Investigating the survival, growth and osmoregulatory responses of blue swimmer crab, *Portunus pelagicus*, early juveniles to salinity and ammonia challenge

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Statement of contribution of others

Firstly, I would like thank my supervisor Dr. Chaoshu Zeng who has provided excellent guidance throughout my experiments and has greatly helped in editing each Chapter of this thesis as well as other publications. I am grateful to him for giving me the opportunity to pursue my higher education at James Cook University (JCU). I would also like to thank Noordiyana Mat Norrdin for her assistance with designing and creating the diets necessary for the experiments in Chapter 7 as well as data interpretation and support throughout my studies. Finally, I would also like to thank Xugan Wu for analysing the HUFA content of the diets in Chapter 7 and for excellent discussions regarding experimental design.

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Declaration on Ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Human (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology recieved clearance from the James Cook University Experimentation Ethics Reveiew Committee (approval numbers A1056 and A1183).

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Abstract

The blue swimmer crab, *Portunus pelagicus*, is a portunid crab native throughout the Indo-Pacific region and their harvests support important commercial fisheries as well as being an emerging aquaculture species. However, basic information on this species relevant to fisheries management and aquaculture production is limited. Two of the most important abiotic factors that can affect the productivity and physiology of aquatic crustaceans include salinity and ammonia-N. Salinities fluctuate widely in open aquaculture systems or estuaries and the ability of aquatic animals to thrive in such conditions is highly species-specific, depending on their osmoregulatory abilities. Ammonia-N, on the other hand, is the first nutrient in the nitrification cycle and, compared to nitrite and nitrate, is often the most toxic to crustaceans leading to reduced growth, physiological disruption or death. Accumulation of ammonia-N in closed aquaculture systems is ubiquitous concern, while in nature, crustaceans can experience elevated levels when burying for prolonged periods or in ecosystems receiving excessive anthropogenic discharge. Although salinity and ammonia-N may appear unrelated, the coping processes to salinity stress and elevated ammonia-N expsoure are both closely linked via the highly important gill enzyme Na^+/K^+-ATPase activity. Furthermore, since these two factors may simutaneously challenge aquatic animals in either aquaculture systems or nature, the main focus of this thesis will investigate the effects of salinity and ammonia-N, alone and in combination, on the survival, growth, osmoregulation and coping responses of *P. pelagicus* juveniles. In addition, the last chapter will examine whether dietary highly unsaturated fatty acids (HUFA) and phospholipids (PL) supplemenation can improve the survival, growth and haemolymph ion maintenance of *P. pelagicus* juveniles at osmotically stressful conditions.

Chapter 2 was designed to investigate salinity levels of 5, 10, 15, 20, 25, 30, 35, 40 and 45 ‰ on the survival and growth of early *P. pelagicus* juveniles over 45 days and at the end of the experiment the haemolymph osmolality was measured to determine their osmoregulatory abilities. Results showed salinities of $\leq$ 15 ‰ and 45 ‰ significantly reduced survival while salinities of $\leq$ 15 ‰ and $\geq$ 40 ‰ significantly reduced the growth of *P. pelagicus* juveniles, indicating that this species is highly sensitive to salinities outside an optimal range of 20 - 35 ‰. Based on the haemolymph osmolality, the reduced performance of the crabs at these salinities may be attributed to their relatively weak osmoregulatory abilities.

In Chapter 3, the acute ammonia-N tolerance of *P. pelagicus* through juvenile development was determined and, to explain potential ammonia-N toxicity differences, gill histopathological changes were observed. Crabs at juvenile stages 1, 3, 5 and 7 were exposed to 0, 10, 20, 40, 60, 80 and 100 mg l^{-1} ammonia-N and at 12-h intervals mortalities were measured for 96-h and then fixed to examine any histopathological gill damage. Results showed that *P. pelagicus* juveniles are highly tolerant to ammonia-N and this ability which increased through juvenile development possibly due to less ammonia-N induced gill damage at older juvenile stages.

Since ammonia-N tolerance was linked with gill damage, Chapter 4 was performed to determine any correlation between gill damage and coping responses, if gill damage is reversible and potential causes for the high ammonia-N tolerance of *P. pelagicus* juveniles. Crabs exposed to sub-lethal ammonia-N levels of 20 and 40 mg l^{-1} were measured for haemolymph osmolality, Na^{+}, K^{+}, Ca^{2+}, pH and ammonia-N levels, total haemocyte counts (THC) and then fixed for gill histological examination at hourly intervals for 48-h. Following 48-h, crabs were transferred to pristine seawater and again measured for these parameters at hourly intervals for 96-h. Ammonia-N had no significant effect on haemolymph osmolality,

ion or pH, while haemolymph ammonia-N levels remained substantially lower than the test media. Further, ammonia-N exposure quickly caused gill damage along with adaptive/healing responses including significanlty higher THC and haemocytes within the gill lamallae. Upon transfer to pristine seawater, signs of gill healing became apparent and by 96-h, the gills were almost completley healed likely facilitated by haemocyte increases. Such findings likely explain the high ammonia-N tolerance of *P. pelagicus*, although this response has not yet been confirmed with other species.

These findings suggest that osmoregulation of *P. pelagicus* was undisrupted by ammonia-N and that haemolymph ammonia-N regulation may have been accomplished via ammonia-N excretion. To confirm this, Chapter 5 was set up to expose *P. pelagicus* juveniles to 0, 20, 40, 60, 80, 100 and 120 mg l^{-1} ammonia-N at salinities of 15, 30 and 45 ‰ for 96-h and then measured for haemolymph osmolality, Na^+, K^+, Ca^{2+} and ammonia-N levels, ammonia-N excretion and gill Na^+/K^+-ATPase activity. Low salinities of 15 ‰ significantly increased posterior gill Na^+/K^+-ATPase activity, compared to 30 and 45 ‰, while anterior gill Na^+/K^+-ATPase activity was unaffected by salinity. However, anterior gill Na^+/K^+-ATPase activity significantly increased with increasing ammonia-N, whereas for the posterior gills, this result only occurred at 30 ‰. Furthermore, ammonia-N excretion and haemolymph ammonia-N levels significantly increased and decreased, respectively at 15 ‰, compared to those at 45 ‰. This likely explains the general pattern of increased ammonia-N toxicity at decreasing salinities to various crustaceans and, furthermore, ammonia-N excretion is unlikely to be the sole mechanism for haemolymph ammonia-N regulation.

During ammonia-N excretion, NH_4^+ can substitute for K^+ via Na^+/K^+-ATPase activity and elevated levels of both these ions has been demonstrated to increase Na^+/K^+-ATPase activity on dissected crustacean gills. While this suggests that ammonia-N excretion and

osmoregulation would remain undisrupted in living crustaceans, this has yet to be proven. Therefore, Chapter 6 was set up to expose *P. pelagicus* juveniles to 0, 20, 40, 60, 80 and 100 mg l^{-1} ammonia-N at low (4.25 mM), normal (8.50 mM) and high (12.75 mM) K^+ levels of seawater for 96-h. After 96-h, haemolymph osmolality, Na^+, K^+, Ca^{2+} and ammonia-N levels, ammonia-N excretion, gill Na^+/K^+-ATPase activity and gill histopathological changes of the crabs were measured. In the presence of ammonia-N, both low and high K^+ significantly reduced both ammonia-N excretion and gill Na^+/K^+-ATPase while haemolymph ammonia-N levels of the crabs significantly increased. An *in vitro* experiment was then performed, at different ammonia-N and K^+ combinations, to compare gill Na^+/K^+-ATPase activity responses with the *in vivo* experiment. The *in vitro* experiment revealed no significant K^+, ammonia-N or interactive effect on gill Na^+/K^+-ATPase activity. Greater histological gill damage and/or adverse physiogical consequences to living crabs may explain this discrepency and therefore *in vitro* investigations may be needed to test their applicability on living animals.

Finally, in Chapter 7, seperate experiments were set up to determine whether dietary highly unsaturated fatty acids (HUFA) and phospholipids (PL) can improve the survival, growth and haemolymph ion maintenance of early *P. pelagicus* juveniles at salinities of 14, 30 and 42 ‰. Results showed that increased dietary HUFA significantly improved growth and haemolymph ion maintenance of *P. pelagicus* juveniles at both 14 and 42 ‰, to become comparable with those cultured at 30 ‰. In contrast, dietary PL supplementation had no effect on overcoming the negative growth effects of high salinity and no significant effect on haemolymph ions. However, increased dietary PL supplementation significantly improved survival rates, particularly at 14 and 42 ‰. These findings demonstrate that the productivity of *P. pelagicus* juveniles can be significantly improved through both increased dietary HUFA and PL supplementation when subjected to osmotically stressful conditions for prolonged periods.

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General introduction

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1. Introduction

1.1. The blue swimmer crab, *Portunus pelagicus*

The blue swimmer crab, *Portunus pelagicus*, is a portunid crab native throughout Indo-Pacific estuaries and oceans and can grow up to 140 mm in carapace width within 12 months (Potter et al. 1983; Kangas, 2000). The males are characterised as having a blue/purple carapace with long chelipeds while the females are more drab in color with smaller chelipeds (Figure 1). Their commercial harvests in this region support important fisheries due to their high meat yield and good taste and their popularity is growing fast with estimated landings increasing from 94,700 tonnes in 1990 to 150,000 tonnes in 1998 (Otto et al. 2001). Apart from the traditional sale as hard-shell crabs, there has been a substantial increase in their utilisation for pastuerised canned crabmeat. Exportation of this product to the United States, Japan and Singapore reportedly generates a multi-million dollar annual revenue for Indonesia alone (Muna, 2005; Setyadi and Suanto, 2005). Meanwhile, interest in soft-shell crab production is growing since this lucrative niche industry can fetch up to AU $60/kilo (Muna, 2005; Setyadi et al. 2005). To meet increasing market demands, *P. pelagicus* are being cultured in Australia using recirculating systems or lined ponds. In recirculating systems, cannibalism is prevented through their individual culture in compartments and the efficient and expedient harvesting of newly molted soft-shell crabs is made possible by a periodic robotic monitoring system (O'Neill, 2003). In addition, lined ponds are also being used to free-range

produce *P. pelagicus* where pre-molt crabs are individually transferred to cages and newly molted crabs are quickly harvested (Walker, 2006).

Figure 1: A copulating pair of male (top) and female (bottom) *Portunus pelagicus* crabs.

Currently, however, blue swimmer crabs are largely sourced from fisheries which is unreliable and seasonal (Otto et al., 2001) and therefore the aquaculture interest of this species is growing due to a number of beneficial characteristics including their relative ease of hatchery production (Walker, 2006), fast growth rates (Josileen and Menon, 2005), year round spawning and high market price (Romano and Zeng, 2008).

In spite of their commercial importance, limited information exists concerning this species and therefore basic information relevant to their aquaculture is considered essential for the aquaculture industry to expand. Two of the most important and limiting abiotic factors on aquaculture systems are salinity and ammonia. Furthermore, the coping mechanisms involved with these factors are linked via osmoregulation (Lucu and Towle, 2003; Weihrauch et al. 2004), which is often highly species-specific (Allan et al. 1990; Péqueux, 1995; Romano and Zeng, 2007a). Therefore knowing the optimal levels, tolerance limits and the associated

physiological responses of *P. pelagicus* to these two important environmental parameters will yield useful information beneficial to the aquaculture industry.

1.2. Crustacean osmoregulation and general principles

Extracellular osmoregulation is the relationship between the osmolality (*i.e.* the total amount of osmolytes such as Na^+ and Cl^- and often expressed as milli osmoles kg^{-1}) of the solvent (or environment) and the haemolymph (or blood) of the aquatic animal (Péqueux, 1995). Typically this is measured from the haemolymph osmolality/ions of the animal with respect to the environmental osmolality/ions. When the environmental osmolality is higher or lower than that of the haemolymph, this condition is termed hyper-osmotic or hypo-osmotic, respectively. At a hyper-osmotic condition an aquatic animal must compensate for a continual influx of salts, which is termed “hypo-osmoregulation”, while at a hypo-osmotic condition the aquatic animals must compensate for a continual loss of salts, which is termed “hyper-osmoregulation”. The point at which the external and haemolymph osmolality are equal is termed "iso-osmotic" (Péqueux, 1995). Although, generally, the salinity at or near the iso-osmotic point indicates the optimal salinity range for the survival and growth of aquatic animals this is not always the case. Furthermore, even at the iso-osmotic point aquatic animals still osmoregulate to some degree (and therefore requires energy) since the ionic composition of the haemolymph is often different from the environment (Péqueux, 1995). An example is from Table 1, modified from Chen and Chia (1997), clearly is showing that the haemolymph ions from the mud crab, *Scylla serrata*, at an iso-osmotic point of 33.4 ‰ can be substantially different from the external salinity. This, therefore, demonstrates that their haemolymph ions are regulated substantially below that of the environment (evident by the iso-ionic point being lower than the iso-osmotic point) in spite of being in an iso-osmotic environment.

Table 1: The various iso-ionic points (mmol l^{-1}) from haemolymph of *S. serrata* adults when exposed to an iso-osmotic condition of 33.4 ‰ (or 968.6 mOsm kg^{-1}). The equivalent salinities, from the various iso-ionic points, are provided to illustrate that the haemolymph ions of *S. serrata* can be substantially lower than those at a salinity of 33.4 ‰. Table modified from Chen and Chia (1997).

Haemolymph ions	Iso-ionic point (mmol l^{-1})	Equivalent salinity (‰)	Ion concentration at a salinity of 33.4 ‰
Cl^-	365	24.0	514
Na^+	322	22.3	483
K^+	8.4	22.2	12.6
Ca^{2+}	11.3	33.4	11.3
Mg^{2+}	28.7	21.3	43

The optimal salinity levels of aquaucultured animals are not always clear-cut as either freshwater or full strength seawater (*i.e.* 30 - 35 ‰), but is often somewhere between these two extremes. Furthermore, the iso-osmotic point (and therefore the associated hypo- and hyper-osmotic conditions) can vary drastically among marine/estuarine species, likely reflecting their evolutionary histories (Péqueux, 1995). For example, based on survival and growth, the optimal salinity range for the mud crab, *Scylla serrata* is 10-25 ‰ with an isosmotic point of 33.4 ‰ (Chen and Chia, 1997; Ruscoe et al. 2004) whereas the marine shrimp *Penaeus indicus* has a higher optimal salinity range of 20-30 ‰ but with a lower isosmotic point of 26 ‰ (Parado-Estepa et al. 1987; Kumlu and Jones, 1995) (for other examples see Table 2).

Table 2: The optimal salinity ranges and isosmotic points for various euryhaline crustacean species.

Species	Optimal salinity	Isosmotic point	Reference
Crabs			
Callinectes sapidus	25-30 ‰	27-35 ‰	Cadman & Weinstein, 1988; Guerin & Stickle,1997a
Callinectes similis	>10 ‰	>35 ‰	Guerin and Stickle, 1997a,b
Scylla serrata	10-25 ‰	33.4 ‰	Chen & Chia, 1997; Ruscoe et al. 2004
Prawns			
Penaeus paulensis	15-25 ‰		Lemos et al. 2001
P. monoceros	30-40 ‰		Kumlu et al. 2001
P. stylirostris	≈30 ‰*	26.2 ‰	Lemaire et al., 2002; Spanopoulos-Hernandez, et al. 2005
P. semisulcatus	30-40 ‰		Soyel & Kumlu, 2003
P. latisulcatus	22-34 ‰	28.8-31.7 ‰	Sang & Fotedar, 2004
P. vannamei	25 ‰ [1] 33-40 ‰ [2]	27.7 ‰ [3]	[1]Bray et al. 1994 [2]Ponce-Palafox et al. 1997 [3] Castille & Lawrence, 1981
P. indicus	20-30 ‰	26 ‰	Parado-Estepa et al., 1987; Kumlu & Jones, 1995
P. setiferus	≈35 ‰*	24-25 ‰	Rosas et al. 1999
P. japonicus	35 ‰*	30-35‰	Cheng & Chen, 2002; Setiarto et al. 2004
P. chinensis		28-24 ‰	Chen & Lin, 1994a
Macrobrachium rosenbergii	0 – 4 ‰ [3]	17-18 ‰ [1] 14.5-15.6 ‰ [2] 17 ‰ [3]	[1] Sandifer et al. 1975 [2] Cheng et al. 2003 [3] Singh, 1980
Cherax tenuimanus	10 ‰		Rouse & Kartamulia, 1992

*Optimal salinities determined based on reduced DO consumption and ammonia-N excretion which generally indicates reduced metabolic demands necessary to maintain ion homeostasis (Guerin and Stickle, 1997b).

It should be noted here that a wide range of factors can affect osmoregulation such as the molting stage, developmental stage, sex, season, temperature, nutritional status, pollutants and even the location (Neufeld et al. 1980; Péqueux, 1995; Charmantier et al. 1988; Lignot et al. 2000). Only some of these will be discussed in more detail later since a comprehensive description of all these factors is beyond the scope of this thesis. However, these factors should be noted since it may explain variability in haemolymph osmolality/ion composition (and sometimes even optimal salinity levels) between experiments of the same species.

While osmoregulation for aquatic animals is a crucial process, the efficiency or ability is largely species-dependent while the strength and strategy depends on external salinities. The species-specific ability to osmoregulate, which is defined as the degree to which the haemolymph osmolality can remain independent of the external osmolality (Péqueux, 1995) (Figure 2), is influenced by various factors on the gills which will be discussed in more detail in section 1.3. However, the important point to note is the osmoregulatory ability of crustaceans will, in turn, greatly dictate the salinities in which they can successfully inhabit (Péqueux, 1995; Henry, 2001). In an aquaculture context, this likely has productivity implications.

Two strategies for osmoregulation exist which are a "compensatory process" and "limiting process" (Péqueux, 1995). A "compensatory process" is a strategy whereby the maintenance of haemolymph osmolality/ions is accomplished via the active movement of solutes to counterbalance their passive diffusion and a "limiting process" is a strategy whereby the maintenance of haemolymph osmolality/ions is accomplished by adjusting the permeability of the boundary structures (*i.e.* the gills) (Péqueux, 1995). These methods have been investigated for many years, from both a molecular and physiological approach, and although many processes are known, there are still many unknowns particularly regarding the limiting process.

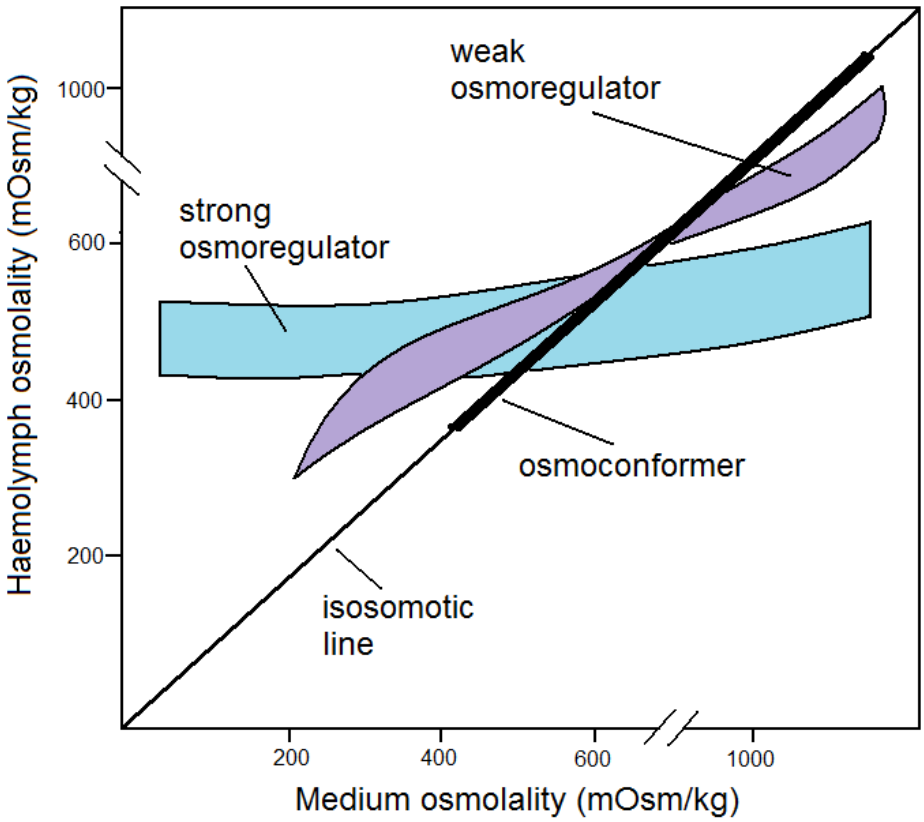

Figure 2: Patterns of haemolymph osmoregulation in crustaceans. The deviations from the isosmotic line indicate the strength of their osmoregulatory ability. Figure modified from Péqueux (1995).

1.3. Osmoregulation – compensatory process

The gills of crustaceans are multifunctional organs since they are largely responsible for acid-base balance, gas exchange, ammonia-N excretion and ion transport (Péqueux, 1995). Although, to a much lesser extent, the antennal and maxillary glands are involved with Mg^{2+} and Ca^{2+} regulation (Péqueux, 1995; Freire et al. 2008). Due to the relatively impermeable exoskeleton of crustaceans, the gills play a dominant role in many crucial physiological processes since these structures are the main boundary surfaces between the haemolymph and environment (Péqueux, 1995). Therefore, the osmoregulatory strength of crustaceans are greatly influenced by gill enzymatic affinities for ions, abundance of ion transport sites and permeability properties (Piller et al. 1995; Castilho et al. 2001; Henry, 2001). Specifically, in

brachyuran crabs, the posterior gills are predominantly specialised for osmoregulation, whereas the anterior gills are predominately involved in gas exchange (reviewed by Péqueux, 1995; Lucu and Towle, 2003; Tresguerres et al. 2008). This distinction is reflected with morphological differences where posterior gills have thicker epithelium, greater distribution of mitochondria, presence of ion transport cells as well as greater increases in transport activity as a function of salinity (Towle and Weihrauch, 2001). On each gill lamellae there are two sides, the basolateral membrane which faces the haemolymph and the apical membrane, which faces the environment. Accordingly, ions are transported from the haemolymph to the gills via basolaterally located exchangers, and apically located exchangers then transport these ions to the environment, or vice versa (depending on the salinity condition and the transport mechanism involved) (Péqueux, 1995; Towle and Weihrauch, 2001; Lucu and Towle, 2003).

Although the exact mechanisms of ion transport are still not completely understood and the location of exchangers cannot always be generalised to all crustaceans, the main mechanisms known to date are: Na^+/K^+-ATPase (exclusively basolaterally located), Na^+, K^+, $2Cl^-$ co-transporters (basolaterally and apically located for secretion and absorption, respectively), Cl^-/HCO_3^- (apically located), Na^+/NH_4^+ (apically located), Na^+/H^+ (apically located) and vaculor-type (V-type) H^+-ATPase (apically located) (Weihrauch et al. 2004; Tsai and Lin, 2007) (Figure 3). Often, studies used to investigate these processes utilise prefused gills, apply known inhibitors to the targeted channel and then measure potential electrical differences to determine their importance (and extent) for ion transport. Among these transport mechanisms, ouabain sensitive Na^+/K^+-ATPase activity is by far the most studied since it is often the most dominant mechanism for regulating Na^+, K^+ and Cl^- ions (and among active transport, 80 % of total ATPase activity is due to this mechanism) (Castilho et al. 2001), it fuels other transport mechanisms (Henry, 2001) and, in addition, ATPase dependent ion transport is

perhaps the most easy to measure. The principle of this process in crustaceans is as follows: 2 K^+ ions are transported outwards from the haemolymph, into the cytoplasm, in exchange for 3 Na^+ ions inwards to the haemolymph at the expense of ATP hydrolysis (Péqueux, 1995). Due to this unequal electrochemical gradient, since more Na^+ ions enter than K^+ ions causing the haemolymph side to become positive, results in Cl^- uptake via Cl^- channels on the basolateral membrane (Riestenpatt et al. 1996). Therefore, Na^+/K^+-ATPase activity directly and indirectly regulates haemolymph monovalent ions of Na^+, K^+ and Cl^-, which are the main ions that contribute to osmolality (approximately 90 % of the total haemolymph ions in crustaceans) (Péqueux, 1995). It is unclear if this process is reversible, however, it has been tentatively suggested (McLaughlin et al. 1996; Lucu and Towle, 2003; Towle pers. com.) and will be discussed later (page 13). Due to the continual excretion of K^+, which could potentially lead to haemolymph hypokalemia (*i.e.* reduced haemolymph K^+) which is well known to be lethal to crustaceans (Romano and Zeng, 2007c), it is believed that K^+ is "recycled" via a K^+ "leak" pathway (or K^+ channels) on the basolateral membrane or may provide the K^+ source for apically located Na^+, K^+, $2Cl^-$ co-transporter (Riestenpatt et al. 1996). For the Na^+, K^+, $2Cl^-$ co-transporter, two types exists, one that secretes ions (on the basolateral side) and one that uptakes these ions (on the apical side). Less is known regarding this transport mechanism, and most studies have focused on identifying the location and presence of this mechanism under different environmental conditions. However, it is believed that all three ions are electrochemically neutral and transported in one direction (depending on the location), does not require ATP and plays a lesser role in ion transport than other transport mechanisms, particularly for more marine species (Lucu and Towle, 2003) (Figure 3).

While Na^+/K^+-ATPase activity is the main driving force for ion transport in estuarine/marine crustaceans, this is believed to be insufficient for Na^+ uptake in freshwater

crustaceans exposed to dilute media (Tsai and Lin, 2007). In this case, V-type H^+-ATPase activity creates an electrochemical gradient by transporting H^+ to the environment, thereby allowing environmental Na^+ uptake, via apically located Na^+/H^+ transportors. Meanwhile, this also creates a HCO_3^- gradient therefore enhancing Cl^- absorption from the environment via apically located HCO_3^-/Cl^- exchangers (Genovese et al. 2005). Freswhater crustaceans utilise this trasnporter for osmoregulation and is apically located, wherease euryhaline/marine crustaceans likely utilise this more for acid/base balance and ammonia-N excretion and this enzyme is cytoplasmically located (Weihrauch et al. 2001; Genovese et al. 2005; Tsai and Lin, 2007).

