that digestive enzyme activity can be a good indicator for appropriate prey density to use in hatchery culture.

The digestive enzyme responses of Zoea II and Megalopae of *S. serrata* to different types of prey helped identify the relative nutritional values of such feeds to the larvae. The digestive enzyme responses of Zoea II larvae to feeding with rotifers or *Artemia* largely reflected the relative proximate contents of these two most commonly used hatchery foods. Similarly, the enzyme activities of mud crab megalopae fed either MBD or *Artemia* reflected differences in digestibility and nutritional quality of *Artemia* as a live prey versus the MBD as a formulated diet. There were huge differences in amylase activity detected between megalopae fed the MBD and *Artemia* indicating a possible significant role of carbohydrates in megalopal nutrition, and carbohydrate deficiency in the MBD used.

7.2.4 Chapter 6 - Changes in digestive enzyme activities related to the moulting cycle and larval ontogeny of *Scylla serrata* and *Panulirus ornatus*

The results of investigations into changes in activities of the major digestive enzymes relating to the moult cycle of Zoea 1 and Megalopa (postlarvae) of *S. serrata* as well as Stage 1 and Stage II phyllosoma of *P. ornatus* provided clues to the major nutrients utilized during the recurring episodes of moult-related changes in feeding activity during larval development. During the moult cycle of *S. serrata* Zoea I, newly hatched zoea appeared to initially spare proteins and rely mainly on carbohydrates and lipids for energy, but as the moulting cycle progressed, all three major nutrients were utilized while the larvae were actively feeding. On the other hand, newly moulted megalopae seemed to initially utilize carbohydrates and proteins while sparing lipids, but as the moult cycle advanced, all three major nutrients were utilized. During the second half of the moulting cycle, however, megalopae relied more on lipids.

During the moulting cycle of *P. ornatus* Stage I phyllosoma, newly hatched phyllosoma initially utilized carbohydrates and proteins to a greater extent, however, as larvae developed towards moulting, utilization of lipids gradually increase. During the initial phase of the moult cycle of Stage II phyllosoma, the pattern of enzyme activities showed increased utilization of carbohydrates and lipids, and reduced utilization of proteins. But during the second half of the moulting cycle of Stage II phyllosoma, lipids were increasingly utilized for energy as the larvae approached ecdysis.

Changes in the activities of the major digestive enzymes during larval development revealed ontogenetic improvements in the digestive capacities of both *S. serrata* and *P. ornatus*. Comparison of specific and total activities showed that total activity appeared to be a better way of expressing the changes in enzyme activities during larval ontogeny. In *S. serrata* larvae, the total activities of major digestive enzymes clearly increased with larval

development but more dramatic improvements occurred at Zoea IV, Zoea V and Megalopal stages. In phyllosoma Stages I to V of *P. ornatus*, the activities of all three major digestive enzymes increased with larval development, especially at Stage IV and V, where increases in protease and esterase activities were more than doubled, suggesting significant improvements in digestive capacity. Such a result may imply that there is a better chance of success in introducing formulated diets at, or beyond, Stage V.

7.3 Overall Discussion and Conclusions

The results of this study showed that analysis of digestive enzyme activities is a useful method for the study of larval nutrition. Overall, this research suggests that digestive enzyme activities do not just reveal the digestive capacity of larvae, but can also be used as an indicator of the nutrient requirements of larvae. The results of Chapter 3 highlight the importance of lipid and protein reserves in developing embryos of *Scylla serrata* and *Panulirus ornatus*. Considering that nutritional reserves in eggs largely reflect broodstock nutrition, such findings could have practical implications on broodstock feeding and nutrition, which should focus on diets containing high levels of these reserves. The results also showed the relative importance of the various (major nutrients) components of larval diets at various stages of larval development and through associated moult cycles of *S. serrata* and *P. ornatus*. They further illustrated the ability of these larvae to respond to different conditions of food availability by adjusting the relative activity levels of the major digestive enzymes to selectively catabolise specific nutrients for energy, growth and development.

Interestingly, the larvae of *S. serrata* and *P. ornatus* appear to have developed a similar strategy towards the utilization of proteins, which appeared as the major energy source of the newly hatched larvae when food is unavailable. In cases where food was

available, the larvae of *S. serrata* appeared to spare proteins by immediately decreasing protease activity while increasing amylase and esterase activities. This suggested that proteins from ingested prey were stored and utilized later towards moulting when feeding was reduced. In the case of fed *P. ornatus* larvae, however, huge increases in amylase activities compared to protease activity suggested that carbohydrates from prey were utilized to a greater extent while proteins were conserved.

The results also showed that carbohydrates generally served as the most important energy source that the larvae is primarily utilizing when food was available. But when feeding was delayed, both *S. serrata* and *P. ornatus* larvae showed a capacity to compensate for delayed food intake by primarily increasing their amylase activities. Prioritising the use of carbohydrates is probably a strategy employed by larval crustaceans because they have limited capacity for storing carbohydrates (Anger, 1986).

The activity levels of the major larval digestive enzymes also varied in response to both quantity and quality of prey provided to the larvae of both *S. serrata* and *P. ornatus*, hence it appears that digestive enzyme activities can be used to identify optimal feeding densities and nutritional quality of various live foods for larvae. For example, significant differences in amylase activities indicated noteworthy differences in nutritional quality between the live prey (*Artemia*) and the formulated food (MBD) fed to *S. serrata* megalopae, particularly in terms of carbohydrate contents. This indicates the possible significant role of carbohydrates in megalopal nutrition and points to the need for further investigations in this area, particularly considering that the dietary carbohydrate requirements of larvae have so far received little research attention compared to proteins and lipids.

The activities of the major digestives enzymes in *S. serrata* and *P. ornatus* larvae were influenced by moult cycles and these changes in enzyme activities provided clues about the nutrients prioritised for metabolism during the moult cycle. It is noteworthy that increased

utilization of lipids occurs towards the end of the moulting cycles which included periods of reduced food intake and total cessation of feeding. This highlights a need to ensure that these crustaceans obtain optimal lipid reserves as this would be crucial for moulting success (Ceccaldi, 1997; Chang, 1995).

Consistent, high-efficiency hatchery production depends on the availability of nutritionally-appropriate feeds that support rapid growth and health (Southgate & Partridge, 1998). Formulated diets have several advantages over live foods (Southgate & Partridge 1998; Southgate, 2003), and have been used successfully with crustacean larvae including those of *S. serrata* (Genodepa *et al*., 2004b; Holme *et al*. 2006a, 2006b). But the timing of introduction of formulated foods within a hatchery feeding protocol is a crucial question for larval crustaceans because of their limited digestive capacity (Ceccaldi,1989; Icely & Nott, 1992; Ceccaldi, 1997; Jones *et al.*, 1997a, b). In this study, notable increases in total activities of the major digestive enzymes at certain stages during larval ontogeny of *S. serrata* and *P. ornatus* provided important information to guide appropriate timing for the introduction of suitable formulated diets. All these confirm the usefulness of digestive enzymes as a tool for gauging the digestive capacity of marine larvae (Hammer *et al*. 2000; Lemos *et al*. 2000; Furne *et al*. 2005) and assisting development of more appropriate larval feeding protocols.

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