Na^+ and Cl^- ions are also regulated, indirectly, during respiration. When CO_2 is produced from crustaceans, this combines with water to form carbonic acid (H_2CO_3), which then dissociates to HCO_3^-. It is the stable HCO_3^- (an amphoteric species) that is the main form in which CO_2 is then transported throughout the haemolymph of crustaceans. This reversible hydration/dehydration is catalysed by the zinc containing enzyme carbonic anhydrase (CA) on the gills in the following formula of $H^+ + HCO_3^- \leftrightarrow CO_2 + H_2O$ (Henry, 2001). This enzyme is located on both the anterior and posterior gills, however, CA activity is influenced by salinity on the posterior gills (being more involved for osmoregulation), whereas CA activity on the anterior gills is salinity independent (and therefore involved in gas exchange and acid/base balance) (Henry, 2001; Genovese et al. 2005). In order to excrete CO_2 to the environment for respiration, membrane associated CA (on the basolateral membrane) converts HCO_3^- back to CO_2, which more readily diffuses across the gill membrane. However, cytoplasmic CA (located mostly on the posterior gills) is salinity dependent since it is responsible in hydrating CO_2 back to H^+ and HCO_3^- which can be used as an anti-porter for Na^+ and Cl^- via apically located Na^+/H^+ (or V-type H^+-ATPase) and Cl^-/HCO_3^- transportors, respectively (Henry, 1988;

Genovese et al. 2005; Serrano et al. 2007). The transport of H^+ and HCO_3^- not only functions as anti-porters for Na^+ and Cl^- ions but also for pH regulation in crustaceans (Henry, 2001; Serrano et al. 2007). It should be noted that unlike Na^+/H^+ transport which is electroneutral in vertebrates, it is believed that two Na^+ ions are exchanged for one H^+ in crustaceans (Towle and Weihrauch, 2001) (Figure 3). Furthermore, while most CA activity for marine crustaceans occurs on the gills, CA activity is higher in the antennal gland for freshwater crayfish (Henry, 2001). Figure 3 provides a diagram for a proposed model for ion exchange in crabs including when NH_4^+ can substitute for K^+ during ammonia-N excretion.

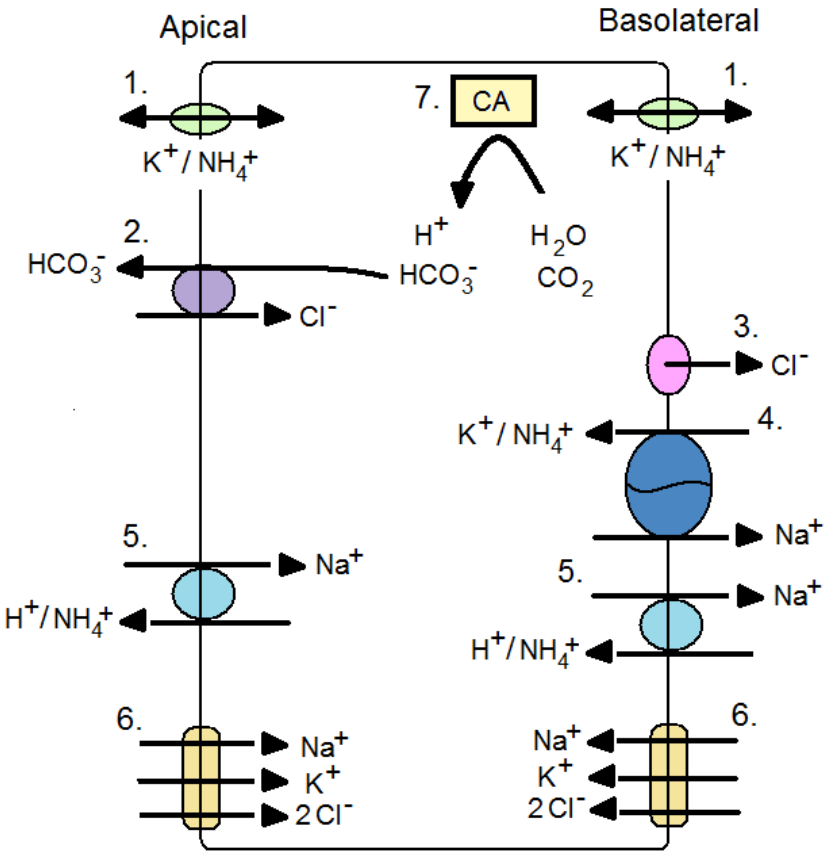

Figure 3: The transporters and enzymes involved in ion transport in the gills of crustaceans, modified from Towle and Weihrauch (2001) and Weihrauch et al. (2004). The direction of ions will vary depending on salinity and the localization of transporters which may vary between species. Note: often NH_4^+ can substitute for K^+ in ion transport. (1) K^+ channels; (2) HCO_3^-/Cl^- exchanger; (3) Cl^- channels; (4) Na^+/K^+-ATPase; (5) Na^+/H^+ exchanger; (6) $Na^+/K^+/2Cl^-$ co-transport; (7) carbonic anhydrase (CA).

At hypo-osmotic conditions, estuarine/marine crustaceans generally increase CA and Na^+/K^+-ATPase activity to increase ion uptake for haemolymph ion homeostasis (Holliday, 1985; Piller et al. 1995; Castilho et al. 2001; López-Mañanes et al. 2002; Genovese et al. 2004, 2005; Torres et al. 2007; Tsai and Lin, 2007; Lucu et al. 2008; Huong et al. 2010). It has been previously demonstrated that gill Na^+/K^+-ATPase activity can significantly increase during short-term exposure (hours to days) or long-term exposure (weeks to years) at low salinities (Palacios et al. 2004a; Lucu et al. 2008). It is believed that long term exposure (weeks to a year) leads a *de novo* synthesis of new proteins and a cellular differentiation of pre-existing cells to ion-transporting cells (Lucu and Towle, 2003; Torres et al. 2007). The environmental cues responsible for triggering gill Na^+/K^+-ATPase activation were determined when Lucu et al. (2008) subjected the shore crab, *Carcinus aestuarii*, to three salinity conditions of 10 ‰ (hypo-osmotic), 38 ‰ (hyper-osmotic) and a third treatment that was originally 10 ‰, but a non-electrolyte sucrose was added to increase the "salinity" to 38 ‰. Interestingly, gill Na^+/K^+-ATPase activities were similar between salinities of 38 ‰, with or without sucrose, whereas at 10 ‰ the gill Na^+/K^+-ATPase activity significantly increased. This therefore demonstrated that decreases in external osmolality, rather than NaCl concentrations (which are the dominate ions in seawater), triggers gill Na^+/K^+-ATPase activity in crustaceans (Lucu et al. 2008).

Investigations regarding ion transport at hyper-osmotic conditions (or hypo-osmoregulation) in estuarine/marine crustaceans are substantially less, and therefore not as well understood (Péqueux, 1995; Roy et al. 2007; Freire et al. 2008). Furthermore, unlike the response to hypo-osmotic conditions, gill CA and/or Na^+/K^+-ATPase activity in aquatic crustaceans at hyper-osmotic conditions is more variable having been shown to increase (Kamemoto, 1991; Holliday et al. 1990; McLaughlin et al. 1996), decrease (Castiho et al. 2001;

Torres et al. 2007) or have no significant change (Genovese et al. 2004, Chung and Lin, 2006; Lucu et al. 2008) when subjected to such conditions. Torres et al. (2007) suggested that decreased gill Na^+/K^+-ATPase activity at hyper-osmotic conditions is likely a response to decrease in Na^+ transport into the haemolymph since reduced Na^+ absorption would be beneficial for crustaceans in a high salt environment. In contrast, Kamemoto (1991) and McLaughlin et al. (1996) both demonstrated that gill Na^+/K^+-ATPase activity in crabs can increase at hyper-osmotic conditions. Although ion transport direction was not determined in these latter studies, but only the activity, the results led to the tentative suggestion that Na^+/K^+-ATPase activity could be involved in the active Na^+ excretion to the environment (McLaughlin et al. 1996; reviewed by Lucu and Towle, 2003). Further evidence was found when Martinez et al. (1998) demonstrated with the mangrove crab, *Ucides cordatus*, that among 7 pairs of gills, Na^+/K^+-ATPase activity from gill 5 increased at hypo-osmotic conditions whereas Na^+/K^+-ATPase activity from gill 6 increased at hyper-osmotic conditions leading to the suggestion that gill 5 and 6 were responsible for NaCl uptake and excretion, respectively. In another study, hyper-osmotic conditions of 45 ‰ caused the expression of Na^+/K^+-ATPase α-subunit on gill pair 7 (out of a total of 9) for the shore crab, *Pachygrapsus marmoratus*, and was suggested that this gill pair secretes ions at high salinities (Jayasundara et al. 2007). While evidence from both Martinez et al. (1998) and Jayasundara et al. (2007) suggest gill Na^+/K^+-ATPase activity can operate in reverse, this has not yet been proven (Towle pers. comm.). Similarly, CA activity of *Litopenaeus vannamei* increased at hyper-osmotic conditions of 45 ‰ also led to the suggestion that this may be responsible for NaCl secretion to the environment (Roy et al. 2007). However, again, since only the total activity (or acidity) was measured but not ion direction, this is considered as still speculation.

A seemingly more common and supported response of estuarine/marine crustaceans, to counter hyper-osmotic conditions, is the production or release of "non-essential" free haemolymph amino acids (FAA) (an organic osmolyte) (Gerard and Gilles, 1972; Dalla Via, 1986; Bishop and Burton, 1993; Silvia et al. 2004; Roy et al. 2007). The reason is that increased haemolymph FAA content leads to increased haemolymph osmotic pressure in the animal thereby decreasing ion diffusion from the environment across the gills (Gerard and Gilles, 1972; Roy et al. 2007). However, an exception to this response has been reported for the Australian freshwater crayfish, *Cherax destructor* (Dooley et al. 2000) and may be linked with the utilisation of differing strategies for freshwater species than estuarine/marine crustaceans.

An alternative strategy to regulate inorganic (*i.e.* ions) or organic (*i.e.* free amino acids) osmolytes is by altering gill permeability, which are the main boundary structures for crustaceans, and is otherwise known as a limiting process. However, generally, less is known regarding this process.

1.4. Osmoregulation – limiting process

An important distinction between crustaceans and other aquatic animals such as fish, is possessing a hard calcified shell which have osmoregulatory implications. Although this shell is not completely impermeable, and the permeability qualities certainly increase during molting, the location of the most ion diffusion occurs across the gill membranes rather than the body (Péqueux, 1995; Rasmussen and Anderson, 1996). Therefore, decreasing gill permeability can be a highly effective method to reduce ion diffusion and water influx rather than solely relying on the more energetically demanding mechanisms of ion transport. Indeed

this is believed to be a crucial mechanism that facilitates the successful long term inhabitation of crustaceans in more diverse environments (Péqueux, 1995).

It has been previously demonstrated in various crustaceans including the Atlantic ditch shrimp, *Palaemonetes varians*, the brown shrimp, *Crangon crangon*, the deep water rose shrimp *Parapenaeus longirostris* (Campbell and Jones, 1990), the shore crab, *Carcinus maenas*, the velvet swimmer crab, *Necora puber* (Rainbow and Black, 2001) and the blue crab *Callinectes sapidus* (Li et al. 2006) that gill permeability can decrease (either short term or long term) in response to low salinity exposure. However, the degree of decreasing permeability is largely species-specific, and furthermore, such a response is not universal. For example, Rainbow and Black (2001) detected no permeability change of the Chinese mitten crab, *Eriocheir sinensis*, when exposed to low salinities and suggested that this species, which is perhaps one of the most euryhaline crustaceans, had a permeability sufficiently low enough that a further decrease would not lead to any advantage.

The mechanisms involved in decreasing gill permeability during short term exposure to osmotically stressful conditions is believed to be the result of closing pore membranes while a long term adaptation are gill membrane fatty acid compositional changes (Morris et al. 1982). In the case of the latter, it has been demonstrated that cell membranes that contain a high amount of long chain saturated fatty acids, such as linoleic acid (18:2*n*-6), have decreased ion and water permeability whereas those with a higher unsaturation index (*i.e.* higher double bonds) and *n*-3 group of polyunsaturated fatty acids (PUFA) have increased ion and water permeability (Morris et al. 1982; Porter et al. 1996). Since the gill (and body) content of freshwater animals (or estuarine animals subjected to lower salinities) often have substantially less PUFA and longer chain fatty acids than marine animals may explain the general phenomenon of the substantially low permeability of freshwater crustaceans (Rainbow and

Black, 2001; Hurtado et al. 2007). For example, water and Na^+ permeability in freshwater crustaceans is between 50 to 500 fold less, respectively, than in marine crustaceans (Péqueux, 1995) which often leads to a 10 fold osmotic difference between the haemolymph and environment for crustaceans living in freshwater compared to marine crustaceans living in seawater. Consequently, such a difference for freshwater crustaceans would necessitate a less energetically costly method for long term hyper-osmoregulation which may be accomplished by decreasing gill permeability via gill lipid compositional changes (Rasmussen and Andersen, 1996).

Changes to the lipid composition of the gill basolateral membrane, which faces the haemolymph, has been suggested to also influence Na^+/K^+-ATPase activity. However much of the literature has focused on freshwater fish transitioning to saltwater. For example, since the transition of the Atlantic char, *Salvelius alpinus*, from freshwater to saltwater is accompanied with an 8 fold increase to gill Na^+/K^+-ATPase activity, but only a 3 fold increase in the membrane protein number (*i.e.* transporting sites), prompted questions as to whether lipid changes could influence the efficiency of enzymatic activity (Bystriansky and Ballantyne, 2007). A correlation was subsequently demonstrated between increased gill Na^+/K^+-ATPase activity as the cholesterol level decreased and the percentage phosphatidylethanolamine (PE) and linoleic acid (18:2n6) increased on the basolateral membrane (Bystriansky and Ballantyne, 2007). Similarly, in another study, PE of the whole gill tissue increased at increasing salinities in the saltwater acclimated trout, *Oncorhynchus mykiss*, which was suggested to enhance ion transport via the stabilisation of membrane bound proteins (Hansen et al. 1995). In contrast, for the saltwater acclimated American eel, *Anguilla rostrata*, increased gill Na^+/K^+-ATPase activity was not accompanied with any basolateral membrane lipid changes (Crockett, 1999). However, since the apical membrane lipid composition was not measured (due to inherent

difficulties in measuring apical enzyme markers) Crockett (1999) suggested that this may have a role in salinity adaptation such as lipid changes to decrease permeability. In another study on the closely related European eel, *A. anguilla*, the lipid composition of whole gill content did not change when subjected to long term exposure to either low or high salinities despite increased gill Na^+/K^+-ATPase activity (Hansen and Grosell, 2004). It seems reasonable to assume that these contradictory results may indicate species-specific differences, between freshwater and marine species, and perhaps even differing roles and importance of compensatory and limiting processes in different species.

1.5. Alterations/disturbances to osmoregulation

Now that the mechanisms of osmoregulation have been described, changes to this process can be more clearly interpreted when measuring haemolymph osmolality, ions, ammonia-N or gill Na^+/K^+-ATPase activity and ammonia-N excretion. The two factors of high aquaculture importance that not only influence, but are intimately linked, with this process are nutrition and ammonia. These two will be separately described in the following paragraphs and will be the main focus for experimentation throughout this thesis.

1.5.1. Nutrition

It is well known that osmoregulation requires energy in the forms of protein (Rosas et al. 1999; Setiarto et al. 2004; Silvia et al. 2004) and lipids (Lemos et al. 2001; Luvizotto-Santos et al. 2003; Palacios et al. 2004b; Sang and Fotedar, 2004), which can contribute to reduced survival and growth of crustaceans at osmotically stressful conditions. Therefore, it seems reasonable to assume a dietary modification approach could potentially be an effective method to improve osmoregulation, and therefore survival and growth, at sub-optimal salinity levels.

Although this area is only recently being explored, initial research was focused on improving the ability of crustaceans to withstand sudden salinity shock tests (SSST) after being fed diets supplemented with either highly unsaturated fatty acids (HUFA) (Palacios et al. 2004b) or phospholipids (PL) (Coutteau et al. 1996; Camara et al. 1997; Wu et al. 2007). The rationale for using these nutrients is HUFA can potentially modulate the fatty acid composition on the gill membrane (Hurtado et al. 2007), gill surface area and gill Na^+/K^+-ATPase activity (Palacios et al. 2004) while phospholipids (PL) are integral for cell membrane stability, which could potentially reduce gill permeability (Coutteau et al. 1996). Furthermore, and importantly, these lipids either cannot be synthesized *de novo* by crustaceans or to the degree necessary to satisfy their metabolic needs (Palacios et al. 2004b; Coutteau et al. 1996). Often, although not always, dietary HUFA supplementation improves survival to SSST, however, since these tests are primarily designed to test animal quality at the previous culture salinity level, but not their long term performance at osmotically stressful conditions (reviewed by Palacios and Racotta, 2007), caution needs to be exercised when interpreting these data. More recently it was determined that following SSST, *L. vannamei*, post-larvae that were fed diets containing elevated dietary HUFA levels had enhanced gill Na^+/K^+-ATPase and carbonic anhydrase activity (Palacios et al. 2004). Subsequently the same authors then conducted experiments to determine if HUFA supplementation can improve survival, growth and osmoregulation of the animal subjected to chronically low (5 ‰) and high (50 ‰) salinities (Hurtado et al. 2006, 2007). Interestingly, it was shown that high dietary HUFA supplementation was only successful at increasing growth rates of *L. vannamei* at high salinities (Hurtado et al. 2006). Furthermore, in a similar study by the same authors, no significant dietary effect was detected on the haemolymph osmolality, haemolymph FAA, gill Na^+/K^+-ATPase activity or gill water content (Hurtado et al. 2007). These seemingly contradictory results were explained by an

enhanced limiting process (Hurtado et al. 2007), however, their data did not appear to fully support such a suggestion which will be discussed in more detail in Chapter 7.

Although studies on dietary PL to crustaceans at different salinities have largely been limited to SSST (Camara et al. 1997; Wu et al. 2007), Roy et al. (2006) investigated whether dietary PL can improve survival and growth of *L. vannamei* at low salinities characterised with high Na^+/K^+ ratios. However, it was determined that no dietary benefit of PL on the survival or growth of *L. vannamei* (Roy et al. 2006) which may be related to their strong osmoregulatory abilities (leading to no further improvement) or the unique culture conditions of the water with unusually high Na^+/K^+ ratios. Although there obviously appears to be limitations to a dietary modification approach to chronic salinity (or ion) stress, previous research has mainly been conducted on one species (*i.e. L. vannamei*) with a high osmoregulatory ability (Castille and Lawrence, 1981). For other species with weaker osmoregulatory abilities the results may be quite different since there may be a greater potential for improvement and it appears to be an area for further research.

1.5.2. Ammonia

While dietary modifications may improve osmoregulation, other factors of aquaculture importance such as temperature, pH, ammonia, nitrite, nitrate and potassium can also reduce the osmoregulatory abilities of the cultured animals (Péqueux, 1995; Romano and Zeng, 2007a,b,c). However, among these factors, ammonia-N (with "ammonia-N" referring to both NH_4^+ and NH_3) is arguably the most important for a number of reasons. Firstly ammonia-N is perhaps one of the most limiting factor in closed aquaculture systems since this toxicant is the first stage of the nitrification cycle, and among the nitrogenous pollutants, is often the most toxic (Meade and Watts, 1995; Romano and Zeng, 2007a,b,c; Romano and Zeng, 2009a).

Furthermore, in addition to ammonia-N being a ubiquitous concern for aquaculturists as well as being ecologically relevant in aquatic ecosystems (Rebelo et al. 1999; Dave and Nilsson, 2005), the mechanisms involved to actively regulate haemolymph ammonia-N levels are intimately linked with osmoregulation via gill Na^+/K^+-ATPase activity (Weihrauch et al. 2004). Since these mechanisms of aquatic crustaceans are predominately performed on the gills (Weihrauch et al. 2004), which are likely more vulnerable to aquatic pollutants due to their constant contact with the external medium (Romano and Zeng, 2009b), investigations on ammonia-N induced gill damage may provide insight to modes of ammonia-N toxicity. Finally, since benthic crustaceans can experience heightened levels of localised ammonia-N, with levels in ocean sediments reaching as high as 39.2 mg l^{-1} due to the high organic content (Weihrauch et al. 1999) along with the continual excretion of metabolic by-products led to the suggestion this gave rise to the ability to actively excrete ammonia-N (Weihrauch et al. 2004). Prior to discussing the role of ammonia-N on osmoregulation, a brief description of its characteristics and source in an aquaculture settings will be provided first.

Ammonia-N is produced from the decomposition of organic matter (*e.g.* uneaten food) and from the branchial excretion of animals (Timmons et al. 2002; Weihrauch et al. 2004). In an aquaculture setting, the continual production of ammonia-N often requires the use of biofilters to convert ammonia-N to the less toxic form of nitrate and/or periodic water exchanges to alleviate its accumulation (Timmons et al. 2002). Although these procedures can be effective in minimising ammonia-N accumulation, it is costly and periodic spikes as well as elevated chronic levels may still occur on closed aquaculture systems which can have significant productivity implications (Timmons et al. 2002). Ammonia exists in two forms, the unionized NH_3 and the ionized NH_4^+ and it is the unionized form that is considerably more toxic since it can diffuse across the lipid bilayers of gills more easily (Evans and Cameron,

1986). These ratios of NH_3 and NH_4^+ can be quantified based on salinity, temperature and pH level in the water (Whitfield, 1974).

However, since benthic crustaceans can frequently experience elevated levels of localised ammonia-N (Weihrauch et al. 1999), it is believed that this common behavior necessitated an adaptive mechanism to remove excessive ammonia-N buildup in the haemolymph (Weihrauch et al. 2004). This process is believed to be predominately via active ammonia-N transport, which is linked with mechanisms of osmoregulation on both the anterior and posterior gills (Weihrauch et al. 2004) despite their specialization for osmoregulation and gas exchange, respectively. Based on the current model, as the more permeable NH_3 diffuses across the gills and into the haemolymph, NH_3 is protonated to NH_4^+ (Weihrauch et al. 2002; 2004). The K^+ ions from the haemolymph are then substituted for NH_4^+ via basolaterally located Na^+/K^+-ATPase activity and transported to the cytoplasma where NH_4^+ dissociates to NH_3 and H^+. NH_3 diffuses into vesicles, is acidified by V-type H^+-ATPase (thus preventing their diffusion outwards from these vesiciles) and NH_4^+ is transported to the apical membrane, via these vesicles, and excreted to the environment via apically located Na^+/ NH_4^+ transport in exchange for Na^+ ions. Due to the removal of H^+ ions in the haemolymph when NH_3 is protonated to NH_4^+, this can lead to an increased haemolymph pH at increasing environmental ammonia-N levels as observed for the mud crab *S. serrata* early juveniles (Romano and Zeng, 2007a). However, excessively high ammonia-N levels may disrupt this process causing haemolymph Na^+ reduction as reported for the American clawed lobster, *Homarus americanus* (Young-Lai et al., 1991), the Kuruma shrimp, *M. japonicus* (Chen and Chen, 1996), the burrowing crab, *Neohelice* (*Chagmagnathus*) *granulata* (Rebelo et al. 1999), the freshwater crayfish, *Pacifastacus leniusculus* (Harris et al. 2001) and *S. serrata* (Romano and Zeng, 2007a). Interestingly, while reduced haemolymph Na^+ levels were directly linked with reduced

haemolymph osmolality in *H. americanus* (Young-Lai et al. 2001) and *P. japonicus* (Chen and Chen, 1996) a similar phenomenon was not detected for *S. serrata* (Romano and Zeng, 2007a) since their osmolality remained unchanged indicating a possibility of this species to compensate for Na^+ loss by operating other ion transport mechanisms or to increase haemolymph free amino acids (FAA). For example, it was previously demonstrated that ammonia-N induced disruptions to ion transport caused a significant increase to haemolymph FAA in the black tiger shrimp, *Penaeus monodon* to maintain stable haemolymph osmotic pressure (Chen and Chen, 2000).

While the mechanisms between ammonia-N excretion and osmoregulation are linked, it has been routinely found that the ammonia-N tolerance of penaeid shrimp including *Penaeus penicillatus* (Chen and Lin, 1991), *P. chinensis* (Chen and Lin, 1992b), *P. semisculcatus* (Kir and Kumlu, 2006) and *L. vannamei* (Lin and Chen, 2001; Li et al. 2007) increase at increasing salinities. One exception to this pattern was detected for *Neohelice* (*Chagmagnathus*) *granulata*, since ammonia-N toxicity increased at both low and high salinities (Rebelo et al. 2000). However, since an acclimation period was absent in Rebelo et al. (2000)'s experiment, sudden osmotic shock may have contributed to their result. Lin and Chen (2001) suggested that higher ammonia-N toxicity at decreasing salinities was the result of higher ammonia-N intake at low salinities, leading to increased haemolymph ammonia-N levels. However no experiment has been performed to confirm this. Interestingly, however, in the absence of added ammonia-N, it was later demonstrated that haemolymph ammonia-N increases at increasing salinity levels for *M. japonicus* (Lee and Chen, 2003). This is likely a species-specific response since an opposite effect was obtained for higher decapods including the blue crab, *Callinectes sapidus*, (Magnum et al. 1976) and *S. serrata* (Chen and Chia, 1996) where haemolymph ammonia-N increased at low salinities. It was believed that, since no ammonia-N was added

and a gradient could not cause increased ammonia-N influx (or uptake) from the environment, this was the result of a deamination of haemolymph FAA at low salinities (Magnum et al. 1976; Chen and Chia, 1996) since, as previously mentioned, this strategy of an increase to haemolymph FAA is performed to increase osmotic pressure in most estuarine/marine crustaceans at high salinities (Gerard and Gilles, 1972; Roy et al. 2007). Furthermore, another potential cause for higher haemolymph ammonia-N levels at low salinities may be due to metabolic changes. It was demonstrated that at high salinities, *S. serrata* shifted nitrogen metabolism from ammoniotelism (*i.e.* production of haemolymph ammonia) to ureotelism (*i.e.* production of haemolymph urea) (Chen and Chia, 1996). Although these experiments were performed in ammonia-N free water at different salinities, and therefore the responses may be different if animals are exposed to elevated ambient ammonia-N levels, the studies by Chen and Chia (1996) and Chen and Chen (2000) may provide a clue to the relationship between ammonia-N toxicity and salinity. If, at high salinities, crustaceans convert haemolymph ammonia-N to urea (Chen and Chia, 1996) along with an increase of haemolymph FAA (Chen and Chen, 2000) than this may decrease the diffusion of ammonia-N to the haemolymph and once ammonia-N inevitably diffuses through (at a presumably lower rate) becomes converted to the less toxic urea at a greater rate. Although this is pure speculation, and the mechanisms regulating haemolymph ammonia-N and salinity levels are mixed and likely highly species-specific, no experiment at different ammonia-N and salinity combinations have been performed (*e.g.* Li et al. 2007).

Interestingly, Borgmann and Borgmann (1997) investigated the ions responsible for influencing ammonia-N toxicity by using the freshwater amphipod *Hyalella azeteca* as a model species for experiments at different Na^+ and K^+ levels. The results showed that while elevated Na^+ ions (from 0.1 mM to 10 mM at a K^+ level of 0.05 mM) increased the ammonia-N

tolerance over three fold, the greatest tolerance was obtained when the highest tested amounts of Na^+ and, especially K^+, were present at 10 and 0.5 mM, respectively. Although many physiological parameters (*e.g.* haemolymph ammonia-N/ions, ion transport, enzyme activity) were not measured to explain such a finding, Borgmann and Borgmann (1997) suggested this result may be due to the prevention of ammonia-N influx (since ammonia-N and hydrated K^+ have the same ionic radius) or increased active ammonia-N excretion via basolaterally located Na^+/K^+-ATPase (where NH_4^+ substitutes for K^+) and apically located Na^+/NH_4^+ transport.

Unfortunately since the experiment by Borgmann and Borgmann (1997), no similar toxicity tests have been conducted on either freshwater or estuarine/marine species, which obviously for the latter, may cause different results. Recently, however, numerous *in vitro* experiments with dissected gills revealed that increases to combined K^+ and ammonia-N can significantly increase gill Na^+/K^+-ATPase activity in the freshwater shrimp, *Macrobrachium olfersii* (Furriel et al. 2004), the swimming crab, *Carinus danae* (Masui et al. 2002; 2005) the hermit crab, *Clibanarius vittatus* (Gonçalves et al. 2006), the blue crab, *Callinectes ornatus* (Garçon et al. 2007) and the swimming crab, *Callinectes danae* (Masui et al. 2002; 2005; 2009). Evidence from these *in vitro* studies indicated that gill Na^+/K^+-ATPase activity increased to accommodate both their osmoregulatory and ammonia-N excretion requirements by either NH_4^+ binding to already available specific sites on the gill (Furriel et al. 2004) or that new binding sites for NH_4^+ became exposed (Garçon et al. 2007). Based on these findings, and if *in vitro* and *in vivo* conditions on gill Na^+/K^+-ATPase activity are representative of each other, may indicate osmoregulation and ammonia-N excretion would remain undisrupted. However, such comparisons, or even *in vivo* investigations, have not yet been performed and therefore appears to be an urgent area for research.

The ability of crustaceans to cope with elevated ammonia-N exposure is not only limited to their efficiency/rate of active ammonia-N excretion, but also to gill permeability which is a similar principle to a limiting process for osmoregulation. Using perfused gills, Weihrauch et al. (1999) performed a study on the effects of elevated ammonia-N levels on active ammonia-N excretion rates, gill permeabilities and then haemolymph ammonia-N from three crab species, *Cancer pagurus*, *Carcinus maenas* and *E. sinensis*. These crabs were chosen since they represented weak, medium and strong osmoregulators, respectively and were acclimated for one month to salinities of 35 ‰, 10 ‰ and 0.5 ‰, respectively. It was demonstrated that among all three crabs, the haemolymph ammonia-N levels were not significantly different from each other, however, the methods to accomplish ammonia-N regulation differed (Weihrauch et al. 1999). The gills of *C. pagurus*, followed by *C. maenas*, were significantly "leakier" to NH_4^+ than *E. sinensis*, and this was subsequently compensated by significantly higher rates of active ammonia-N excretion from both *C. pagurus* and *C. maenas* (Weihrauch et al. 1999). These authors suggested two potential explanations for this result. Firstly, that increased salinities would, in turn, increase gill permeability and therefore a greater reliance on active ammonia-N excretion would be required to counter increased ammonia-N influx. However, this suggestion is not entirely supported by the results since different species were exposed to one salinity level. Furthermore, this does not appear to explain the general pattern of increased ammonia-N toxicity at low salinities, presumabley due to increased ammonia-N uptake, observed in numerous crustaceans species (Chen and Lin, 1991; Chen and Lin, 1992b; Kir and Kumlu, 2006; Lin and Chen, 2001; Li et al. 2007). The second suggestion was that crabs exhibiting weak osmoregulatory abilities, and therefore high gill permeabilities, such as *C. pagurus*, need to rely on a stronger ability to excrete ammonia-N across a gradient, whereas crabs with strong osmoregulatory abilities and lower gill

permeabilities, such as *E. sinensis*, would simply rely on lower NH_4^+ gill permeability to prevent excessive ammonia-N build-up in the haemolymph. This latter suggestion appears more convincing and indicates species-specific differences which is more consistent with existing literature. However, experiments investigating various coping processes of the same species at different salinity and ammonia-N combinations may provide supporting evidence to either of the two suggestions.

1.6. Summary and reasons for using *Portunus pelagicus* as experimental animals

Throughout this literature review an attempt was made to explain the relevance of osmoregulation to aquaculture, the many processes/strategies involved, the factors that may influence this activity, and illustrate areas that warrant investigation. Furthermore, this crucial process for all aquatic crustaceans, regardless of salinity levels, can have complex interactions with a variety of factors including (but not limited to) nutrition and ammonia-N. Indeed, in the case of the latter, despite substantial research (*e.g.* Péqueux, 1995; Rasmussen and Andersen, 1996; Towle and Weihrauch, 2001; Lucu and Towle, 2003; Kirschner, 2004; Weihrauch et al. 2004; Forest and von Vaupel Klein, 2006; Kennedy and Cronin, 2007; Freire et al. 2008), many areas or processes are still not well understood while the effects of nutrition on osmoregulation are, at the best, limited.

The blue swimmer crab, *Portunus pelagicus*, appears to be a good candidate for experimentation since, despite the high commercial importance of this species throughout the Indo-Pacific region (Kangas, 2000; Otto et al. 2001; Muna, 2005; Setyadi and Suanto, 2005), limited information is available regarding their basic biology relevant to both the aquaculture and fisheries industry. Since, as previously mentioned, salinity and ammonia-N are important

environmental factors these will be separately investigated in Chapters 2 and 3, respectively to provide baseline information. In Chapter 2, investigations will be made to determine the optimal salinity level for the survival and growth of *P. pelagicus* juveniles over a broad salinity range as well as to quantify their osmoregulatory abilities. Chapter 3 will measure the acute ammonia-N tolerance of *P. pelagicus* through juvenile development and if any correlation in tolerance is linked with ammonia-N induced gill damage. Chapter 4 will examine if ammonia-N influences osmoregulation and pH balance of *P. pelagicus* juveniles, if any potential change is correlated with gill damage and if gill damage is eventually reversible. Chapters 5 and 6 will examine the effect of ammonia-N at either different salinity or K^+ combinations on osmoregulation, ammonia-N excretion and haemolymph ammonia-N of *P. pelagicus* juveniles to investigate their adaptive responses. Chapter 7 will deal with dietary highly unsaturated fatty acids (HUFA) and phospholipids (PL) to determine if these can improve the survival, growth and haemolymph ion maintenance of *P. pelagicus* juveniles at sub-optimal salinities. Finally, Chapter 8 will summarise the major findings of the experiments, how they are all related and their implications to the aquaculture and fisheries industry. In addition, it is believed that this thesis will also make significant contributions to the fields of crustacean physiology, nutrition and toxicology.

Chapter 2

The effects of salinity on the survival, growth and haemolymph osmolality of early juvenile blue swimmer crabs, *Portunus pelagicus*

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1. Introduction

To understand the efffects of salinity on *Portunus pelagicus* juveniles, it is first necessary to measure their survival, growth and, particularly, the osmoregulatory abilities of this species at different salinities that may be found in estuarine and/or aquaculture systems. While this experiment has obvious implications for aquaculture, fisheries and ecology, this Chapter will also provide the foundation for future experiments by determining what optimal salinities are required, which salinities will stress but not induce crab mortalities as well as their osmoregulatory abilities to place future findings within the context of this important characteristic.

The importance of salinity cannot be overstated since this is one of the most important abiotic and ubiquitous factors that can greatly influence both the productivity and physiology of aquacultured animals. Although generally most aquatic animals have a certain degree of euryhalinity (Péqueux, 1995), optimal salinity levels for survival and growth can be highly variable depending on the species (Rouse and Kartamulia, 1992; Bray et al. 1994; Kumlu and Jones, 1995; Kumlu et al. 2001; Soyel and Kumlu, 2003; Ruscoe et al. 2004). Furthermore, osmotic stress can lead to other underlying and undesirable consequences such as increased respiration (Chen and Chia, 1996; Spanopoulos-Hernández et al. 2005) and increased ammonia-N excretion (Chen and Lin 1994b; Lemos et al. 2001; Silvia et al. 2004; Shinji et al.

2009) which may, in a closed system, affect water quality. Therefore, determining the optimal salinity level for a targeted aquaculture species will likely benefit the industry

Determining the optimal salinity range of animals is often achieved by evaluating their survival and growth, however, measuring the haemolymph osmolality can be a powerful tool in understanding and quantifying the osmoregulatory abilities of crustaceans at various salinity levels (reviewed by Lignot et al. 2000). Currently no laboratory based experiments have been performed concerning the effects of salinity on early *P. pelagicus* juveniles, however, there is evidence to suggest they are sensitive to hypo-osmotic conditions. For example, it has been reported that a mass emigration from estuaries occurs immediately preceding the wet season in Western Australia (Lestang et al. 2003a,b). While this evidence is anecdotal and may be related to other factors, it does provide a solid basis for investigation to clearly due to the many implications to ecology, aquaculture and fisheries. The experiments were hence conducted and designed to measure the effects of salinity on the survival, growth and haemolymph osmolality of early *P. pelagicus* juveniles.

2. Material and Methods

2.1. Source of crabs

Blue swimmer crab broodstock were caught form estuaries in Townsville, north Queensland, Australia using baited traps. The broodstock was then labeled and maintained in outdoor 1,000-L oval recirculating tanks (temperature maintained at 28 ± 2°C and a salinity of 32 ± 2 ‰) at the Marine Aquaculture Research Facility Unit (MARFU), James Cook University. Each day the broodstock were fed to satiation on an alternation of prawns, mussels and squid. When a berried female (*i.e.* crab that has visible eggs on the abdomen) was observed it was disinfected in a static formalin bath (concentration 50 µl l^{-1}) for 6-h.

After disinfection, the berried female was transferred indoors and individually held in a 300-L tank for hatching at a temperature of 26 ± 1°C and salinity of 34 ± 1.5 ‰. The tank water was aerated and continually circulated through 3 cartridge filters (10, 5 and 1 µm) and a UV sterilizer. The berried female was not fed and faeces and discarded eggs were daily siphoned from the tank and accompanied with a 10 % water exchange.

Upon hatching the aeration was removed and the active larvae were siphoned from the hatching tank. Larvae were stocked at approximately 500 larvae L^{-1} and cultured at a salinity of 25 ‰ and temperature of 29 ± 1°C. Newly hatched larvae were initially fed rotifers (*Branchionus* sp.) at 20-40 individuals ml^{-1} and the rotifer density was maintained by daily additions of microalgae *Nannochloropsis* sp. (concentration of approximately 3 -5 million cells ml^{-1}). From the Zoea II stage onwards, *Artemia* (*INVE, AAA*) nauplii were hatched and daily added to the larval tanks, without enrichment, and the *Artemia* density was successively increased from an initial 1-2 individuals ml^{-1} to 3-5 individuals ml^{-1} by the time the megalopae stage was reached. Daily water exchanges were initially between 10 and 15 % at the Zoea I and II stages, respectively and then increased to 20-50 % from the Zoea III stage onwards.

Upon larval metamorphosis to the first crab stage (C1) they were transferred to two 300-L recirculating holding tanks at a temperature of 27 ± 2°C and salinity of 32 ± 2 ‰. All crabs were fed a combination of frozen *Artemia* nauplii and ongrown adults and gradually weaned onto a formulated crumble feed produced by Ridley (Australia) designed for the black tiger shrimp, *Penaeus monodon* (43 % protein, 6 % fat, 3 % fibre). They were then exclusively fed the shrimp feeds until the experiment commenced.

2.2. Experimental design and set up

Two separate salinity experiments with similar design and set-ups were conducted for 45 days in succession from the 5th and 6th spawning from the same female, respectively. The salinity treatments in the first experiment were 10, 15, 25 and 40 ‰ and for the second experiment of 5, 20, 30, 35 and 45 ‰. Each treatment was triplicated and each replicate consisted of 10 crabs individually cultured in round black plastic containers (diameter 16 cm × height 19 cm). The black containers had numerous 3.75 mm holes and were bathed in a 300-L tank (diameter 1.1 m × height 0.4 m) filled with 200-L of pre-adjusted seawater.

A total of 12 tanks (4 salinity treatments × 3 replicates) were used in the first experiment and 15 tanks (5 salinity treatments × 3 replicates) were used in the second experiment. All tanks were arranged in a random block in parallel rows underneath a shed area. A clear plastic lid was placed on top of each tank to reduce evaporation and heat loss and the water was vigorously aerated to maintain adequate dissolved oxygen (DO) levels and water mixing. A thermostat controlled immersion heater was used to maintain water temperatures above 26°C in each tank throughout the experiments and a natural photoperiod was maintained.

The salinity experiments commenced when the majority of crabs produced in the hatchery molted to the crab 4 stage (C4), approximately 2 weeks post settlement. Healthy and intact C4 juveniles were randomly chosen from the holding tanks and weighed (mean weight: experiment 1 = 0.027 ± 0.001 g and experiment 2 = 0.028 ± 0.001 g) prior to being individually placed in a black container. All containers were labelled to enable tracking of consecutive molts from each crab. Salinity acclimation involved a step-wise increase/decrease at a rate of 3 ‰ h^{-1} from the original salinity (32 ‰) through the addition of either condensed seawater (brine) or de-chlorinated freshwater.

At the early stage of the experiments, a 10 % daily water exchange was performed for each 300-L tank. However with the growth of the crabs, the water exchange was successively increased to approximately 80 % by the end of the experiment. The culture water used throughout the experiments were obtained from filtered natural source seawater (32 ± 2 ‰) and adjusted with either de-chlorinated freshwater or brine produced through evaporation. Every 2 weeks a 100 % water exchange was performed and all containers and tanks were sterilized with a chlorine solution.

Throughout the experiment, crabs were fed the Ridely shrimp feeds daily to satiation. Uneaten food, faeces and debris were daily siphoned and checks were made in the morning for any mortalities, including incidences of "molt death syndrome" (MDS). MDS was defined as a mortality resulting from an inability of a crab to completely shed the old exoskeleton during molting and diagnosed as an incomplete disengagement of the old shell from a dead crab. Successful molts were recorded for the intermolt period as well as the carapace length and width of the molt using a digital caliper (0.01 mm) (Mitutoyo, Japan). The carapace length was defined as the distance from the longest rostral spike to the abdomen and the carapace width was defined as the distance between the tips of the two lateral spines. To allow adequate water uptake and recalcification (Cadman and Weinstein, 1988) 4-day post molt crabs were measured for wet weights using a digital scale (Adventurer Pro, Australia). Prior to weighing, each crab was blotted dry with a tissue and placed on the zeroed scale in a small container filled with culture water.

At the end of the experiments, the final sizes of all surviving crabs were measured. Unfed intermolt crabs in each salinity treatment were sampled for osmolality in accordance with Lignot et al. (2000). To obtain haemolymph osmolality measurements, a syringe was inserted through the proximal arthodial membrane at the base of the right second walking leg.

Each aliquot of haemolymph was immediately analysed on a cryoscopic osmometer (Osmomat 030; Gonotec) for osmolality. For dry weight measurements, all crabs of at least 4-day post molt were placed in an oven at 60°C for 48-h and dry weight measurements were made using a digital scale (Adventurer Pro, Australia).

Every morning the salinity was checked with a hand refractometer (Iwaki, Japan) and adjusted if required. Every second day the pH (WP-80; TPS) and dissolved oxygen (DO) levels (WP-82Y; TPS) were digitally measured. Every week the ammonia levels were measured with a test kit (Aquarium Pharmaceuticals). Throughout both experiments the ammonia ranged from 0.001 to 0.1 mg l^{-1}, pH between 7.7 to 8.3 and the DO remained above 6 mg l^{-1}. Temperatures were measured with min./max. thermometers. The mean temperature during the first experiment was 30 ± 4°C and during the second experiment was 31 ± 4°C as the former was conducted during cooler months.

2.3. Data analysis

To assess differences in growth rates over time the carapace size or wet weight increases at each crab stage was obtained by subtracting the new carapace size/wet weight by the old carapace size/wet weight at each molt. The specific growth rate (SGR) of all surviving crabs was calculated using the following formula:

$$SGR = (\ln W_f - \ln W_0)/t \times 100$$

where SGR is the specific growth rate; W_f is the final size/weight; t is the days of culture; W_0 is the initial size or weight.

Final survival, SGR, mean intermolt period, carapace size and wet/dry weights of the juveniles at each salinity treatment were analysed using a one-way ANOVA after confirmation of normality and homogeneity of variance. Log or arcsine transformation of data was performed before further analysis whenever variances were not homogenous. If any significant differences were detected ($p < 0.05$), differences among treatments were identified using Tukey's HSD test (Zar, 1999). Direct comparisons of survival and growth data between the first and second experiment were not performed since these experiments were run at different times and the data could be compounded by batch variations. A linear regression was used to determine the relationship between the haemolymph and medium osmolality (mOsm kg^{-1}). The isomotic line was plotted as the point where the haemolymph osmolality equaled the medium osmolality. All statistical analysis was performed using SPSS statistical package version 11.0.

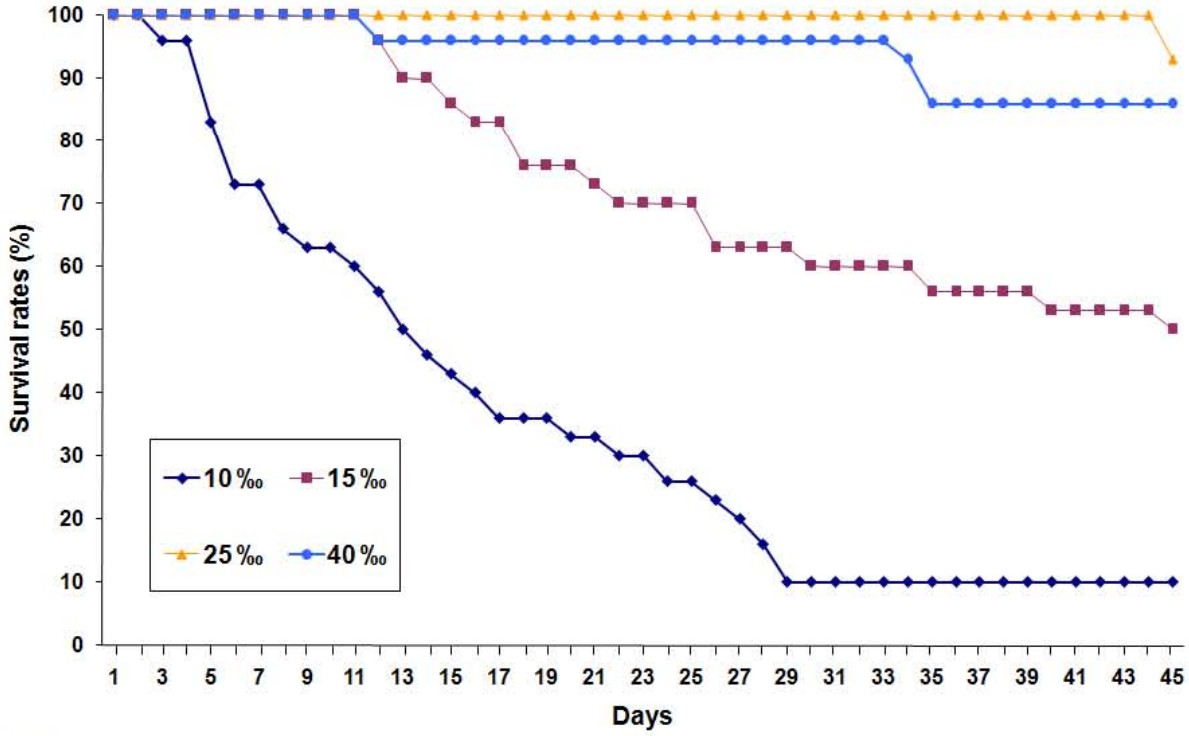
3. Results

3.1. Survival

The data from the two experiments showed that salinity significantly affected the survival of early *P. pelagicus* juveniles (Fig. 1). Among the 4 treatments in experiment 1, the survival rate at 25 ‰ was the highest (93.3 ± 6.7 %), followed by 40 ‰ (86.6 ± 8.8 %), 15 ‰ (50.0 ± 5.58 %) and 10 ‰ (10.0 ± 5.8 %) (Fig. 1A). Statistical analysis showed that the mortality rate was significantly higher ($p < 0.01$) at 15 ‰ than those of 25 or 40 ‰. No significant differences ($p > 0.05$) were detected between 25 and 40 ‰ (Table 1).

Among the 5 salinity treatments in experiment 2, the survival rate at 20 ‰ was the highest (93.3 ± 6.7 %) followed by 30 ‰ (63.3 ± 16.7 %), 35 ‰ (63.3 6.7 %), 45 ‰ (43.3 ± 3.3 %) and 5 ‰ (0 %) (Fig. 1B). A total morality occurred at 5 ‰ on day 20, which was significantly higher ($p < 0.01$) than any other treatment. The mortality rate was significantly

higher ($p < 0.01$) at 45 ‰ compared to 20 ‰, while the 30 and 35 ‰ treatments were not significantly different ($p > 0.05$) from either the 25 or 45 ‰ treatment (Table 1).



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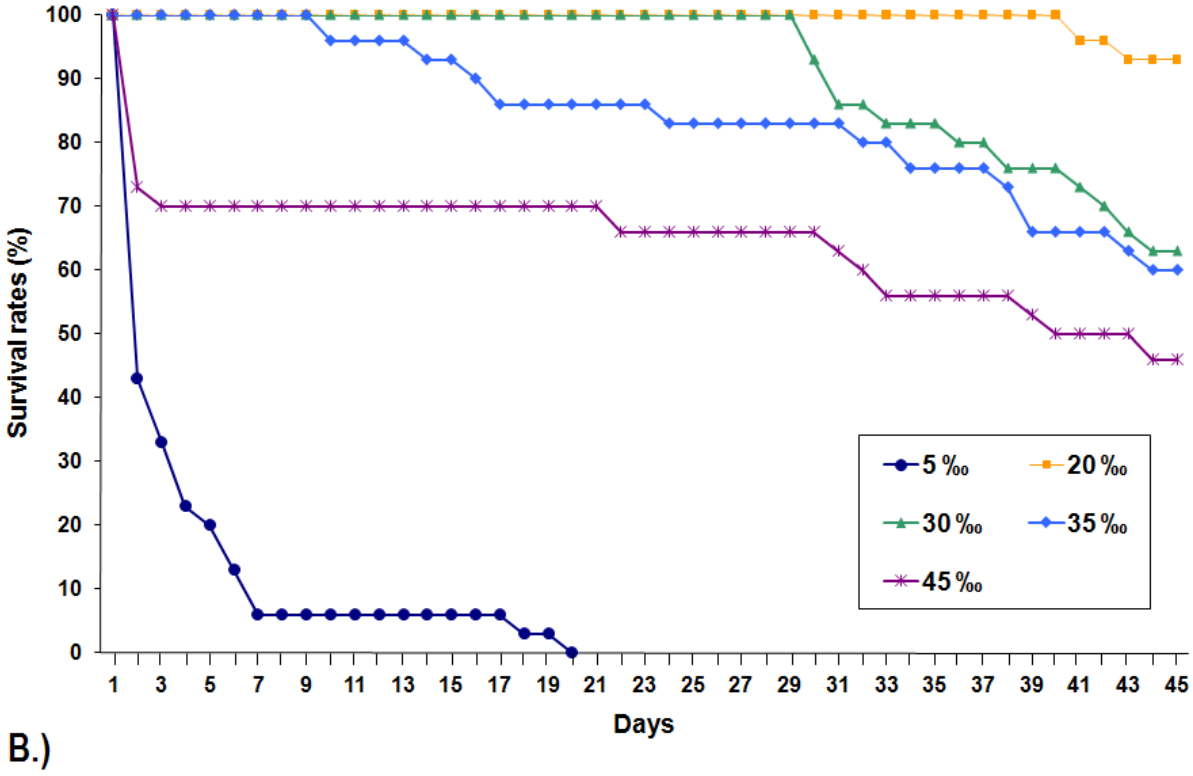

Figure 1: The survival of early *Portunus pelagicus* juveniles under various conditions in experiment 1 (A) and experiment 2 (B).

The majority of mortalities in the salinity treatments that occurred during the experiment were often due to "molt death syndrome" (MDS) (Table 1). The highest incidence of MDS occurred at 15 ‰.

Table 1: Incidences of "molt death syndrome" (MDS) and the total number of mortalities of early *Portunus pelagicus* juveniles in each salinity treatment.

Experiment 1	Incidence of MDS	Total mortality	Experiment 2	Incidence of MDS	Total mortality
Salinity (‰)			Salinity (‰)		
10	13	27 ^{c}	5	8	30 ^{c}
15	15	15 ^{b}	20	2	2 ^{a}
25	2	2 ^{a}	30	9	10 ^{ab}
40	4	4 ^{a}	35	11	12 ^{ab}
			45	7	16 ^{b}

* The different superscripted letters indicate significant differences ($p < 0.05$).

3.2. Growth

In experiment 1, the mean intermolt period of the C5 crabs cultured at 10 ‰ was significantly longer ($p < 0.01$) compared to those at 15, 25 and 40 ‰. However from the C6 stage onwards, no further statistical tests on growth or development could be performed for the 10 ‰ treatment due to a reduced number of surviving crabs. No significant difference in the mean intermolt period was detected ($p > 0.05$) among the 15, 25 and 40 ‰ treatments (Table 2). In experiment 2, the mean intermolt period at 45 ‰ was significantly longer ($p < 0.01$) at each crab stage compared to that of 30 ‰. It was also significantly longer ($p < 0.01$) than those at 20 and 35 ‰ at the C5 and C6 stage. No significant difference was detected ($p > 0.05$) among the 20, 30 and 35 ‰ treatments (Table 2). A complete morality occurred at 5 ‰ and there were no successful molts.

Table 2: The mean (± SE) intermolt period (days) of early *Portunus pelagicus* juveniles cultured at different salinities.

Crab stage				
Experiment 1	10 ‰	15 ‰	25 ‰	40 ‰
C5	10.6 ± 0.7 **b** *	8.5 ± 0.2 **a**	7.8 ± 0.2 **a**	8.4 ± 0.2 **a**
C6	7.5 ± 0.2 **	7.8 ± 0.2 **a**	7.7 ± 0.2 **a**	8.1 ± 0.2 **a**
C7	11.0 ± 0.5 **	8.8 ± 1.1 **a**	8.5 ± 1.1 **a**	8.7 ± 0.9 **a**
C8	n/a	10.0 ± 0.3 **a**	10.0 ± 0.3 **a**	10.0 ± 0.3 **a**
Experiment 2	20 ‰	30 ‰	35 ‰	45 ‰
C5	6.1 ± 0.1 **a**	5.4 ± 0.7 **a**	5.5 ± 0.7 a	7.4 ± 0.2 **b**
C6	6.6 ± 0.2 **a**	6.9 ± 0.2 **a**	6.9 ± 0.2 a	8.6 ± 0.5 **b**
C7	10.1 ± 0.4 **a,b**	9.6 ± 0.5 **a,b**	8.6 ± 0.3 a	10.8 ± 0.3 **b**
C8	9.7 ± 0.3 **a,b**	9.2 ± 0.3 **a,b**	9.2 ± 0.3 a	10.7 ± 0.2 **b**

* The different superscripted letters indicate significant differences ($p < 0.05$).
** Statistics not performed due to a reduced sample of surviving crabs.

The mean carapace length and width increase at the first molt (molting to the C5 stage) in experiment 1 was significanlty less ($p < 0.01$) at 10 ‰ compared to all other salinity treatments (Fig. 2A & B). However, no significant difference ($p > 0.05$) in the mean wet weight increase was detected (Fig. 2C). After the C5 to C6 stage the mean carapace size and wet weight increase was often significanlty less ($p < 0.01$) at 40 ‰ than at 25 ‰ (Fig. 2A-C). The mean carapace length and width increase at the C7 to C8 stage was significanlty less ($p < 0.01$) at 15 ‰ than at 25 ‰ (Fig. 2A and C).

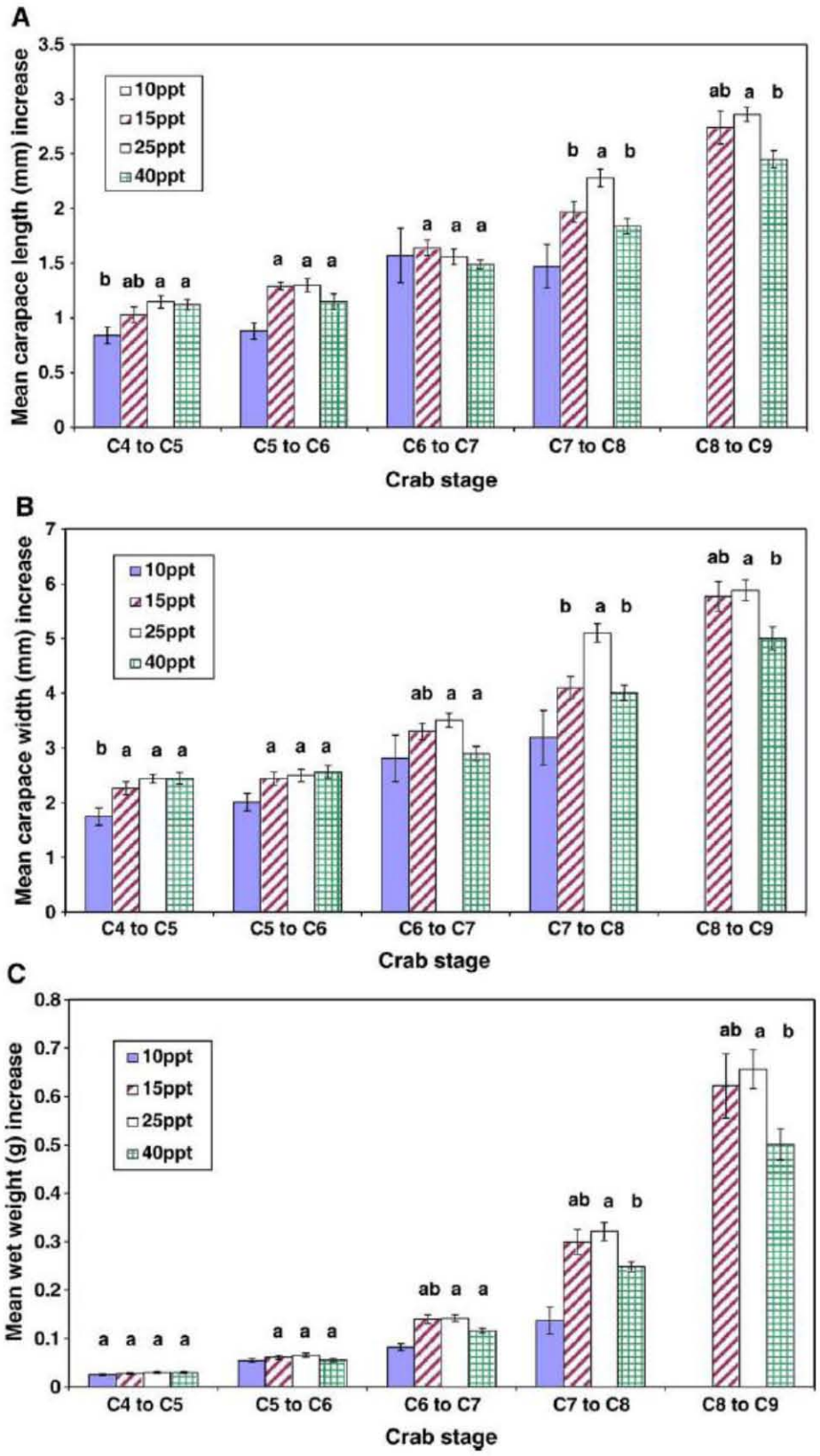

Figure 2: Mean increase in carapace length (mm) (A) carapace width (mm) (B) and the wet weight (g) (C) in early Portunus pelagicus juveniles cultured under different salinites in experiment 1. Statistical tests were not performed beyond the C6 stage at 10 ‰ due to a reduced sample size (n = 3). Different letters indicate significant differences ($p < 0.05$).

Among the salinity treatments in experiment 2, the mean carapace length and wet weight increase were the lowest at 45 ‰ for all crab stages and the differences from the other treatments were often significant ($p < 0.05$) (Fig. 3A & C). With the exception of the molt to the C4 to C5 and C5 to C6 stages, the carapace width increase at 45 Among the salinity treatments in experiment 2, the mean carapace length and wet weight increase were the lowest at 45 ‰ for all crab was significanlty less at each molt ($p < 0.01$) compared to those at 20, 30 and 35 ‰ treatments at any crab stage (Fig. 3A and C).

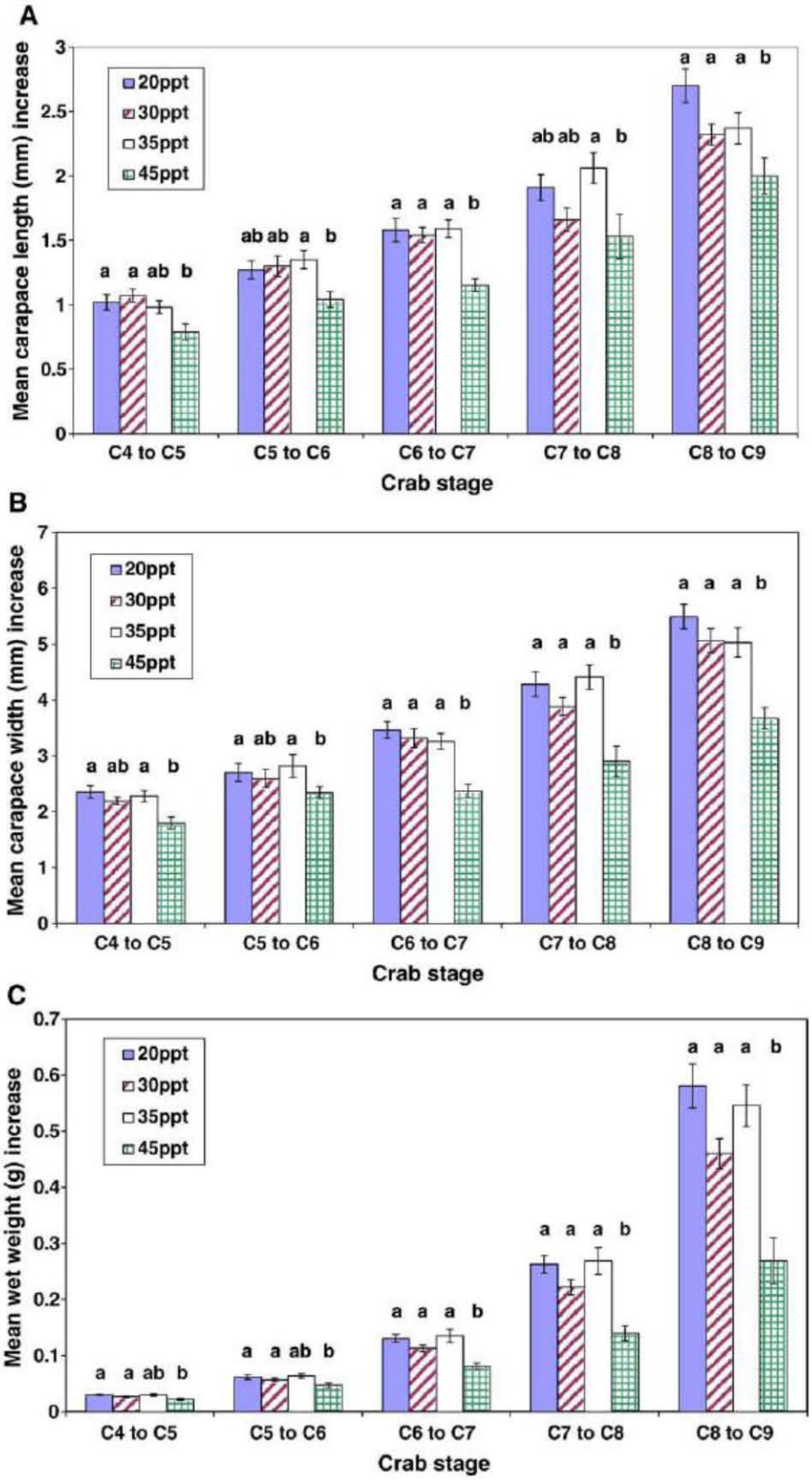

Figure 3: Mean increase in carapace length (mm) (A), carapace width (mm) (B) and the wet weight (g) (C) in early Portunus pelagicus juveniles cultured under different salinities in experiment 2. Different letters indicate significant differences ($p < 0.05$).

Among the 4 salinity treatments in experiment 1, no significant difference ($p > 0.05$) in the final dry weight was detected, although the percentage dry weight was significantly higher ($p < 0.05$) at 40 ‰ than at 15 ‰ (Table 3).

Among the 4 salinities tested in experiment 2, the final dry weight was significantly lower ($p < 0.05$) at 45 ‰ than at 20, 30 and 35 ‰. The final dry weight was significantly was significantly lower at 20 ‰ than at 35 ‰, while no significant differences ($p > 0.05$) were detected between 30 and 35 ‰. The percentage dry weight was not significantly different ($p > 0.05$) between all treatments of the experiment (Table 3).

Table 3: The final mean dry weight (g) (± SE) and percentage dry weights of early *Portunus pelagicus* juveniles cultured at different salinities.

Experiment 1				
Salinity	10 ‰	15 ‰	25 ‰	40 ‰
Final dry weight	0.080 ± 0.002 **	0.354 ± 0.044 ^{a}*	0.379 ± 0.029 ^{a}	0.309 ± 0.029 ^{a}
% dry weight	26.1 ± 2.1 **	27.9 ± 0.6 ^{b}	30.1 ± 0.7 ^{ab}	31.1 ± 0.7 ^{a}
Experiment 2				
Salinity	20 ‰	30 ‰	35 ‰	45 ‰
Final dry weight	0.295 ± 0.022 ^{b}	0.361 ± 0.028 ^{ab}	0.411 ± 0.056 ^{a}	0.130 ± 0.050 ^{c}
% dry weight	29.5 ± 0.8 ^{a}	28.9 ± 0.8 ^{a}	31.2 ± 1.0 ^{a}	31.2 ± 0.8 ^{a}

* The different superscripted letters indicate significant differences ($p < 0.05$).
** Statistics not performed due to a reduced sample of surviving crabs.

The specific growth rates (SGR) of carapace length, carapace width and wet weight in experiment 1 were the highest at 25 ‰ and lowest at 10 ‰, although the later treatment was not included in statistical analysis due to the low number of surviving crabs at the end of the experiment. The SGR for carapace length and wet weight was significantly lower ($p < 0.05$) at 40 ‰ than both the 15 and 25 ‰ treatments, while the SGR for carapace width was significantly less ($p < 0.05$) at 15 and 40 ‰ than at 25 ‰ (Table 4). In experiment 2, the SGR for carapace length, carapace width and wet weight was significantly less ($p < 0.01$) at 45 ‰

than those of the 20, 30 and 35 ‰ treatments, while no significant differences were detected among the 20, 30 and 35 ‰ treatments (Table 4).

Table 4: The mean specific growth rates (SGR ± SE) of the carapace length (mm), carapace width (mm) and wet weight (g) of early *Portunus pelagicus* juveniles cultured at different salinities.

Experiment 1				
Salinity	10 ‰	15 ‰	25 ‰	40 ‰
SGR carapace length	1.62 ± 0.12 **	2.60 ± 0.05 ^{a,b} *	2.75 ± 0.06 ^{a}	2.45 ± 0.05 ^{b}
SGR carapace width	1.94 ± 0.18	2.88 ± 0.05 ^{b}	3.14 ± 0.06 ^{a}	2.82 ± 0.05 ^{b}
SGR wet weight	5.081 ± 0.381 **	8.162 ± 0.110^{a,b}	8.614 ± 0.182 ^{a}	7.692 ± 0.134 ^{b}
Experiment 2				
Salinity	20 ‰	30 ‰	35 ‰	45 ‰
SGR carapace length	2.67 ± 0.07 ^{a}	2.81 ± 0.06 ^{a}	2.77 ± 0.08 ^{a}	2.04 ± 0.07 ^{b}
SGR carapace width	3.08 ± 0.08 ^{a}	3.18 ± 0.07 ^{a}	3.14 ± 0.07 ^{a}	2.29 ± 0.06 ^{b}
SGR wet weight	8.693 ± 0.220 ^{a}	9.041 ± 0.172 ^{a}	8.952 ± 0.239 ^{a}	6.551 ± 0.864 ^{b}

* The different superscripted letters indicate significant differences ($p < 0.05$).
** Statistics not performed due to a reduced sample of surviving crabs.

3.3. Haemolymph osmolality

Figure 4 shows the mean haemolymph osmolality (± SE) of the crabs reared under different salinities at the end of the experiments. The haemolymph osmolality of the juvenile crabs showed a clear and significant ($p < 0.05$; $r^2 = 0.950$) positive linear relationship ($Y = 0.752 (X) + 278$) to the osmolality of the culture medium. The iosomotic point equaled 1106 mOsm kg^{-1}, which is equivalent to a salinity of approximately 38 ‰.

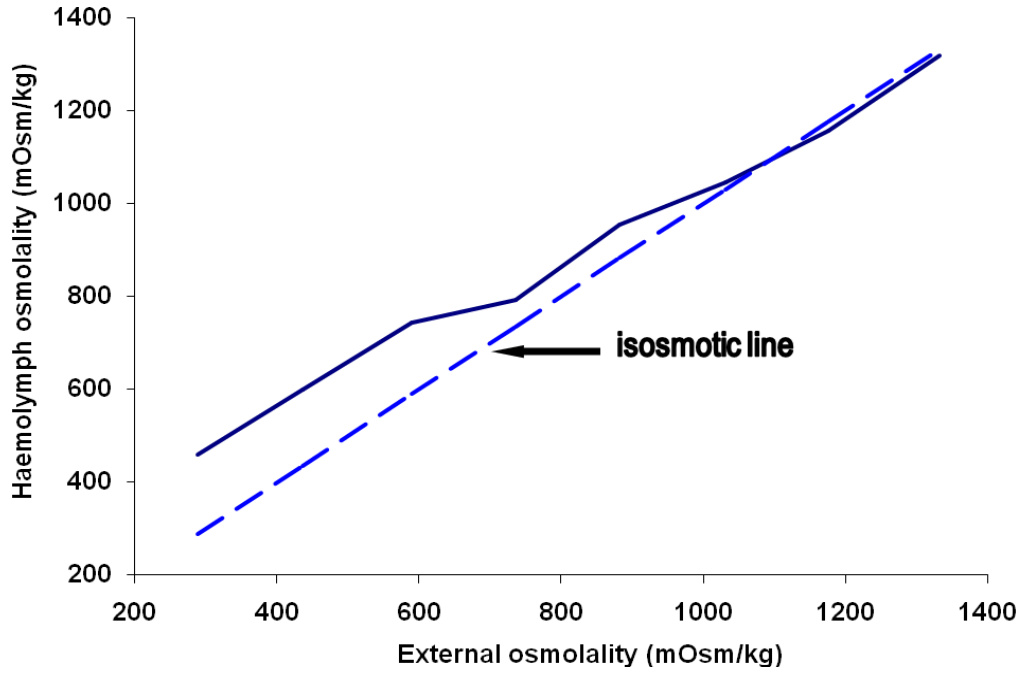

Figure 4: The relationship between the haemolymph osmolality (± SE) and the medium osmolality of early *Portunus pelagicus* juveniles (mean weight = 1.225 ± 0.056) cultured at different salinities.

4. Discussion

The results of the current experiments indicate that a salinity range outside 20 to 35 ‰ can significnatly reduce the survival, growth and development of early *P. pelagicus* juveniles. When an acclimation rate of 3 ‰ h^{-1} was adopted, initial high mortalities were recorded at 5 and 45 ‰. This is in contrast to early juvenile mud crabs (*Scylla serrata*) which reportedly experienced no inital mortalites at the same salinities when an accelerated acclimation rate of 5 ‰ h^{-1} was applied (Ruscoe et al. 2004).

After the inital acclimation period, significanlty sustained mortalities continued to occur at 5, 10 and 15 ‰ indicating that early *P. pelagicus* juveniles have difficulty in adapting to prolonged exposures to low salinity conditions. Furthermore, at a salinity of 5 ‰, a complete mortality occurred on day 20. Interestingly the majority of the sustained deaths in all salinity treatments were due to “molt death syndrome” (MDS), which has been previously linked with

high temperature, genetic factors (Rouse and Kartamulia, 1992), inadequate nutrition (Bowser and Rosemark, 1981; Gong et al. 2004) and, for early *P. pelagicus* juveniles, elevated nitrite-N levels (Romano and Zeng, 2009b) .

The SGR of early *P. pelagicus* juveniles cultured at the higher salinities (40-45 ‰) were significantly less in both experiment. However the salinity effects of 45 ‰ on growth and development were more immediate since the mean intermolt period was significantly longer and the carapace size and wet weight increases were significantly lower at each molt compared to those in the 20, 30 and 35 ‰ treatments. Similarly, immediate effects on growth and development were detected at 10 ‰ with a significantly prolonged intermolt period and reduced carapace size increase at the first molt. Other salinity experiments on early juveniles of several other portunid crabs, including *Callinectes sapidus*, *C. similis* (Guerin and Stickle, 1997a,b) and *S. serrata* (Ruscoe et al. 2004) have shown no significant growth differences when cultured between a salinity range of 10-40 ‰. This suggests that early *P. pelagicus* juveniles have a substantially lower tolerance to extreme salinities than other portunid crabs, and this finding is supported by the fact that early *P. pelagicus* juveniles exhibit weak osmoregulatory abilities form the haemolymph osmolality data obtained in this study.

The osmoregulatory ability of aquatic animals can be determined by measuring the haemolymph osmolality at various salinity conditions and compared with the osmolality of the medium (Lignot et al. 2000). The current study showed that the blue swimmer crab haemolymph exhibited a positive linear relationship with the medium osmolality. Similar results have been reported for penaeid prawn (Chen and Lin, 1994a,b; Sang and Fotedar, 2004; Setiarto et al. 2004) and other portunid crabs (Chen and Chia, 1997; Guerin and Stickle, 1997a,b). While the juvenile crabs exhibited hypo-osmoregulation above the isosmotic line (*i.e.* > 38 ‰) and hyper-osmoregulation below the isosmotic line (*i.e.* < 38 ‰), studies have

shown that *S. serrata* (Chen and Chia, 1997), *C. sapidus* and *C. similis* (Guerin and Stickle, 1997a,b; Li et al. 2006) juveniles are comparatively stronger hyper-osmoregulators than *P. pelagicus* juveniles. This is indicated by the smaller deviation from the isosmotic line and steep slope (Chen and Lin, 1994a,b; Lignot et al. 2000) between salinities of 10 and 15 ‰ for *P. pelagicus.*

It is well known that hyper-osmoregulation in crustaceans requires energy in the form of protein (Rosas et al. 1999; Setiarto et al. 2004; Silvia et al. 2004) or lipids (Lemos et al. 2001; Luvizotto-Santos et al. 2003; Palacios et al. 2004; Sang and Fotedar, 2004). The percentage dry weights may reflect this since it can indicate increased water content and fewer energy reserves (Sang and Fotedar, 2004) that are channelled for growth. Reduced energy reserves likely occurred with the juveniles cultured at low salinities (*i.e.* 10 and 15 ‰) as they had lower percentage dry weights and significantly reduced survival and growth. It is perhaps worth noting that after approximately 2 weeks of low salinity exposure, the juveniles were observed to have focal carapace discoloration of various sizes. This may indicate reduced phenoloxidase activity that is known to occur at low salinities (Lamela et al. 2005) that is responsible for melanin synthesis (Adachi et al. 2005).

It may be argued that salinities closer to the iso-osmotic points would result in decreased metabolic demands and therefore increased growth as observed with penaeid shrimps (Chen et al. 1995). However portunid crabs including *C. sapidus, C. similis* (Guerin and Stickle, 1997a,b) and *S. serrata* (Chen and Chia, 1997; Ruscoe et al. 2004) reportedly have optimal salinity levels below their iso-osomtic points. This experiment also confirms this relationship. Péqueux (1995) has suggested that at high salinities the haemolymph osmolality of "weak osmoregualtors" may exhibit a close parallel association with the iso-osmotic line due to either a disruption in osmoregulation or a strategy to reduce the osmotic gradient between

the haemolymph and environment. The latter appears to be the case in this study. The early *P. pelagicus* juveniles cultured from salinities of 35 ‰ and above exhibited a reduced deviation from the iso-osmotic line. Furthermore, these juveniles had decreased percentage water contents, which have been similarly reported for *Peneaus chinensis* (Chen and Lin, 1994b; Chen et al. 1995), *P. setiferus* (Rosas et al. 1999) and *P. latisulcatus* (Sang and Fotedar, 2004) at high salinities. Decreased percentage water content may be the result of increased free amino acids to counteract high salt loads (Luvizotto-Santos et al. 2003; Silvia et al. 2004; Wang et al. 2004) that are likely essential for growth and molting. However, other reports have suggested that reduced growth at higher salinity conditions may also be attributed to reduced feed assimilation or consumption (Kumlu and Jones, 1995; Lemos et al. 2001; Sang and Fotedar, 2004). While feeding rates were not quantified in this study, it was observed that the crabs cultured at high salinities, particularly at 45 ‰, were less active upon the introduction of food. These two factors may have synergistically contributed to the significantly reduced growth of early *P. pelagicus* juveniles at salinities above the iso-osmtoic point.

One of the interesting findings in this experiment was the high incidence of MDS in all the salinity treatments. It has been demonstrated by Gong et al. (2004) that the incorporation of dietary trace metals and lipids significantly reduced the incidence of MDS in the shrimp *Litopenaeus vannamei* by increasing their osmoregualtory capacity. Therefore, since early juvenile blue swimmer crabs exhibit weak osmoregulatory abilities and their current production largely relies on commercial shrimp feeds, it appears that designing a species-specific diet may be necessary to reduce MDS and improve growth over a broad range of salinities.

The results of the current experiments demonstrate that salinity can have an immediate and significant effect on survival and growth and a salinity range between 20 and 35 ‰ is recommended for the culture of early juvenile blue swimmer crabs. Furthermore, the natural

distribution range of *P. pelagicus* and the behavior of mass emigration from Western Australian estuaries appears to reflect this salinity range. This suitable salinity range of early *P. pelagicus* juveniles, as revealed by the present study, has significant implications for aquaculture, since it can be utilised for various purposes including farm site selection and the maintenance of salinity levels within a recirculating system to maximize productivity.

Chapter 3

Ontogenetic tolerance changes to acute ammonia exposure and associated gill histological alterations to early *Portunus pelagicus* juveniles

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1. Introduction

As previously demonstrated in Chapter 2, salinity has a significant effect on the survival and growth on early *P. pelagicus* juveniles, likely due to their weak osmoregulatory abilities. While this provided baseline information, this Chapter will similarily be used as a foundation for future experiments by determing their ammonia-N tolerance through juvenile development and if any change is correlated with ammonia-N induced gill damage. Again, by providing a context, this will allow a better understanding of how ammonia-N tolerance and osmoregulation of crustaceans are influenced while also having implications for future expeirmental design, particularly when simutaneously investigating salinity and ammonia-N on this crab species.

Ammonia-N is perhaps the most limiting factor in a closed aquaculture system, and substantially more toxic than nitrite or nitrate (Meade and Watts, 1995; Romano and Zeng, 2007a,b,c). Furthermore, on these systems, a rapid build-up can occur from the decomposition of organic matter and the branchial excretion of aquatic animals (Timmons et al. 2002; Freire et al. 2008). While ammonia has two forms, ionised (NH_4^+) and un-ionised (NH_3), the un-ionised form is considered more toxic to aquatic animals since it can easily diffuse across cell membranes (Chen and Kou, 1993; Kir and Kumlu, 2006). The proportion of the more toxic un-ionised form is well known to increase as the temperature increases, salinities decrease, and

especially, as the pH increases (Chen and Lin, 1991; Chen and Kou, 1993) which may severely damage various organs including the gills (Rebelo et al. 2000).

The gills are important organs for crustaceans since they are largely responsible for gas exchange, ammonia-N excretion, osmoregulation and acid-base balance (Péqueux, 1995; Weihrauch et al. 2002, 2004). It has been demonstrated that acute ammonia-N exposure can alter the gill physiology and morphology of the fish pacamã, *Lophiosilurus alexandri* (Cardoso et al. 1996) and the estuarine crab, *Neohelice* (*Chasmagnathus*) *granulata* (Rebelo et al. 2000). Such histological changes included necrosis, epithelial damage (Cardoso et al. 1996) and lamellae collapse (Rebelo et al. 2000). The physiological consequences of these changes are likely to affect aquaculture productivity due to reduced gas exchange (Rebelo et al. 1999, 2000), impaired osmoregulation (Young-Lai et al. 1991; Chen and Chen, 1996), reduced growth (Chen and Lin, 1992a; Neil et al. 2005) and, in severe cases, mortality of the animal (Ostrensky and Wasielesky, 1995; Zhao et al. 1997; Kir and Kumlu, 2006).

Ammonia-N tolerance in crustaceans appears to be species-specific (Allan et al. 1990) as well as their tolerance variation through ontogenetic development (Chin and Chen, 1987; Young-Lai et al. 1991; Ostrensky and Wasielsky, 1995; Zhao et al. 1997; Cavalli et al. 2000; Neil et al. 2005). For example, while Young-Lai et al (1991) detected a significantly positive relationship in ontogenetic ammonia-N tolerance at different life stages of the clawed lobster *Homarus americanus*, Ostrensky and Wasielesky (1995) found no significant correlation of ammonia-N tolerance and development with the São Paulo shrimp, *Penaeus paulensis*.

Determining the ammonia-N tolerance of a targeted culture species and the tolerance through ontogenetic development have obvious aquaculture management implications. The experiments were hence designed to measure the actue ammonia-N tolerance through the early

juvenile ontogeny of *P. pelagicus* as well as to investigate the effects of ammonia-N exposure on the gill structure to help explain any potential changes in tolerance.

2. Materials and Methods

2.1. Source of crabs

The crabs were larvicultured according to the protocol described in Chapter 2. Upon larval metamorphosis to the first crab stage, which was defined as the C1 stage, the salinity in the larval rearing tanks were gradually increased to 30 ‰ over the course of 1-h. After acclimation, 240 C1 crabs were randomly chosen for the larval culture tanks and individually placed in round plastic 5-L capacity containers.

The remaining crabs were transferred to outdoor recirculating tanks at a salinity of 30 ± 2 ‰ and a temperature of 27 ± 2°C. Initially numerous hides were provided to reduce cannibalism and the crabs were fed a combination of frozen *Artemia* nauplii (INVE, AAA) and formulated crumble feed (Ridely). At the second crab stage (C2) and onwards, the crabs were exclusively fed the crumble feed. When the crabs reached the C3 stage, they were individually placed in plastic containers (diameter 16 cm × height 19 cm) and their molts and stages were monitored daily. In each container, there were numerous 3.75 mm holes to facilitate adequate water exchanges. When the desired juvenile stage was reached the crabs were then moved indoors, acclimated and starved for 24-h prior to the commencement of the experiment. All juvenile crabs used in this study were sourced from two separate females and equally used in each experiment.

The carapace size of the crabs were measured using a digital caliper (0.01 mm) (Mitutoyo Absolute digimatic, Japan). The carapace length (CL) and carapace width (CW) were defined according to the description in Chapter 2. The wet weights of the crabs were

measured using a digital scale (0.001 g) (Adventurer Pro) before each experiment commenced. Prior to weighing, the crabs were blotted dry with a tissue and placed on a zeroed scale in a small container of water. Table 1 shows the mean CL, CW, wet weight and the average development duration to each crab stage from the day of metamorphosis to the C1 stage.

Table 1: The mean carapace length (CL ± SE) and width (CW ± SE), wet weight (± SE) and average developmental duration from the crab 1 (C1) stage to each of the four *Portunus pelagicus* juvenile crab stages.

Crab stage	CL (mm)	CW (mm)	Wet wt. (g)	Days from metamorphosis to C1 stage
C1	2.45 ± 0.01	3.12 ± 0.02	0.002 ± 0.001	--
C3	4.12 ± 0.03	7.94 ± 0.09	0.028 ± 0.001	7
C5	7.45 ± 0.08	13.30 ± 0.21	0.187 ± 0.002	22
C7	11.53 ± 0.19	18.31 ± 0.60	0.732 ± 0.036	40

2.2. Experimental design and set-up

A total of 960 crabs were used for the acute ammonia toxicity experiments, with 240 crabs used for each juvenile stage. During the experiments, each juvenile was individually kept in a 5-L capacity container (diameter 21 cm × height 20 cm). The container was filled with 2-L of test solution for the C1 and C3 crab experiments and increased to 4-L of test solution for the C5 and C7 crab experiments. All containers were bathed in four 1000-L oval tanks with the water temperature maintained at 28 ± 0.5°C through air conditioning and submersible heaters. Each container was gently aerated by an air tube connected to a fine-tipped glass pipette.

Natural source seawater (5 µm filtered and UV sterilized) was used for all experiments and salinities were pre-adjusted to 30 ‰ using de-chlorinated freshwater. The pH was measured (WP-80; TPS) and pre-adjusted to 8.10 through the addition of sodium hydroxide (NaOH) pellets. A total of 7 ammonia-N treatments (5, 10, 20, 40, 60, 80 and 100 mg l^{-1}) and a

control (no ammonia-N added) were set up for each juvenile stage. A total of 30 crabs were individually placed in separate containers for each ammonia-N treatment and control (7 ammonia-N treatments and a control × 30 crabs = 240 crabs). Therefore, each crab acted as a replicate. Ammonia stock solutions were made daily by dissolving 38.2 g ammonium chloride (NH_4Cl) (analytical grade) in 1-L of distilled water which was then diluted to create the desired ammonia-N concentrations. Each container received a 100% daily water exchange, according to the "static renewal method" described by the American Public Health Association (1985) and no food was provided during the experiments. The photoperiod was L:D = 14:10 with a light intensity between 132 - 170 lux as measured by a lux meter (TPS. MC-88 Light meter, US).

To determine the LC_{50} values, mortality observations were made at 12-h intervals up to 96-h. Death was assumed when no movement or response occurred when mechanically stimulated with a glass rod.

At the end of each experiment all surviving crabs from each treatment and control were fixed for histological examination. Based on previous pilot studies, which utilised 3 days of fixing in either 10 % (v/v) formalin, 10 % (v/v) formalin followed by de-calcification or a 10 % (v/v) FAACC (4 % formaldehyde, 5 % acetic acid and 1.3 % calcium chloride) formalin solution, it was determined that the 10 % (v/v) FAACC formalin solution was the most effective in softening the hard carapace to allow for easier sectioning of the crab. After the 3-day fixing in FAACC, the crabs were transferred to 70 % (v/v) ethanol until further processing. The crabs were then progressively dehydrated at increasing concentrations of alcohol and embedded in paraffin wax. Sections (5 µm) were cut using a rotary Leitz microtome (model 1512, US), stained with hematoxylin and eosin and then examined and digitally photographed under a light microscope.

To confirm the ammonia-N concentrations, three ammonia samples from each container were randomly sampled on the first and last day of the experiment. The Salicyate method was used for all ammonia-N samples and measurements were made on a colorimeter (HACH 10200; US). The results confirmed that all ammonia-N samples were within 0.1 mg l^{-1} of the nominal ammonia-N concentration.

2.3. Data analysis

The LC_{50} values (median lethal concentration), their 95 % confidence intervals of ammonia-N, NH_3-N and the Chi-square analysis goodness of fit were computed using SAS program PROC PROBIT (SAS Institute Inc., 1990) after estimated lines were satisfactory. The un-ionised form (NH_3-N) of ammonia-N was calculated according to Whitfield (1974) based on a salinity of 30 ‰, temperature of 28°C and a pH of 8.1.

3. Results

3.1. Survival

No crabs died in the control (0 mg l^{-1}) or 5 mg l^{-1} ammonia-N during the 96-h experimental period for all four juvenile stages. The 96-h LC_{50} values of ammonia-N (mg l^{-1}) for the C1, C3, C5 and C7 crabs over the 96-h duration are shown in Fig. 1, and at all stages, a significantly negative ($p < 0.01$) relationship was detected between ammonia-N tolerance and exposure duration. The 12-h LC_{50} value of ammonia-N for the C1 and C3 crabs were calculated to be 74.13 and 79.43 mg l^{-1}, respectively (Fig. 1A, B) with a value of 5.50 and 5.68 mg l^{-1}, respectively for NH_3-N (Table 2). In contrast, no 12-h LC_{50} value was obtained for the C5 and C7 crabs since no mortalities were observed over a 12-h exposure duration in any of the ammonia-N treatments (Fig. 1C, D, Table 2). At 100 mg l^{-1}, the highest ammonia-N level

tested, all C1 and C3 crabs died with 12-h of exposure, while all C5 and C7 crabs died within 36-h and 48-h, respectively.

A significantly positive relationship ($p < 0.01$) was detected between the juvenile developmental stages and ammonia-N tolerance: the 96-h LC_{50} value (mg l^{-1}) of ammonia-N was 23.10, 25.23, 37.43 and 50.65 and 1.65, 1.80, 2.68 and 3.62 of NH_3-N for the C1, C3, C5 and C7 crabs respectively. The ammonia-N tolerance from the C1 to C7 crab stage showed an over two-fold increase.

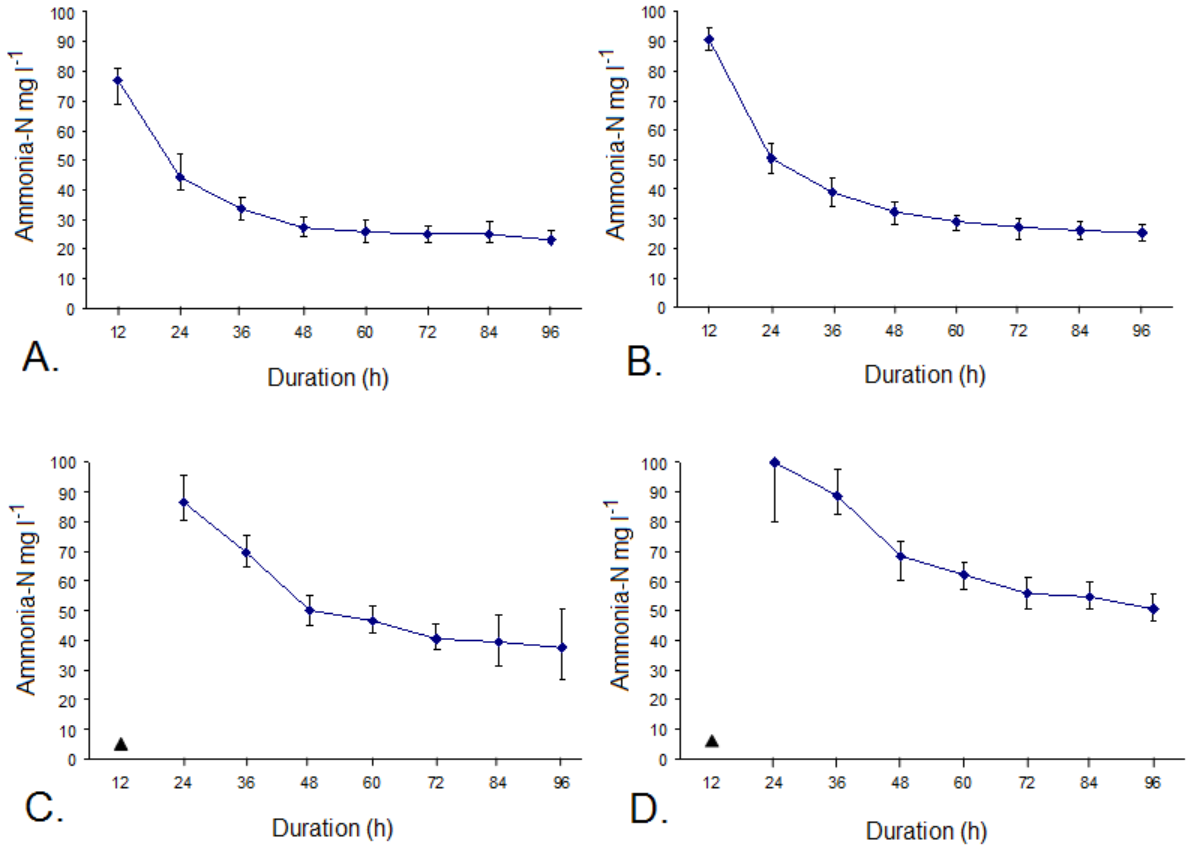

Figure 1: The LC_{50} values (and 95% confidence limits) of ammonia-N (mg l^{-1}) for the blue swimmer crab *Portunus pelagicus* early juveniles. (A) crab 1 stage, (B) crab 3 stage, (C) crab 5 stage, (D) crab 7 stage. ▲ indicates no LC_{50} values obtained as no mortalities occurred within a 12-h exposure period.

Table 2: The LC_{50} values (mg l^{-1}) of NH_3-N and their 95 % confidence intervals (in parenthesis) at the C1, C3, C5 and C7 stages of *Portunus pelagicus* juveniles.

	Duration (hr)							
Crab stage	12	24	36	48	60	72	84	96
C1	5.50	3.16	2.40	1.93	1.85	1.79	1.78	1.65
	(5.17-5.83)	(2.59-3.77)	(2.14-2.68)	(1.69-2.22)	(1.62-2.13)	(1.56-2.07)	(1.56-2.06)	(1.44-1.92)
C3	6.48	3.59	2.78	2.31	2.07	1.93	1.87	1.80
	(6.20-6.77)	(3.25-3.96)	(2.50-3.07)	(2.06-2.56)	(1.84-2.33)	(1.70-2.20)	(1.65-2.13)	(1.59-2.06)
C5	**	6.19	4.97	3.59	3.33	2.90	2.83	2.68
		(5.73-6.79)	(4.57-5.40)	(3.25-3.93)	(3.01-3.66)	(2.60-3.22)	(2.27-3.43)	(1.87-3.58)
C7	**	7.70	6.34	4.89	4.44	4.00	3.91	3.62
		(7.19-9.19)	(5.89-6.95)	(4.53-5.24)	(4.12-4.76)	(3.67-4.33)	(3.58-4.24)	(3.32-3.92)

** indicates no LC_{50} value was obtained due to too few deaths for probit analysis

3.2. Gill histological changes induced by acute ammonia-N exposure

The early *P. pelagicus* juveniles possessed 8 pairs of phyllobrachiate gills characterised by numerous lamellae branching from the branchial (central) stem. Histological examination of the surviving crabs at the end of the 96-h experiment showed that at an ammonia-N concentration of 5 mg l^{-1}, the gills of the C1 crabs underwent epithelial changes and localized infiltration of haemocytes (Fig. 2B). However, at the same ammonia-N concentration, no apparent physiological changes of the gills were observed at the C3, C5 or C7 stage (Fig. 3B). With increasingly higher ammonia-N concentrations the histological gills changes became more prominent and included, in ascending order, of localized infiltration of haemocytes, extensive infiltration of haemocytes, lamellae distortion, a reduction to the haemolymph spaces, disruption of pillar cells and lamellae collapse. For example, at 10 mg l^{-1} ammonia-N, the gill lamellae of the C7 staged crabs showed localized infiltration of haemocytes with normal lamellae structure (Fig. 3C), whereas at 60 mg l^{-1} ammonia-N the gill lamellae of the C7 staged

crabs exhibited extensive infiltration of hemocytes, disrupted pillar cells and lamellae collapse (Fig. 3F) (see figure legends for further details).

While the gill lamellae exposed to higher ammonia-N concentrations were observed to cause increased physiological changes, the severity of changes was less with the more developed crabs at the same ammonia-N concentration (Fig. 2 and 3). For example, at 20 mg l^{-1} ammonia-N, the gill lamellae of the C1 crabs exhibited necrosis, disrupted pillar cells, extensive infiltration of heamocytes and distorted lamellae structure (Fig. 2D), whereas at the same ammonia-N concentration, the gill lamellae of the C7 crabs showed only localized infiltration of haemocytes but intact pillar cells and normal lamellae structure (Fig. 3D).

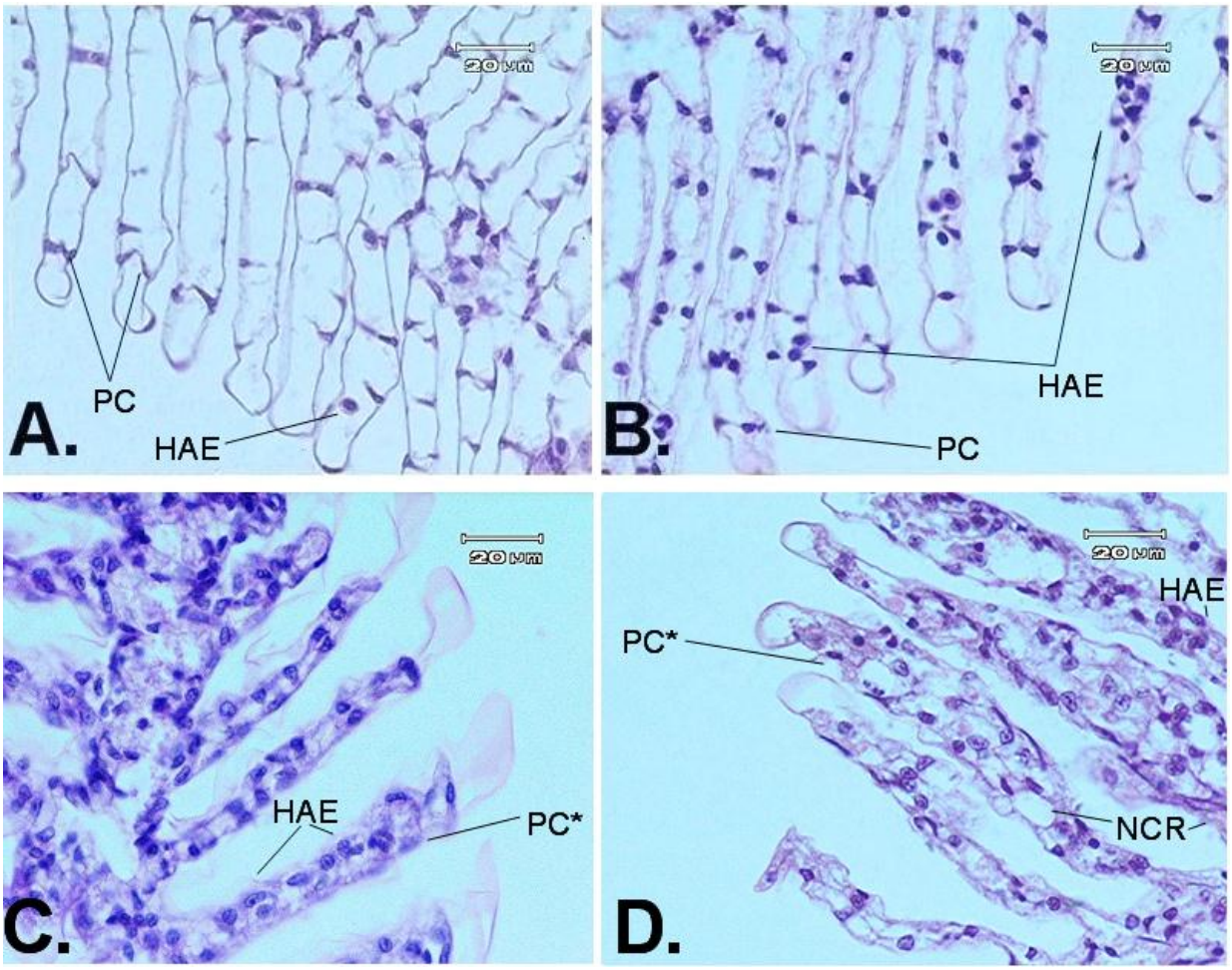

Figure 2: Anterior gill lamellae of the surviving crab 1 stage *Portunus pelagicus* following a 96-h exposure to different ammonia-N concentrations. (A) crabs from the control, showing normal lamellae structure, intact pillar cells (PC) and a haemocyte (HAE); (B) crabs exposed to 5 mg l^{-1} ammonia-N showing intact pillar cells (PC), epithelial sloughing and an increase in haemocytes within the lamellae space (HAE); (C) crabs exposed to 10 mg l^{-1} ammonia-N, showing an increase of heamocytes within the lamellae space (HAE), disrupted pillar cells (PC*) and a thickening/sloughing to the epithelium; (D) crabs exposed to 20 mg l^{-1} ammonia-N, showing necrosis (NCR), extensive infiltration of haemocytes within the lamellae space (HAE), sloughing of the epithelium, disrupted pillar cells (PC*) leading to a complete breakdown to the intralamellae septum. × 40; Scale bars = 20 µm.

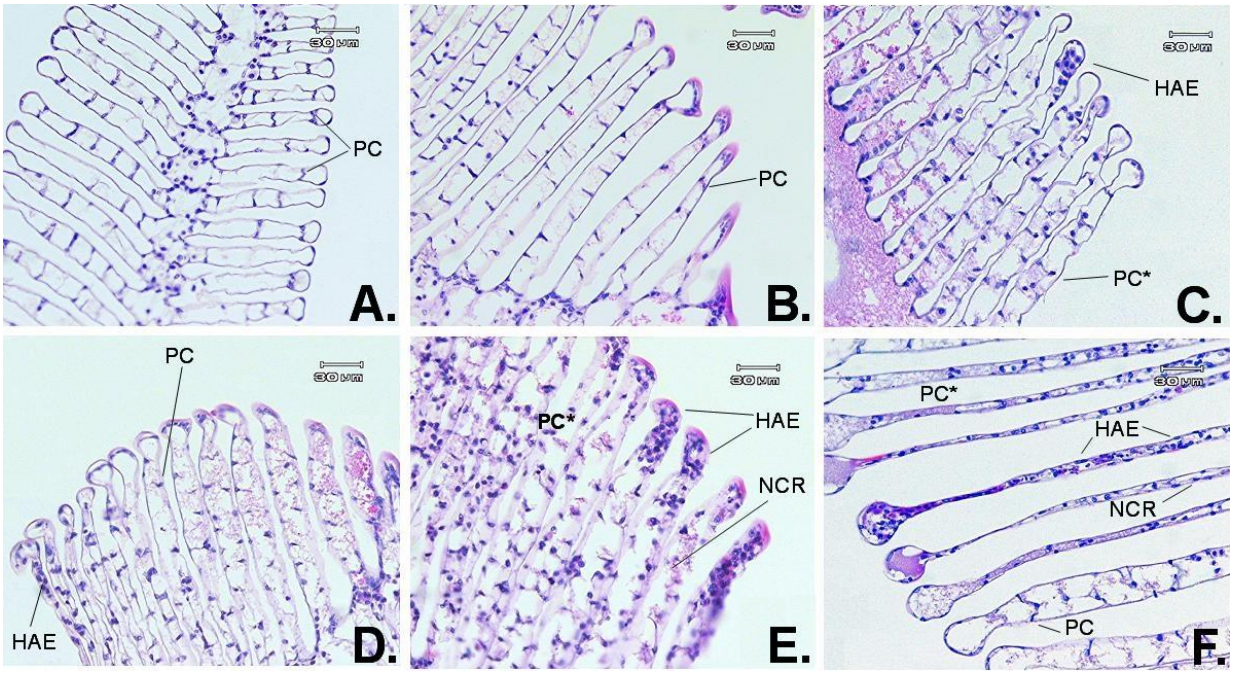

Figure 3: The anterior gill lamellae of the surviving crab 7 stage *Portunus pelagicus* following a 96-h exposure to different ammonia-N concentrations. (A) crabs from the control showing normal lamellae structure and intact pillar cells (PC); (B) crabs exposed to 5 mg l^{-1} ammonia-N, showing normal lamellae structure and intact pillar cells (PC); (C) crabs exposed to 10 mg l^{-1} and (D) crabs exposed to 20 mg l^{-1}, showing intact pillar cells (PC), infiltration of haemocytes (HAE) and slight lamellae distortion; (E) crabs exposed to 40 mg l^{-1} ammonia-N showing disrupted pillar cells (PC*), extensive infiltration of haemocytes (HAE), necrosis (NCR) and lamellae distortion; (F) crabs exposed to 60 mg l^{-1} ammonia-N, showing disrupted pillar cells (PC*), necrosis (NCR), extensive infiltration of haemocytes (HAE) and lamellae collapse alongside swollen lamellae with intact pillar cells (PC). × 20; scale bars = 30 µm.

4. Discussion

The results of the current study demonstrate that the ammonia-N tolerance significantly increases with the ontogeny of early *P. pelagicus* juveniles. While Lin and Chen (2001) have suggested a similar trend may apply for juvenile penaeid species, previous studies on the ammonia-N tolerance of aquatic animals through ontogeny have only focused on comparing differences of various lifecycle phases, *i.e.* a particular larval stage versus a juvenile or adult stage (Chin and Chen, 1987; Young-Lai et al. 1991; Ostrensky and Wasielesky, 1995) or through larval development (Zhao et al. 1997; Cavalli et al. 2000; Neil et al. 2005) with different correlations in ammonia-N tolerance. However, in spite of the limited information

currently available concerning the acute toxicity of ammonia-N to crabs, *P. pelagicus* juveniles appear to have a considerably higher ammonia-N tolerance than other decapods crustaceans, especially shrimp species. This is particularly valid when the relative wet weights of these decapods crustaceans are considered (Table 3). The "safe level", derived by multiplying the 96-h LC_{50} value by an empirical application factor of 0.1 (Sprague, 1971), was 2.31 mg l^{-1} ammonia-N (0.16 NH_3-N mg l^{-1}), 2.52 mg l^{-1} ammonia-N (0.18 mg l^{-1} NH_3-N), 3.74 mg l^{-1} ammonia-N (0.26 NH_3-N mg l^{-1}) and 5.06 mg l^{-1} ammonia-N (0.36 mg l^{-1} NH_3-N), for the C1, C3, C4 and C7 crabs, respectively. Such a higher ammonia-N tolerance may be explained by an ecological adaptation described by Weihrauch et al. (2004). The authors suggested that benthic animals which bury themselves for prolonged periods in the sediment can experience high levels of localised ammonia-N resulting in a physiological need to transport haemolymph ammonia-N against a gradient (*i.e.* to the environment). Such a physiological adaptation likely applies to *P. pelagicus* juveniles and adults since they commonly bury themselves for long durations, particularly during the daytime and winter (Kangas, 2000). It is perhaps worthy to note that the only crustacean, that has been studied to date, which exceeds the ammonia-N tolerance of *P. pelagicus* juveniles, with comparable wet weights, is the mud crab *Scylla serrata* early juveniles (Romano and Zeng, 2007a) (Table 3). However, it was suggested by Romano and Zeng (2007a) that the high ammonia-N tolerance of *S. serrata* likely reflects the habitat differences between these two crab species. For example, *P. pelagicus* typically inhabit sand shores/embayments in clearer waters (Kangas, 2000) whereas *S. serrata* are more abundant on muddy shores/mangrove flats with highly organic, and often anoxic, sediments (Hill et al. 1982; Hyland et al. 1984). Therefore, due to this habitat difference, it may be possible that *S. serrata* experience higher levels of ammonia-N in sediments therefore necessitating a more efficient mechanism to cope with elevated ammonia-N levels.

Table 3: The 96-h LC_{50} values of ammonia-N (NH_3-N + NH_4-N) and un-ionised ammonia (NH_3-N) for various crustacean species.

Species	Life stage	Weight (g)	Salinity (‰)	Ammonia-N (mg l^{-1})	NH_3-N (mg l^{-1})	Reference
Peneaus monodon	Postlarvae		34	11.51	1.04	Chin and Chen (1987)
P. chinensis	Juvenile	0.61	10	28.18	1.94	Chen and Lin (1992b)
			20	38.87	2.46	
			30	42.44	2.47	
P. paulensis	Juvenile	5.45	28	38.72	1.10	Ostrensky and Wasielsky (1995)
	Adult	31.43	28	42.49	1.06	
P. semisulcatus	Postlarvae	0.27	15	7.07	0.34	Kir and Kumlu (2006)
			20	7.11	0.35	
			25	8.94	0.39	
			30	14.51	0.61	
			35	18.72	0.75	
			40	19.06	0.74	
P. penicillatus	Juvenile	0.4 - 0.7	34	38.72	1.11	Chen and Lin (1991)
			25	24.88	0.99	
L. vannamei	Juvenile		15	24.39	1.20	Lin and Chen (2001)
			25	35.40	1.57	
			35	39.54	1.60	
M. ensis	Juvenile		25	35.59	0.87	Nan and Chen(1991)
Eriocheir sinensis	Juvenile	0.06	25	31.6	0.90	Zhao et al (1997)
Homarus americanus	Postlarvae		30	144	2.36	Young-Lai et al (1991)
	Adult		30	219	3.25	
Scylla serrata	Juvenile	0.373	30	95.35	7.10	Romano and Zeng (2007a)
Portunus pelagicus	Juvenile	0.002	30	23.10	1.65	Present study
		0.028	30	25.23	1.80	
		0.187	30	37.43	2.67	
		0.703	30	50.65	3.62	

Following acute ammonia-N exposure, hyperplasia, necrosis and epithelial changes of the lamellae have been reported with pacamã (*L. alexandri*) larvae and alevins (Cardoso et al. 1996) as well as to the estuarine crab (*N. granulata*) adults (Rebelo et al. 2000). Furthermore, Rebelo et al. (2000) reported gill lamellae collapse with *N. granulata* due to pillar cell disruption. The reason for this suggestion is the structural support and directing haemolymph flow that the periodic distribution of pillar cells provide (Harrison and Humes, 1992). A similar morphological change occurred with surviving *P. pelagicus* crabs at the C7 stage when

exposed to 60 mg l^{-1} ammonia-N. However, based on observations at the C1 and C3 crab stage when exposed to 20 mg l^{-1} ammonia-N, it appears that disrupted pillar cells did not necessarily result in lamellae collapse.

The anterior gills, characterised as having a thin epithelium, are believed to be predominately responsible for gas exchange (Péqueux, 1995; Tresguerres et al. 2008). The presence of haemocytes within the anterior gill lamellae, increasing at higher ammonia-N concentrations, may have been a response to phagocytise damaged tissue/cells (Harrison and Humes, 1992). However, at higher ammonia-N concentrations, the extensive prevalence of haemoyctes, along with lamellae collapse, likely reduced haemolymph flow necessary for gas exchange with the gill lamellae. Indeed, this may explain the increased ammonia-N toxicity at low dissolved oxygen concentrations (Rebelo et al. 2000) as reported for *P. monodon* juveniles (Allan et al. 1990) and *H. americanus* adults (Young-Lai et al. 1991). Consequently, this emphasises the need to maintain adequate aeration in closed aquaculture systems, particularly in recirculating systems that may periodically experience heightened ammonia-N.

The ability to regulate haemolymph ammonia-N levels lower than the environment has been demonstrated with numerous decapods crustaceans including the blue crab, *C. sapidus* (Cameron, 1986), the mud crab, *S. serrata* (Chen and Chia, 1996; Romano and Zeng, 2007a), the shore crab, *C. maenas* (Weihrauch et al. 1998), Dungen crab, *Cancer pagurus*, the common shore crab, *C. maenas* and the Chinese mitten crab *E. sinensis* (Weihrauch et al. 1999). A major contributor to this ability is accomplished via active ammonia-N excretion (reviewed by Weihrauch et al. 2004) and a similar ability likely occurred with *P. pelagicus* early juveniles as such a mechanism could explain their capacity to tolerate high ammonia-N concentrations, particularly at the C5 and C7 stage. Therefore, since both the posterior and anterior gills are responsible for active ammonia-N excretion (Weihrauch et al. 2002, 2004) the histological

changes observed at the C1 and C3 stages may explain their significantly lower ammonia-N tolerance compared to the C5 and C7 stages. For example, the C1 crabs exposed to 5, 10 and 20 mg l^{-1} ammonia-N and the C3 crabs exposed to the 10, 20 and 40 mg l^{-1} ammonia-N levels exhibited distinct and severe morphological changes to the epithelium. These changes included detachment, sloughing and thickening (Fig. 2B, C, D). In contrast, the C5 and C7 crabs exposed to 10 and 20 mg l^{-1} ammonia-N had intact epithelium and normal structure (Fig. 3C, D). It may therefore be speculated that more severe morphological changes observed at the C1 and C3 stage may have interrupted active ammonia-N excretion and/or impeded gas exchange, resulting in the significantly lower ammonia-N tolerance at these earlier stages.

In conclusion, the results have demonstrated that *P. pelagicus* juveniles have a comparatively higher ammonia-N tolerance than other crustacean species, that ammonia-N tolerance increases with juvenile ontogenetic development and that acute ammonia-N exposure can severely affect the morphology and physiology of the anterior gill structure. These findings have significant implications for the aquaculture management of this crab species, as well as addressing a need for further physiological investigations.

Chapter 4

Changes to the histological gill structure and haemolymph composition of early blue swimmer crabs, *Portunus pelagicus*, juveniles during elevated ammonia-N exposure and the post-exposure recovery

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1. Introduction

Now that important baseline information has been established from Chapters 2 and 3 revealing that although early *P. pelagicus* juveniles are relatively weak osmoregulators which can be viewed as a significant drawback for aquaculture, their ammonia-N tolerance is comparatively higher than other crustacean species. This prompts some interesting questions, pertinent to the aquaculture industry and general physiology, which include the following.

Does elevated ammonia-N exposure eventually disrupt osmoregulation?
If so, is there any correlation to gill damage?
Can ammonia-N induced gill damage eventually heal when returned to pristine seawater?
What are the potential causes for the high ammonia tolerance?

The following experiment will attempt to answer these questions through simulating a time course experiment of sub-lethal ammonia-N exposure and subsequent post-exposure recovery period in pristine seawater.

Since the gills of crustaceans are in constant contact with the external medium these structures are particularly vulnerable to potential pollutants (Romano and Zeng, 2009b).

Rebelo et al. (2000) and Chapter 3 showed that elevated ammonia-N can rapidly cause severe gill damage to crustaceans which included necrosis, hyperplasia, epithelial damage, pillar cell disruption and lamellae collapse. This can be of particular concern since the gills of aquatic animals are multi-functional organs responsible for many crucial physiological processes including ion exchange, acid/base balance, ammonia-N excretion and respiration (Harrison and Humes, 1992; Péqueux, 1995; Weihrauch et al. 2004). Indeed previous investigations detected significant decreases to haemolymph osmolality and/or Na^+ ions at elevated ammonia-N levels (Young-Lai et al. 1991; Chen and Chen, 1996; Harris et al. 2001; Romano and Zeng, 2007a) which may be linked to gill damage.

Since the gills of crustaceans clearly have important roles, are particularly vulnerable to external pollutants and the damage has been linked to their mortality (Rebelo et al. 2000; Chapter 3), correlating such damage in sequence with their physiological responses may yield important information on the modes and progression of toxicity. However, to date, histological examination of ammonia-N induced gill damage, and associated physiological changes linked with their function, have only focused on a single sampling point of exposure (*e.g.* Rebelo et al. 2000; Miron et al. 2008). Furthermore, it is yet unclear if sub-lethal ammonia-N levels will cause irreparable gill damage to aquatic animals, or if healing is possible, the potential factors involved. A common response of the gills from *P. pelagicus* juveniles at various nutrients is an infiltration of haemocytes (Chapter 3, Romano and Zeng, 2007b; Romano and Zeng, 2009b), which is well known to play a critical role in the removal of necrotic/damaged tissue (Battistella et al. 1996; Johansson et al. 2000). However, this has yet to be quantified and no linkage has been made to the total haemocyte counts (THC) in the haemolymph during and after elevated ammonia-N exposure.

The experiment was hence designed to continuously monitor the haemolymph osmolality, Na^+, K^+, Ca^{2+}, pH, ammonia-N, total haemocyte counts (THC), as well as the histopathological gill changes of early *P. pelagicus* juveniles over 48-h of exposure to sub-lethal ammonia-N levels and the subsequent post-exposure recovery period for 96-h in pristine seawater (no added ammonia-N). In addition, two higher ammonia-N levels were used to induce crab morbidity to determine if haemolymph osmolality, Na^+, K^+, Ca^{2+} and pH levels would eventually be disrupted to potentially explain causes for lethality.

2. Materials and Methods

2.1. Experiment 1

2.1.1. Source of crabs

The crabs were larvicultured according to the protocol described in Chapter 2. Upon metamorphosis to the C1 crab stage, the crabs were further cultured according to the protocol previously described in Chapter 3. When the crabs reached the C5 – C6 stage (mean carapace width = 18.76 ± 0.26), approximately 3 weeks after settlement, the crabs were brought indoors for the commencement of the experiment.

2.1.2. Preparation of test solutions

A 10,000 mg l^{-1} ammonia-N stock solution was made according to Chapter 3 and diluted in seawater to create the desired ammonia-N concentrations of the test solutions used for experiment 1 and 2. The salinity of the source seawater was 36 ‰, which was reduced to 30 ‰ using de-chlorinated freshwater and the ammonia-N, nitrite-N and nitrate-N concentrations were measured and all were below 0.01 mg l^{-1} which were previously analysed by the *Australian Centre for Tropical Freshwater Research* according to APHA (1989). The

pH of each test solution was maintained at 8.1 through the addition of sodium hydroxide (NaOH) pellets and the pH measured using a pH digital meter (WP-80; TPS, Australia).

2.1.3. Experimental design

A total of 288 crabs (C5 stage; mean weight = 0.225 ± 0.012 g), each individually kept within a separate container thus acting as a replicate, were used in the experiment. Among these, 140 replicate crabs were used for each of the two sub-lethal ammonia-N treatments of 0.714 and 2.857 mmol l^{-1} (or 10 and 40 mg l^{-1} ammonia-N), respectively (based on values from Chapter 3), while 8 replicate crabs were used for the control (no ammonia-N added). Each crab was individually placed within a 5-l container, filled with 2-l of the desired test solution, and gently aerated via a fine tipped pipette. Each container received a daily 100% water exchange according to the "static renewal method" described by the APHA (1989). Although all crabs were daily fed formulated crumble feed designed for the tiger prawn, *Penaeus monodon,* (43% protein, 6% fat, 3% fibre), the containers were marked and the crabs were starved 24-h prior to haemolymph sampling. To prevent the decomposition of food affecting the water ammonia-N levels, the feeds were siphoned out after 3-h which was sufficiently long enough for the crabs to cease eating. All containers were held within six 1,000-L freshwater baths and the temperature was maintained at 28 ± 0.5°C through submersible heaters and air conditioning.

At time intervals of 0, 1, 3, 6, 12, 24, 36 and 48-h, the haemolymph from 8 intermolt replicate crabs exposed to either 0.714 or 2.857 mmol l^{-1} ammonia-N treatments (10 and 40 mg l^{-1} ammonia-N, respectively) were obtained via a syringe inserted through the proximal arthropodal membrane at the base of the right second walking leg. Haemolymph samples from 5 crabs were then measured for haemolymph osmolality and Na^+, K^+ and Ca^{2+} levels while haemolymph samples from the other 3 crabs were used to measure the haemolymph pH, total

haemocyte counts (THC) and ammonia-N levels. To determine haemolymph Na^+, K^+ and Ca^{2+} levels a haemolymph sample of 20 µl was immediately diluted with 2 ml of distilled water and analysed on flame photometer (Sherwood 410, Cambridge, UK). To determine the haemolymph osmolality, an aliquot of haemolymph (50 µl) was immediately analysed on a cryoscopic osmometer (Osmomat 030; Gonotec). The haemolymph pH was measured using a pH digital meter (WP-80; TPS, Australia) equipped with a micro-pH electrode (MI-710, Microelectrodes Inc., US) after a two-point calibration with precision buffers. Following the measurement of pH, the haemolymph was divided into two portions to be used for either determining the haemolymph THC or ammonia-N levels. To measure the haemolymph THC, anticoagulant (citrate concentrated solution, 4% w/v) was added at a ratio of 1:9 (anticoagulant:haemolymph) and the haemocytes were counted on a haemocytometer under a light microscope (magnification × 10). The haemolymph samples for measuring the ammonia-N were immediately diluted with distilled water, frozen at -20°C and analysed within 2 days by the *Australian Centre for Tropical Freshwater Research* (ACTFR) using the Nesslerization method (4500 NH_3-G) according to APHA (1989). To determine the actual ammonia-N concentrations during the experiment, 3 samples from each sub-lethal ammonia-N treatment were taken on the first and last day of the experiment and similarly analysed for ammonia-N at the ACTFR. The mean values of ammonia-N did not deviate from the stated concentrations by more than 5 %. Between the two days, the means of the actual concentrations in each treatment did not deviate by more than 3 % and the average of these actual values, of 0.706 and 2.798 mmol l^{-1} ammonia-N and will be used throughout to indicate these two sub-lethal ammonia-N treatments.

After each haemolymph sampling, 4 crabs were immersion fixed in a 10% (v/v) FAACC formalin solution (4 % formaldehyde, 5 % acetic acid and 1.3 % calcium chloride) for

3 days and transferred to 70 % (v/v) ethanol until further processing. For the histological examination of the gills, the crabs were progressively dehydrated at increasing concentrations of alcohol and embedded in paraffin wax. Sections (5 µm) of the crab were cut using a rotary Leitz microtome (model 1512, US) and stained with hematoxylin and eosin. The anterior and posterior gill structures were examined and digitally photographed under a light microscope.

All crabs subjected to haemolymph sampling were removed from the experiment and not used a second time. Following the 48-h exposure to either 0.714 or 2.857 mmol l^{-1} ammonia-N, all remaining crabs were immediately transferred to pristine seawater with no added ammonia-N to initiate a 96-h recovery period. To determine the duration required to stabilise haemolymph osmolality, Na^+, K^+ and Ca^{2+}, pH and ammonia-N levels, as well as normalisation of the gill structure post sub-lethal ammonia-N exposure, 8 crabs were sampled at 1, 3, 6, 12, 24, 36, 48, 72, 84, 96-h, respectively adopting the same procedure mentioned above. However, during this recovery experiment, once the haemolymph ammonia-N levels had returned to those of the pre-exposure level, no further analysis was performed.

2.2. Experiment 2

To determine if osmo-ionoregulation and haemolymph pH regulation in early *P. pelagicus* juveniles is interrupted by ammonia-N induced morbidity, 16 crabs were exposed to lethal concentrations of 7.142 and 10.714 mmol l^{-1} ammonia-N (or 100 and 150 mg l^{-1} ammonia-N) (Chapter 3), and another 16 crabs served as a control (no ammonia-N added). Similar experimental protocols and the same crab stage/batch were used as those of experiment 1 (see 2.1.1). However hourly observations for moribund crabs were made until 24-h only (48-h in experiment 1). Morbidity was diagnosed as the crabs exhibiting severe disorientation (*e.g.* an inability to remain upright) and/or little movement when gently stimulated with a glass rod.

When a moribund crab was observed, it was sampled immediately for haemolymph osmolality, Na^+, K^+, Ca^{2+} and pH levels measurements using the methods described in 2.2.2. For purposes of comparison, a control crab was simultaneously sampled for analysis. To determine the ammonia-N levels of the water, 3 samples from each treatment were measured using the methods described in 2.2.2. The actual values in the 7.142 and 10.714 mmol l^{-1} ammonia-N treatments were 7.036 and 10.518 mmol l^{-1} ammonia-N, respectively and these values will be used throughout the text.

2.3. Data analysis

The haemolymph ammonia-N, Na^+, K^+ and Ca^{2+} levels are expressed as mmol l^{-1}. To convert mmol l^{-1} to mg l^{-1}, multiply mmol l^{-1} by the respective molecular weights. In the case of ammonia-N conversions to mg l^{-1}, the molecular weight of nitrogen is used.

Since the damage to the anterior and posterior gills following elevated ammonia-N exposure appeared similar, only the anterior gill structural changes were quantified. To quantify these histopathological changes, from each ammonia-N treatment and exposure/recovery duration, 15 lamellae were randomly chosen from each of the 4 replicate crabs for measurements of lamellae diameter (based on the distance from each epithelia to quantify swelling or collapse) and number of haemocytes present within the lamellae. The lamellae diameter was measured using a micrometer (1 µm) and the number of haemocytes was counted within each lamellae. The data from the 15 lamellae, of each replicate crab, were then pooled for statistical analysis.

To determine any significant effects ($p < 0.05$) of the ammonia-N levels and exposure/recovery duration on the haemolymph composition or quantified gill histological measurements either a one-way or a two-way ANOVA was used. To determine any significant

effects ($p < 0.05$) of ammonia-N induced morbidity on the haemolymph osmolality, Na^+, K^+, Ca^{2+} and pH levels a one-way ANOVA was used. Differences between treatments were determined using Duncan's Multiple Range Test (Duncan, 1955) using the SPSS statistical software version 16.0.

3. Results

3.1. Haemolymph osmolality, Na^+, K^+, Ca^2, pH, THC and ammonia-N

In both experiment 1 and 2, the haemolymph osmolality and Na^+, K^+, Ca^{2+} levels of the crabs remained above that of the external medium at a salinity of 30 ‰ (compostion of seawater presented in Table 1) indicating hyperosmo-ionoregulation (Fig. 1A, B, C, D; Table 1). The results of experiment 1 showed that exposure to sublethal ammonia-N levels of 0.706 and 2.789 mmol l^{-1} up to 48-h had no significant effect ($p > 0.05$) on the haemolymph osmolality, Na^+, K^+, Ca^{2+} or pH levels. The haemolymph osmolality and pH of the crabs from the experiment 1 ranged from 896 to 915 (mOsm kg^{-1}) (Fig. 1A) and 7.4 to 7.9 (Fig. 2), respectivley, while mean haemolymph Na^+, K^+ and Ca^{2+} levels fluctuacted from 508 to 516, 10 to 15 and 10.8 to 14 mmol l^{-1}, respectively throughout the 48-h exposure and subsequent 96-h recovery period (Fig 1B, C, D).

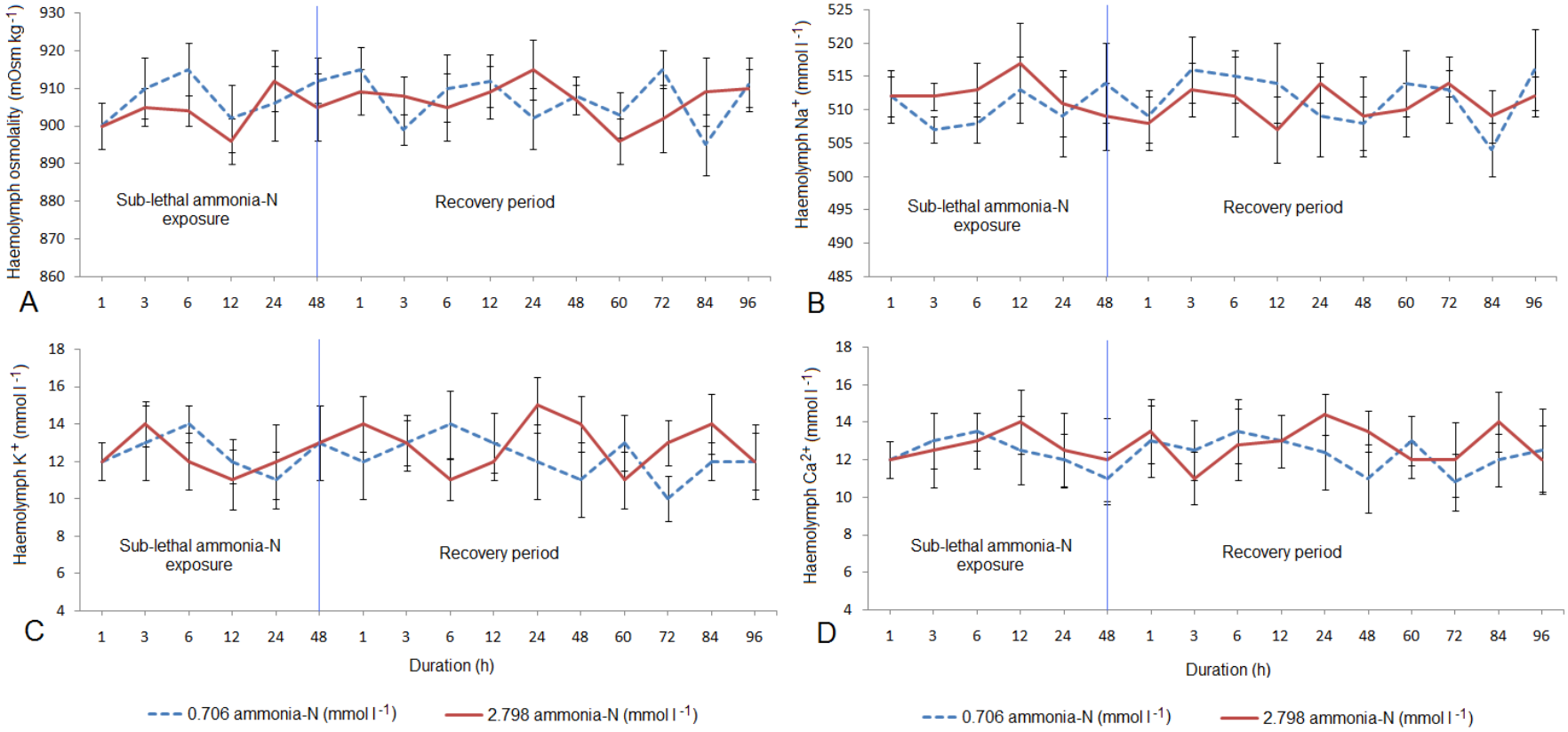

Figure 1: The mean haemolymph (A) osmolality (mOsm kg^{-1}), (B) Na^{+}, (C) K^{+} and (D) Ca^{2+} levels (mmol l^{-1}) (± SE) of early *Portunus pelagicus* juveniles exposed to two sub-lethal ammonia-N levels of 0.706 (dashed line) and 2.798 (solid line) mmol l^{-1} over 48-h and post-exposure recovery period. No significant time or ammonia-N effect were deteced ($p > 0.05$).

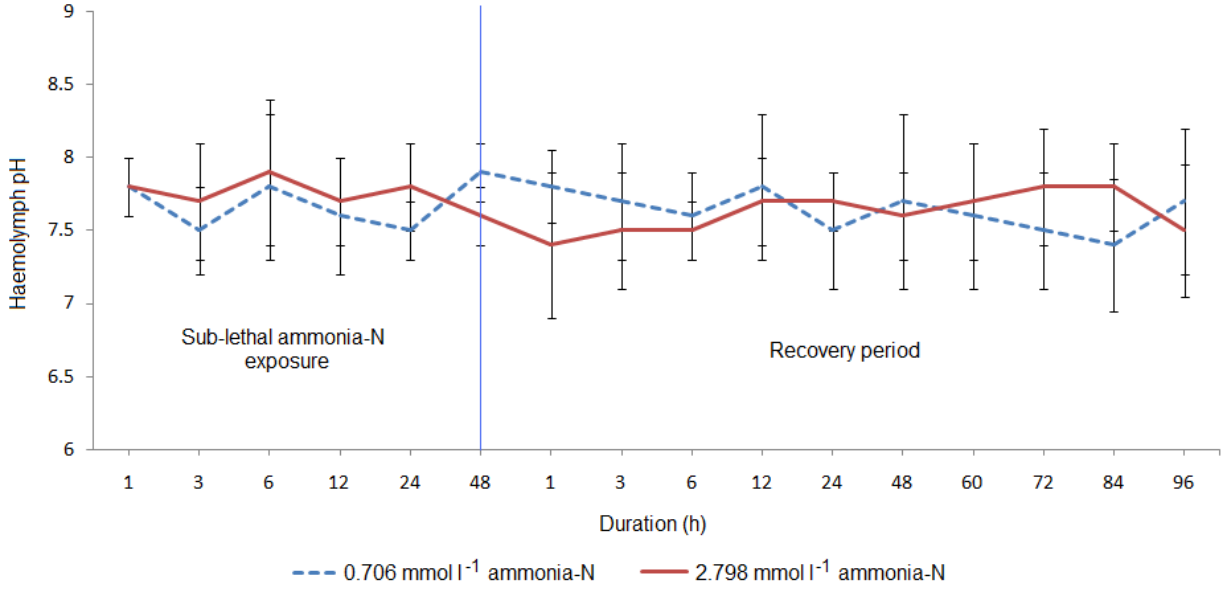

Figure 2: The mean haemolymph pH (± SE) of early *Portunus pelagicus* juveniles exposed to two sub-lethal ammonia-N levels of 0.706 (dashed line) and 2.798 (solid line) mmol l^{-1} over 48-h and post-exposure recovery period. No significant time or ammonia-N effect were deteced ($p > 0.05$).

Similarily in experiment 2, moribound crabs induced by exposed to subtantially higher lethal ammonia-N levels of 7.036 and 10.518 mmol l^{-1} also showed no significant change ($p < 0.05$) on their haemolymph Na^+, K^+, Ca^{2+} and pH when compared to the control crabs (Table 1). The mean haemolymph osmolality and the haemolymph Na^+, K^+, Ca^{2+} levels of the moribound crabs were 908 ± 5.96 (mOsm kg^{-1}), and 510.2 ± 5.2, 12.0 ± 0.3, 12.0 ± 0.4 mmol l^{-1}, respectively, while the mean pH was 7.59 ± 0.23 (Table 1). A two-way ANOVA detected no significant time (of exposure and recovery duration), ammonia-N level or interaction effect ($p > 0.05$) on the haemolyph osmolality, Na^+, K^+, Ca^{2+} or pH levels.

Table 1: The osmolality, Na^+, K^+, Ca^{2+} and pH levels of the seawater (30 ‰) and in the haemolymph (± SE) of the control and moribund early *Portunus pelagicus* crabs induced by exposure to lethal concentrations of ammonia-N (7.142 and 10.714 mmol l^{-1}).

Osmolality (mOsm kg^{-1}), ionic composition (mmol l^{-1}) and pH of seawater at a salinity of 30 ‰					
	Osmolality	Sodium	Potassium	Calcium	pH
	840	393.1	8.5	9.5	8.1
Osmolality (mOsm kg^{-1}), ionic composition (mmol l^{-1}) and pH of the crab haemolymph					
Ammonia-N treatment	Osmolality	Sodium	Potassium	Calcium	pH
Control	908 ± 5.96 **a***	510.2 ± 5.2 **a**	12.0 ± 0.3 **a**	12.0 ± 0.4 **a**	7.59 ± 0.23 **a**
7.036 mmol l^{-1}	903 ± 9.5 **a**	513.0 ± 6.3 **a**	12.8 ± 2.16 **a**	12.2 ± 0.9 **a**	7.69 ± 0.20 **a**
10.518 mmol l^{-1}	907 ± 8.3 **a**	512.8 ± 7.3 **a**	12.5 ± 1.76 **a**	12.2 ± 1.0 **a**	7.61 ± 0.18 **a**

*Same superscript letters within each column indicates no significant differences ($p > 0.05$).

In contrast to the haemolymph osmolality and ions, both the haemolymph ammonia-N levels and THC of the crabs exposed to the sub-lehtal ammonia-N levels showed an increasing trend with both the ammonia-N concentration and duration of exposure. The mean haemolymph ammonia-N levels of the control crabs (no added ammonia-N) was low at 0.69 × 10^{-3} mmol l^{-1}. However, within one hour of exposure to 0.706 and 2.798 mmol l^{-1} ammonia-N the haemolymph ammonia-N levels significantly increased ($p < 0.01$) (Fig. 3). The highest haemolymph ammonia-N levels of 0.361 and 1.692 mmol l^{-1} occurred at 48-h, which was the longest exposure duration, at 0.706 and 2.798 mmol l^{-1} ammonia-N, respectively (Fig. 3). However, within one hour of post-exposure recovery from 2.798 mmol l^{-1} ammonia-N, the haemolymph ammonia-N significantly decreased ($p < 0.01$) from the 48-h exposure peak. By 12-h post-exposure to both sub-lethal ammonia-N levels the haemolymph ammonia-N of the crabs was not significantly different ($p > 0.05$) from those in the control (Fig. 3). A two-way ANOVA detected a significant time (of exposure and recovery duration) and ammonia-N

concentration effect ($p < 0.01$) on the haemolymph ammonia-N levels, however, no significant interaction was detected ($p > 0.05$).

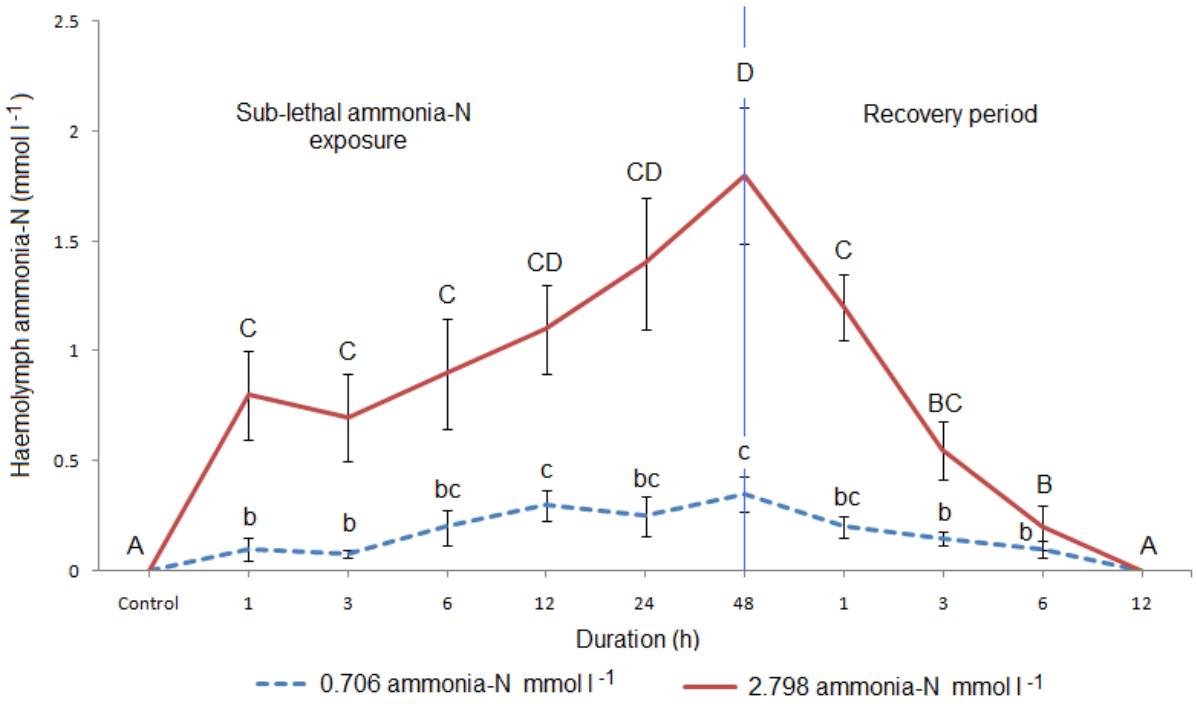

Figure 3: The mean haemolymph ammonia-N levels (mmol l^{-1}) (± SE) of early *Portunus pelagicus* juveniles exposed to two sub-lethal ammonia-N levels of 0.706 (dashed line) and 2.798 (solid line) mmol l^{-1} over 48-h and post-exposure recovery period. Different lower and upper case letters indicate significant differences ($p < 0.05$) within 0.706 and 2.798 mmol l^{-1} ammonia-N, respectively.

The mean haemolymph THC of the control crabs was 29.51 ($\times 10^4$ cells ml^{-1}), however by 12-h and 6-h to f exposure to 0.706 and 2.798 mmol l^{-1} ammonia-N, respectively, the haemolymph THC significantly increased ($p < 0.01$) (Fig. 4). Once this significant THC increase occurred, it continued throughout the exposure to both 0.706 and 2.798 mmol l^{-1} ammonia-N. At the 84-h recovery from post-exposure to both sub-lethal ammonia-N levels, the haemolymph THC of the crabs significantly decreased ($p < 0.05$) to levels that were not significantly different ($p > 0.05$) from those in the control (Fig. 4). A two-way ANOVA detected a significant time effect (of exposure and recovery duration) ($p < 0.05$) on the

haemolymph THC, however, no significant ammonia-N level or interaction effect was detected ($p > 0.05$).

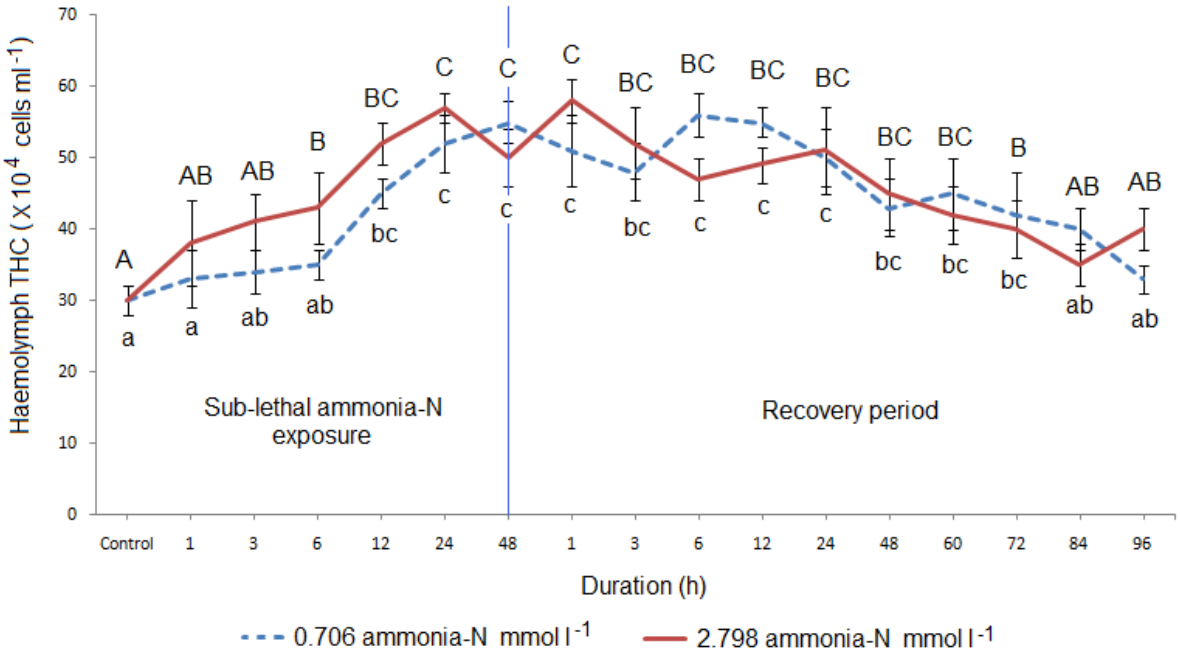

Figure 4: The mean haemolymph total haemocyte count (THC) (± SE) of early *Portunus pelagicus* juveniles exposed to sub-lethal ammona-N levels of 0.706 (dashed line) and 2.798 (solid line) mmol l^{-1} over 48-h and post-exposure recovery period. Different lower and upper case letters indicate significant differences ($p < 0.05$) within 0.706 and 2.798 mmol l^{-1} ammonia-N, respectively.

3.2. Gill histopathological changes

The anterior lamellae exposed of the control crabs showed intact pillar cells (PC), occasional presence of haemocytes (HAE) within the lamellae and a thin epithelium (Fig. 5A, B). Although no mortalities occurred during the 48-h exposure to 0.706 and 2.798 mmol l^{-1} ammonia-N, at 3-h and 1-h, respectively, the gill lamellae width significantly decreased ($p < 0.01$) and the number of haemocytes (HAE) significantly increased ($p < 0.05$) within the gill lamellae of these crabs. Such gill histopathological changes lasted throughout the rest of 48-h exposure period at both sub-lethal ammonia-N levels, although the degree of these changes was greater at the higher ammonia-N level of 2.798 mmol l^{-1} (Table 2). Other histological changes, which were difficult to quantify, also occurred throughout the exposure duration, which

included epithelial damage (*e.g.* sloughing, thickening and detachment) and disrupted/necrotic pillar cells (DPC) leading to lamallae distortion and a complete breakdown in the intralamellar septum (Fig. 6A – 6F).

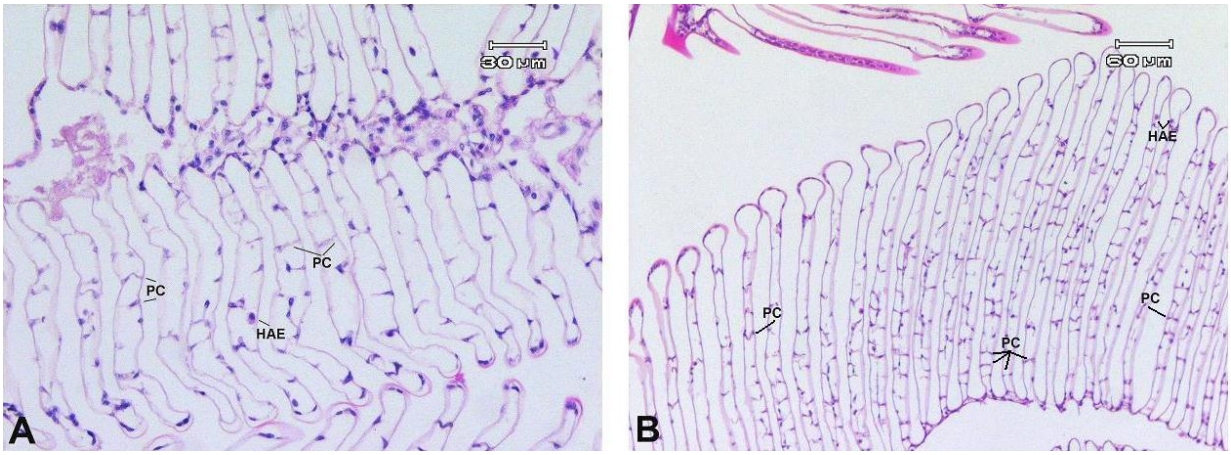

Figure 5: The anterior gills of early *Portunus pelagicus* juveniles showing the lamallae of control crabs. Note the presence of intact pillar cells (PC), low incidences of haemocytes (HAE) within the lamellae and undamaged epithelium. (A) Magnification × 20 and (B) magnification × 10; (A) Bar = 30 µm and (B) 60 µm.

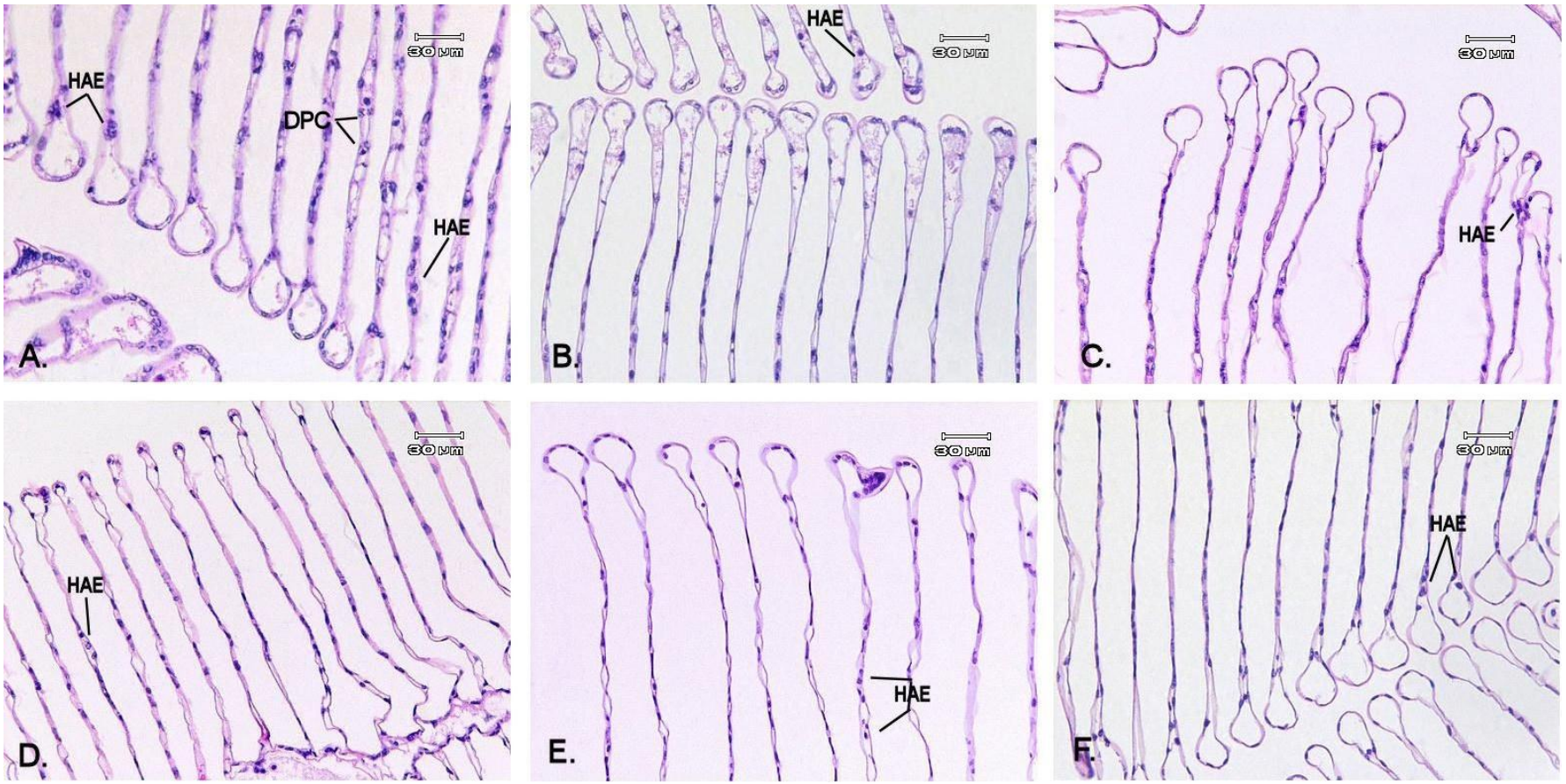

Figure 6: Time sequence to the anterior gill changes of early *Portunus pelagicus* juveniles exposed to 2.857 mmol l^{-1} ammonia-N for (A) 1-h, (B) 6-h, (C) 12-h, (D) 24-h, (E) 36-h and (F) 48-h. Notice the drastic structural changes that occurred at 1-h of exposure which includes lamellae constriction/collapse leading to a reduction in the intralamellar space, disrupted pillar cells (DPC), epithelial thickening and presence of haemocytes (HAE). Also notice the presence of haemocytes (HAE) in each exposure hour. Although disrupted pillar cells are difficult to observe for the subsequent hours of exposure due to the nearly complete collapse of the lamellae, this phenomenon is implied since the primary function is structural support.

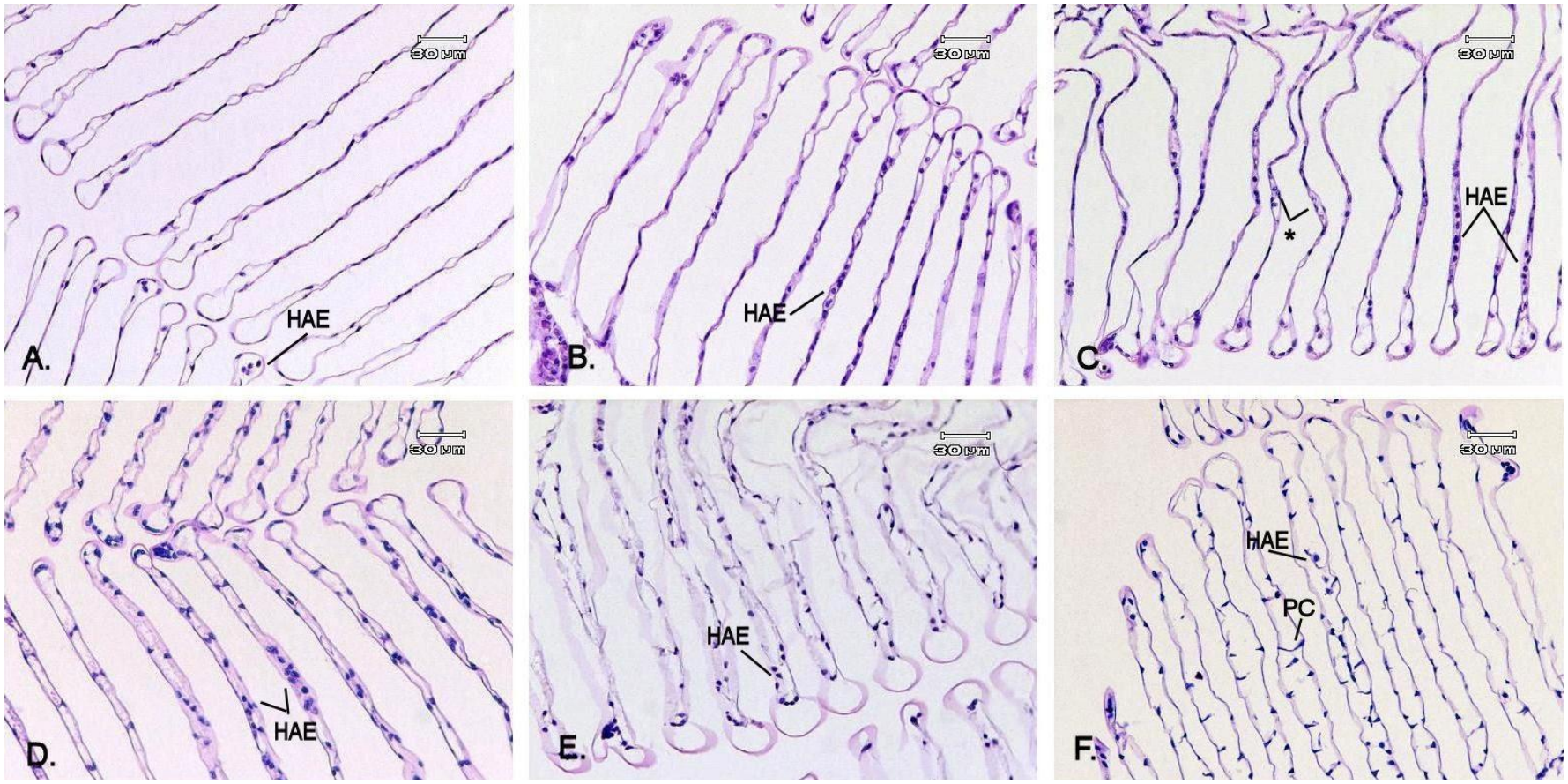

Figure 7: Time sequence to the anterior gill recovery to ammonia-N free seawater of early *Porutnus pelagicus* juveniles from a 96-h exposure to 2.857 mmol l^{-1} ammonia-N at (A) 1-h, (B) 6-h, (C), 12-h, (D), 24-h, (E) 36-h and (F) 48-h. Note the amount of collapsed lamellae in the 1 and 6-h lamellae and some "bubbling" (*) occurring at 12-h recovery where the lamellae are beginning to expand. An expansion of the gill lamellae continues and increases as the recovery time increases to ammonia-N free seawater at 24, 36 and 48-h where a nearly complete recovery has occurred by 48-h. Also note the high prevalence of haemocytes (HAE) in each recovery time.

When the recovery period was initiated, an increase to the gill lamellae width and decrease in the haemocyte number within the gill lamellae occurred and these were more rapid for the crabs exposed to 0.706 mmol l^{-1} ammonia-N than those exposed to 2.798 mmol l^{-1} ammonia-N (Table 2). At 24-h of recovery, the lamellae width of the crabs previously exposed to 0.706 mmol l^{-1} ammonia-N significantly increased ($p < 0.01$) while the haemocyte number decreased ($p < 0.05$) when compared to the control. However for the crabs previously exposed to 2.798 mmol l^{-1} ammonia-N, a substantially longer recovery period of 48-h and 72-h was required for significantly increased lamellae width ($p < 0.01$) and decreased haemocyte number (HAE) ($p < 0.05$) to occur, respectively (Table 2). In addition, coinciding with significant increases to lamellae width and decrease in number of haemocytes within the lamellae, other signs of gill normalisation also occurred, which included pillar cell restructuring, epithelial healing, and a clearing of the intralamellae septum (Fig.7A – 7F). A two-way ANOVA detected a significant treatment and time effect (of both exposure and recovery duration) on the lamellae width and haemocyte number with the lamellae of early *P. pelagicus* juveniles, however no significant interaction ($p < 0.05$) was detected.

Table 2: The mean lamellae width (µm) (± SE) and haemocyte number (± SE) within the anterior lamellae of early *Portunus pelagicus* juveniles exposed to two sub-lethal ammonia-N levels over 48-h and the 96-h post-exposure recovery period.

	Sub-lethal ammonia-N concentration			
	0.706 mmol l^{-1} ammonia-N		2.798 $mmol^{-1}$ ammonia-N	
Exposure duration	Lamellae width (µm)	Haemocyte number	Lamellae width (µm)	Haemocyte number
0-h (control)	15.08 ± 0.82 ^{a}	1.05 ± 0.15 ^{a}	15.08 ± 0.82 ^{a}	1.05 ± 0.15 ^{a}
1-h	11.69 ± 4.59 ^{a,b}	2.32 ± 1.52 ^{a}	4.56 ± 2.91 ^{c}	5.75 ± 0.50 ^{b}
3-h	9.32 ± 3.56 ^{b}	5.48 ± 1.54 ^{b}	3.84 ± 2.14 ^{c}	4.29 ± 0.26 ^{b}
6-h	9.62 ± 3.86 ^{b}	4.68 ± 1.20 ^{b}	3.95 ± 2.08 ^{c}	5.23 ± 1.82 ^{b}
12-h	8.94 ± 2.45 ^{b}	4.87 ± 2.14 ^{b}	2.54 ± 1.23 ^{c,d}	4.80 ± 1.42 ^{a,b}
24-h	8.08 ± 2.60 ^{b}	6.32 ± 2.37 ^{b}	1.01 ± 0.92 ^{d}	4.95 ± 1.10 ^{b}
36-h	7.99 ± 1.68 ^{b}	5.79 ± 2.15 ^{b}	1.13 ± 1.42 ^{d}	5.28 ± 0.95 ^{b}
48-h	8.23 ± 2.26 ^{b}	4.95 ± 2.20 ^{b}	1.03 ± 1.05 ^{d}	5.25 ± 1.20 ^{b}
Post-exposure recovery				
1-h	8.14 ± 2.57 ^{b}	5.82 ± 1.38 ^{b}	1.86 ± 1.09 ^{d}	7.85 ± 0.58 ^{c}
3-h	8.78 ± 3.42 ^{b}	4.76 ± 1.64 ^{b}	2.92 ± 1.76 ^{c}	6.59 ± 1.29 ^{b,c}
6-h	10.54 ± 3.51 ^{a,b}	5.73 ± 2.89 ^{b}	2.24 ± 1.60 ^{c}	4.21 ± 1.57 ^{b}
12-h	11.42 ± 3.09 ^{a,b}	3.54 ± 1.85 ^{a,b}	5.89 ± 2.09 ^{c,b}	4.65 ± 2.38 ^{b}
24-h	12.89 ± 2.78 ^{a}	3.05 ± 1.80 ^{a,b}	9.67 ± 2.89 ^{b}	3.68 ± 2.41 ^{b}
36-h	13.73 ± 2.59 ^{a}	1.75 ± 0.25 ^{a}	12.19 ± 2.11 ^{b}	3.62 ± 1.52 ^{b}
48-h	15.21 ± 1.23 ^{a}	1.60 ± 0.30 ^{a}	16.20 ± 1.65 ^{a}	2.58 ± 2.20 ^{b}
60-h	15.09 ± 1.42 ^{a}	1.05 ± 0.58 ^{a}	15.68 ± 1.05 ^{a}	2.03 ± 1.02 ^{a,b}
72-h	15.34 ± 1.02 ^{a}	1.42 ± 0.28 ^{a}	14.23 ± 1.23 ^{a}	1.26 ± 0.42 ^{a}
84-h	15.19 ± 0.54 ^{a}	1.27 ± 0.30 ^{a}	15.26 ± 1.09 ^{a}	1.17 ± 0.22 ^{a}
96-h	15.10 ± 0.92 ^{a}	1.14 ± 0.25 ^{a}	14.98 ± 1.15 ^{a}	1.21 ± 0.31 ^{a}

* Different letters within each column indicate significant differences ($p < 0.05$).

4. Discussion

Fluctuating ammonia-N levels in an aquaculture setting can be routinely experienced by crustaceans which may affect their productivity and survival (Rebelo et al. 2000; Romano and Zeng, 2007a; Miron et al. 2008; Chapter 3). Since the gills of aquatic animals are particularly susceptible to potential pollutants, the conditions of the current experiment were created to

examine/quantify the effects of sub-lethal ammonia-N exposure and subsequent recovery on the gill structure of early *P. pelagicus* juveniles in sequence with a series of haemolymph parameters closely associated with their function.

The results clearly show that despite exposure to ammonia-N levels substantially below the threshold of lethality (Chapter 3), the gills of early *P. pelagicus* crabs showed drastic histopathological changes within a short period of exposure. These observed changes included epithelial damage/thickening, disrupted pillar cells due to necrosis, increased presence of haemocytes within the lamellae space, lamellae distortion and lamellae constriction/collapse. These findings are in agreement with our previous study on similar sized crabs of the same species during an acute ammonia-N toxicity experiment (Chapter 3). One of the most drastic histopathological changes in the current study was lamellae collapse, which occurred within 3-h and 1-h of 0.706 and 2.798 mmol l^{-1} ammonia-N exposure, respectively, and once this occurred, it persisted to many of the lamellae throughout the remaining duration of exposure (*i.e.* 48-h). This phenomenon within a relatively short time frame and at sub-lethal ammonia-N levels was unexpected since, in our previous acute ammonia-N toxicity experiment utilising a longer exposure duration (of 96-h) and higher ammonia-N levels (up to 7.137 mmol l^{-1}), lamellae collapse was only detected at the highest ammonia-N concentrations close to or at their 96-h LC_{50} values (Chapter 3). Although it may be possible that the longer ammonia-N exposure of our previous experiment allowed time for the crabs to adapt/acclimate at the lower ammonia-N concentrations, since a leveling of LC_{50} values at 60-h onwards due to a reduction in mortality frequency is typical of early *P. pelagicus* (Chapter 3) and other crustaceans (Lin and Chen, 2001), further experimentation on a longer time frame is needed to confirm this.

In spite of lamellae collapse there was, indeed, evidence suggesting a healing response of the crabs since the total haemocyte count (THC) in the haemolymph, which are responsible

for the phagocytosis of foreign material/necrotic tissue (Battistella et al. 1996; Johansson et al. 2000), significantly increased at 12-h and 6-h of exposure to 0.706 and 2.798 mmol l^{-1} ammonia-N, respectively. This rapid immunological response coinciding with a significant increase to the haemocytes within the gill lamellae (at 6-h and 1-h of exposure 0.706 and 2.798 mmol l^{-1} ammonia-N, respectively) may indicate that this was initiated to cope with gill structure damage. The increase of haemocytes within the haemolymph and/or gill lamellae appears to be a common response for early *P. pelagicus* juveniles at increased nutrient concentrations based on our previous investigations following their exposure to elevated NH_4-N, NO_2-N, NO_3-N, KNO_2, KNO_3 and KCl-K levels (Chapter 3; Romano and Zeng, 2007b; Romano and Zeng, 2009b). This may therefore have some potential as a non-specific biomarker for this species to elevated nutrient exposure. Interestingly, these results are in direct contrast with previous reports on other crustaceans including the giant freshwater prawn *Macrobrachium rosenbergii* (Cheng and Chen, 2002), the Kuruma shrimp *Penaeus japonicus* (Jiang et al. 2004), the white shrimp *Litopenaeus vannamei* (Liu and Chen, 2004), the Chinese mitten-handed crab *Eriocheir sinensis* (Hong et al. 2007), the Indian spiny lobster *Panulirus homarus* (Verghese et al. 2007), the blue shrimp *Litopenaeus stylirostris* (Mugnier et al. 2008) and the southern white shrimp *Litopenaeus schmitti* (Rodríguez-Ramos, 2008) since an increase in external ammonia-N levels reportedly caused a significant decrease in haemolymph THC. It is unclear if such a haemolymph THC reduction in these species would diminish their healing capacity of the gills and further investigations may be warranted due to the increasing threat of anthropogenic discharges to various ecosystems.

During the initiated recovery period following their exposure to both sub-lethal ammonia-N exposure after 48-h, obvious signs of gill healing occurred and, to the best of our knowledge, our experiment appears to be the first to describe and quantify such an occurrence.

Based on observations and quantification of the gill lamellae, the minimum duration to allow for a complete recovery from a 48-h exposure to 0.706 and 2.798 mmol l^{-1} ammonia-N was 24 and 48-h, respectively. Although this recovery was clearly dependent on both the previous ammonia-N exposure concentration and duration of recovery, individual variability was also noticed. Nevertheless, the first signs of recovery appeared similar which included localised expansions along the gill lamellae allowing for increases in the intralamellae septum (*i.e.* space within the lamellae). This presumably permitted a further infiltration of haemocytes to heal and phagocytise dead/damaged tissue (Harrison and Humes, 1992). This was generally followed by a greater expansion to the intralamellar septum, epithelial healing/thinning, reduced presence of haemocytes and pillar cell normalisation until the lamellae completely expanded and the structure was restored to the pre-exposed state. While this demonstrates that gill healing is possible following gill damage induced by the temporary exposure to sub-lethal ammonia-N concentrations, it is unclear if exposure to other pollutants being discharged into various ecosystems would elicit a similar healing response and warrants further investigations.

Interestingly, despite severe gill damage occurring soon after the crabs were transferred to sub-lethal ammonia-N levels, which persisted to the end of 48-h exposure, no significant changes to the haemolymph osmolality, Na^+, K^+, Ca^{2+} or pH levels of *P. pelagicus* juveniles were detected while the haemolymph ammonia-N levels remained substantially lower than the external environment. Furthermore, at ammonia-N levels of 10 fold higher, chosen to induce the morbidity of these crabs, a similar result of undisrupted haemolymph osmolality, ions and pH was obtained. Although early *P. pelagicus* juveniles are known to be weak osmoregulators (Chapter 2), the haemolymph ions were still different from the environment indicating ion regulation at a salinity of 30 ‰. Therefore these results suggest that, even for moribund crabs, gill enzymes (*i.e.* carbonic anhydrase and Na^+/K^+-ATPase activity) responsible for transporting

ions, regulating pH and excreting ammonia-N across a gradient were still functioning. Undisrupted haemolymph osmolality and/or Na^+ ions are in contrast to other crustaceans such as the American clawed lobster *Homarus americanus* (Young-Lai et al. 1991), *P. japonicus* (Chen and Chen, 1996), the freshwater crayfish *Pacifastacus leniusculus* (Harris et al. 2001) and the mud crab *Scylla serrata* (Romano and Zeng, 2007a). It is believed that reduced haemolymph Na^+ ions, caused by ammonia-N exposure, was the result of either a breakdown of the NH_4^+/Na^+ excretion process (Young-Lai et al. 1991; Chen and Chen, 1996) or a depolarisation of the cell membrane (Harris et al. 2000). However for the estuarine crab, *Neohelice* (*Chasmagnathus*) *granulata*, no significant reduction in haemolymph Na^+ ions were detected in spite of ammonia-N levels reaching lethal levels and causing severe gill damage including lamellae collapse (Rebelo et al. 2000). These authors suggested that this result may be linked with the relatively strong osmoregulatory abilities of *N. granulata*. However such a suggestion can unlikely be generalised for all crustaceans since, in the current study, *P. pelagicus* experienced no significant change to haemolymph Na^+ levels in spite of being relatively weak osmoregulators (Chapter 2). Further, in contrast, it was previously shown that elevated ammonia-N levels significantly reduced the haemolymph Na^+ ions in *S. serrata* (Romano and Zeng, 2007a) despite this crab species being considered a relatively strong osmoregulator (Chen and Chia, 1997). These results suggest highly species-specific responses/adaptations of crustaceans when challenged with elevated ammonia-N levels and, furthermore, that gill damage may not necessarily be linked with disrupted osmoregulation.

It seems possible that undisrupted osmo-ionregulation may contribute to the relatively high tolerance of *P. pelagicus* juveniles to ammonia-N (Chapter 3), although another likely contributing factor was the substantially lower haemolymph ammonia-N levels of the crabs compared to the external environment throughout the exposure duration. This general finding

has been similarly reported for other crustaceans (*e.g.* Schmitt and Uglow, 1997; Rebelo et al. 1999; Harris et al. 2001; Weihrauch et al. 1999) and attributed to various factors including low gill permeability, active ammonia-N excretion and/or ammonia-N detoxification depending on the species (Schmitt and Uglow, 1997; Weihrauch et al. 1999). However direct comparisons between species is difficult since previous studies utilise different experimental conditions which affect the more permeable (and toxic) NH_3 ratios. Since NH_3 easily crosses gill bilayers, the proposed mechanism for active ammonia-N excretion is the haemolymph protonation of NH_3 to NH_4^+ which is subsequently excreted to the environment (Weihrauch et al. 2004) and such a process was suggested to contribute to significantly increasing the haemolymph pH of early *S. serrata* juveniles (Romano and Zeng, 2007a). However, in the current study, the haemolymph pH of *P. pelagicus* juveniles remained unaltered throughout the sub-lethal ammonia-N exposure or at ammonia-N levels causing morbidity. Rebelo et al. (2000) detected a similar result with *N. granulata* juveniles and suggested a compensatory effect occurred as a result of increasing haemolymph CO_2 (leading to decreased haemolymph pH) closely matching with haemolymph NH_3 protonation (leading to increased haemolymph pH). Obviously, further experimentation is warranted to determine the extent of ammonia-N exposure on haemolymph CO_2/O_2 levels in this species to help explain the exact cause(s) for the unaltered haemolymph pH and potential mechanisms to ammonia-N induced morbidity.

It appears that many of the undisrupted physiological responses to ammonia-N following either sub-lethal or lethal ammonia-N levels likely contribute to the high ammonia-N tolerance of early *P. pelagicus* juveniles. However, the underlying mechanisms leading to this result is still unclear. This therefore led to Chapter 5 to help elucidate these findings by examining, among other parameters, gill Na^+/K^+-ATPase activity and ammonia-N excretion of early *P. pelagicus* juveniles when exposed to different ammonia-N and salinity combinations .

Chapter 5

Ammonia-N, salinity and the combination of both on the associated coping processes of early *Portunus pelagicus* juveniles

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1. Introduction

It has been previously demonstrated that both ammonia-N and salinity are highly important abiotic factors that can potentially affect the productivity and physiology of early *P. pelagicus* juveniles (Chapters 2, 3 and 4). However, simultaneously investigating their combined effects have not yet been performed on either this species or other crustaceans. Such an investigation would be considered desirable since both ammonia-N and salinity may vary substantially in aquaculture systems, as well as in nature. Furthermore, the coping processes associated with elevated ammonia-N exposure and osmoregulation are linked via ouabain sensitive gill Na^+/K^+-ATPase activity.

When crustaceans are exposed to elevated ammonia-N levels this can lead to active ammonia-N excretion which is a two step process. Based on past experiments the current model suggests that NH_4^+ can substitute for K^+ via basolaterally located Na^+/K^+-ATPase activity, and NH_4^+ is then excreted to the environment via an apically located Na^+/NH_4^+ transporters (reviewed by Weihrauch et al. 2004). This likely explains the observed gill Na^+/K^+-ATPase activity increase to elevated ammonia-N levels during both *in vivo* (Chen and Nan, 1992; Wang et al. 2004) and *in vitro* (Furriel et al. 2004; Masui et al. 2002; 2005; Garçon et al. 2007) experiments. However, excessively high ammonia-N levels may disrupt this process (Chen and Nan, 1992; Wang et al. 2004), which was suggested to be the cause of

significantly reduced haemolymph osmolality and/or haemolymph Na^+ ions of crustaceans (Young-Lai et al. 1991; Chen and Chen, 1996; Harris et al. 2001; Romano and Zeng, 2007a).

In addition to ammonia-N potentially altering the gill Na^+/K^+-ATPase activity of crustaceans, salinity is also well known to influence this response and it is therefore possible to gauge both the osmoregulatory and ammonia-N excretion responses of crustaceans via measuring their gill Na^+/K^+-ATPase activity (Weihrauch et al. 2004). Typically at low salinities, gill Na^+/K^+-ATPase activity of estuarine or marine crustaceans increases (Holliday, 1985; Piller et al. 1995; Castilho et al. 2001; López-Mañanes et al. 2002; Genovese et al. 2004; Torres et al. 2007; Lucu et al. 2008) to enhance the rate of haemolymph Na^+ and Cl^- uptake from the environment. In contrast, gill Na^+/K^+-ATPase activity at high salinities is more varied, including increases (Kamemoto, 1991; Holliday et al. 1990; McLaughlin et al. 1996), no significant change (Genovese et al. 2004, Chung and Lin, 2006; Lucu et al. 2008) or decreases (Castiho et al. 2001; Torres et al. 2007). These different findings at high salinities indicate highly species-specific responses as well as the suggestion that Na^+/K^+-ATPase activity may be involved in hypo-osmoregulation (McLaughlin et al. 1996; Lucu and Towle, 2003).

Estuarine systems may routinely present environmental challenges to crustaceans due to wide salinity fluctuations (de Lestang et al. 2003) while the potential to encounter elevated ammonia-N levels is also greater due to anthropogenic discharges and/or burying in sediments with high organic contents (Weihrauch et al. 1999; Dave and Nilsson, 2005). This is likely to be relevant to *P. pelagicus*, due to their wide distribution and commercial importance throughout the Indo-Pacific region where the early juvenile life stages commonly inhabit estuarine systems for months (de Lestang et al. 2003).

While evidence indicates that the mechanisms to cope with elevated ammonia-N and extreme salinity levels in crustaceans are linked, the acute ammonia-N tolerance of penaeid shrimps decrease with decreasing salinities (Chen and Lin, 1991; Chen and Lin, 1992b; Lin and Chen, 2001; Kir and Kumlu, 2006; Li et al. 2007). This prompts interesting questions as to how crabs respond and adapt when subjected to varied salinity and ammonia-N combinations particularly since it has been previously demonstrated that early *P. pelagicus* juveniles are weak osmoregulators (Chapter 2) with a high ammonia-N tolerance (Chapter 3). However, it appears no investigators have yet attempted to link gill Na^+/K^+-ATPase activity, ammonia-N excretion and haemolymph ammonia-N levels from the same crustacean species when simultaneously subjected to various ammonia-N and salinity levels. The aim of the current experiment was to hence investigate the responses of early *P. pelagicus* juveniles at different salinity and ammonia-N combinations, which include haemolymph osmolality, haemolymph Na^+, K^+, Ca^{2+} and ammonia-N levels along with ammonia-N excretion rates and gill Na^+/K^+-ATPase activity.

2. Materials and Methods

2.1. Source of experimental crabs and acclimation

The crabs were larvicultured according to the protocol described in Chapter 2. Upon metamorphosis to the C1 crab stage, crabs were further cultured according to the protocol described in Chapter 3. Once the crabs reached the C7 stage, which took approximately 3 weeks, they were transferred to 300-L tanks for a one week acclimation to 15, 30 and 45 ‰ at a rate of 3 ‰ h^{-1} until the desired levels were reached. Salinities of 15 ‰ and 30 ‰ were created via additions of de-chlorinated freshwater to the source seawater of 32 ‰. The salinity of 45 ‰ was made by additions of brine to the source seawater. Brine was previously prepared by

filling indoor tanks with seawater and evaporation was accelerated through strong aeration and submersible heaters.

During the acclimation period, the crabs were daily fed to satiation using the pellet feeds (Ridley). For the first 2 days a 10 % water exchange was performed, however, when the feeding of early juvenile *P. pelagicus* increased substantially after the first 2 days of acclimation the water exchange was then increased to 80 % for the following 5 days to ensure that ammonia-N levels never exceeded 1 mg l^{-1}. After the one week acclimation period, the crabs were transferred indoors for the commencement of the experiment.

2.2. Preparation of test solutions

The source of seawater had an original salinity of 36 ‰, and an ammonia-N, nitrite-N and nitrate-N level all of 0.01 mg l^{-1}. One day prior to the use of brine, the heaters were removed to allow the water temperature to normalize with the ambient air temperature.

In each of the 3 salinity treatments, there consisted 4 ammonia-N concentrations of 0 (no ammonia-N added), 20, 40, and 60 mg l^{-1} based on values from Chapter 3. To create these ammonia-N concentrations, a 10,000 mg l^{-1} ammonia-N stock solution was made according to Chapter 3. This stock solution was then diluted to the different salinity test solutions to obtain the desired ammonia-N concentration.

2.3. Experimental design and set-up

In each ammonia/salinity treatment consisted of 20 replicate crabs which were individually cultured in separate plastic 5-L capacity containers filled with 4-L of test solution. All containers were arranged in a random block design and bathed in a freshwater bath maintained at 28°C through submersible heaters. Each container received a 100% water

exchange according to the "static renewal method" (APHA, 1989). No food was provided throughout the experiment. The photoperiod was 14:10-h light:dark, with a light intensity between 132 and 170 lux, as measured by a lux meter (MC-88 light meter; TPS).

2.4. Haemolymph osmolality, Na^+, K^+, Ca^{2+} and ammonia-N levels

The haemolymph ion composition and haemolymph ammonia-N levels were measured according to Chapter 4 while the methodology for gill histology were performed according to Chapter 3.

2.5. Ammonia-N excretion rates

Ammonia-N excretion rates of the crabs were measured over a 24-h interval (started at 72 -h and ended at 96-h). At 72-h of the experiment, immediately after the containers received a 100 % water exchange, newly replaced water was sampled from 3 replicate containers (containers sterilized prior to the water exchange) for each ammonia-N/salinity treatment. Each sampled container was then sealed for the following 24-h and at 96-h (*i.e.* 24-h later) the water from the same containers were sampled again. The water was measured for ammonia using the same procedure mentioned above for haemolymph ammonia-N levels. The rate of ammonia-N excretion was calculated using the following formula:

$$((C_{final} - C_{initial}) \times V) / (Wt/h)$$

where C_{final} is the final ammonia-N concentration of the water, $C_{initial}$ is the initial ammonia-N concentration of the water, V is the water volume of the container, Wt is the wet weight (g) of the crab and h is the hour interval between ammonia-N sampling.

Measurements of ammonia-N levels for the blank controls were also conducted in containers without crabs which were used to adjust the readings of ammonia-N excretion rates.

To confirm the actual ammonia-N concentrations used in the experiment, 3 samples were taken from each treatment at 0-h and 72-h (immediately after new test solutions were added to the containers) and measured using the above mentioned procedure. The mean values of the actual ammonia-N concentrations at 0-h and 72-h were within the 5 % of the stated ammonia-N concentrations.

2.6. Preparation of gill homogenate

After the 96-h experiment, the surviving crabs from each treatment were quickly killed and both the anterior (1^{st} - 3^{rd} pair) and posterior (6^{th} - 8^{th}) gills pairs were dissected out, and the gills of the crabs (n = 9 – 15) from the same treatment were pooled to yield 5 replicates for each treatment. The gills were then snap frozen in liquid nitrogen and kept in a -80ºC freezer until processing (within one month). The gills were then thawed on ice and homogenized with 20 twisting strokes in an ice-cold buffer (1:9 w/v) containing 0.25 M sucrose, 6 mM EDTA, 50 mM Tris and 0.1 % (w/v) sodium deoxycholate with a pH of 7.2. The homogenate was then centrifuged at 4°C at 8,900 rpm. The supernatant was removed and centrifuged again at 4°C at 39,000 rpm. No pellets were observed and the supernatant was removed and kept in a -80°C freezer until the assay was conducted (within 1 week).

2.7. Na^+/K^+-ATPase assay on gills

To conduct the assay the supernatant was first thawed on ice. The gill homogenate (67 µl) was then added to two separate media (200 µl). One of the media contained 167 mM NaCl, 50 mM KCl and 33 mM imidazole-HCl at a pH of 7.2 while the other medium contained 217 mM NaCl, 1.67 ouabain and 33 mM imidazole-HCl at a pH of 7.2. Since the second medium

contained no K^+ and ouabain (which is known to inhibit ATPase activity) this medium should not allow a reaction to occur, and thus, serves as a blank.

Both media were pre-incubated in a water bath at 30°C for 10 min., followed by the addition of 67 µl of a solution containing 25 mM Na_2ATP and 50 mM $MgCl_2$ at a pH of 7.2 to each of the two media. The two media were allowed to incubate for 15 min. at 30°C. After 15 min., the reaction was stopped by adding 1.5 ml of "Bonting's" reagent containing 560 mM H_2SO_4, 8.1 mM ammonium molybdate and 170 mM $FeSO_4$. After 20 min., to allow for color formation, the solutions were placed in a two separate cuvettes, and the absorbance was read at 720 nm on a spectrometer after prior readings with phosphate standards. The liberated phosphate was determined by subtracting the readings from medium two (no K^+ and ouabain) with medium one (with K^+ and no ouabain) and the Na^+/K^+-ATPase activity was expressed in µmol P_i h^{-1} mg $protein^{-1}$. See ***Appendix*** for a comprehensive description of the methodology.

2.8. Data analysis

Any significant differences among treatments for the haemolymph osmolality, haemolymph Na^+, K^+, Ca^{2+}, haemolymph ammonia-N levels, ammonia-N excretion rates and gill Na^+/K^+-ATPase activity as well as any potential interaction of ammonia-N and salinity on these measured variables were analyzed using a two-way ANOVA. Significant differences among treatments ($p < 0.05$) were determined using Duncan's multiple range test (Duncan, 1955).

3. Results

3.1. Haemolymph osmolality Na^+, K^+, Ca^{2+} and ammonia-N levels

The mean haemolymph osmolality of the control crabs at salinities of 15 ‰, 30 ‰ and 45 ‰, were 579.8, 938.2 and 1321.2 mOsm kg^{-1}, respectively. Meanwhile, for the control crabs subjected to salinities of 15 ‰, 30 ‰ and 45 ‰, the mean haemolymph Na^+, K^+ and Ca^{2+} was 217, 408 and 575 mmol l^{-1}, 7.6, 12.3 and 15.3 mmol l^{-1}, and 7.2, 12.3 and 15.3 mmol l^{-1}, respectively (Table 2).

A significant salinity effect ($p < 0.01$) was detected on the haemolymph osmolality and haemolymph Na^+, K^+ and Ca^{2+} levels of the crabs since these values decreased as salinities decreased (Table 2). At salinities of 15 ‰ and 30 ‰ the haemolymph osmolality of the crabs were hyper-osmotic to the experimental test solutions while the haemolymph osmolality at a salinity of 45 ‰ was near iso-osmotic. Meanwhile the haemolymph Na^+ levels of the crabs at salinities of 15 ‰, 30 ‰ and 45 ‰ were hyper-ionic, near iso-ionic and hypo-ionic, respectively (Table 2).

Table 1: The osmolality (mOsm kg^{-1}), Na^+, K^+ and Ca^{2+} (mmol l^{-1}) of the seawater at salinities of 15 ‰, 30 ‰ and 45 ‰ and from the haemolymph (mean ± SE) of early *Portunus pelagicus* juveniles after 96-h exposure to different ammonia-N levels (mg l^{-1}) and subjected to salinities of 15 ‰, 30 ‰ and 45 ‰. No significant ammonia-N effect ($p > 0.05$) was detected although a significant salinity effect was detected ($p < 0.05$) indicated by different lowercase letters within each column.

Treatment	Osmolality	Sodium	Potassium	Calcium
15 ‰ salinity	435	197.5	4.5	4.5
Ammonia-N				
0 mg l^{-1}	579.8 ± 10.2 **a**	217.7 ± 7.7 **a**	7.6 ± 0.3 **a**	7.2 ± 0.2 **a**
20 mg l^{-1}	584.0 ± 9.2 **a**	223.7 ± 5.1 **a**	7.4 ± 0.5 **a**	7.3 ± 0.3 **a**
40 mg l^{-1}	582.3 ± 11.8 **a**	222.1 ± 3.8 **a**	7.2 ± 0.2 **a**	7.3 ± 0.2 **a**
60 mg l^{-1}	589.5 ± 5.7 *	231.3 ± 3.2 *	7.5 ± 0.1 *	7.4 ± 0.2 *
30 ‰ salinity	882	393.7	8.0	8.0
Ammonia-N				
0 mg l^{-1}	938.2 ± 10.8 **b**	408.6 ± 8.3 **b**	12.3 ± 0.4 **b**	12.4 ± 0.4 **b**
20 mg l^{-1}	931.9 ± 6.3 **b**	410.2 ± 8.0 **b**	12.5 ± 0.6 **b**	12.1 ± 0.5 **b**
40 mg l^{-1}	944.3 ± 9.3 **b**	404.9 ± 6.3 **b**	12.4 ± 0.4 **b**	12.2 ± 0.4 **b**
60 mg l^{-1}	942.9 ± 11.5 **b**	411.3 ± 5.8 **b**	12.6 ± 0.3 **b**	12.5 ± 0.3 **b**
45 ‰ salinity	1332	590.1	12.0	12.0
Ammonia-N				
0 mg l^{-1}	1321.2 ± 12.5 **c**	575.1 ± 3.2 **c**	15.3 ± 0.3 **c**	15.4 ± 0.3 **c**
20 mg l^{-1}	1319.6 ± 11.9 **c**	587.6 ± 6.4 **c**	15.2 ± 0.4 **c**	15.3 ± 0.4 **c**
40 mg l^{-1}	1319.5 ± 8.7 **c**	566.9 ± 5.2 **c**	15.3 ± 0.4 **c**	15.6 ± 0.3 **c**
60 mg l^{-1}	1325.2 ± 13.5 **c**	578.4 ± 6.6 **c**	15.1 ± 0.2 **c**	15.2 ± 0.5 **c**

* No statistics performed due to an insufficient number of surviving crabs ($n = 2$) sampled.

The mean haemolymph ammonia-N levels of the control crabs subjected to salinities 15 ‰, 30 ‰ and 45 ‰ were 0.03, 0.01 and 0.01 mg l^{-1} ammonia-N, respectively and were not significantly different from each other ($p > 0.05$) (Table 3). However, at 20 mg l^{-1} ammonia-N, the haemolymph ammonia-N of the crabs subjected to a low salinity of 15 ‰ was significantly higher ($p < 0.05$) than those at 30 ‰ and 45 ‰. Meanwhile, when exposed to 40 and 60 mg l^{-1} ammonia-N, the haemolymph ammonia-N levels of the crabs at 15 ‰ were significantly higher than those at 45 ‰ only (Table 3). Furthermore, within the same salinity treatments, the haemolymph ammonia-N levels of the crabs significantly increased ($p < 0.01$) as the ammonia-N levels increased (Table 3).

A two-way ANOVA detected no significant interaction ($p > 0.05$) between the salinity and ammonia-N level on the haemolymph osmolality, haemolymph Na^+, K^+ and Ca^{2+} levels or haemolymph ammonia-N levels.

Table 2: The mean haemolymph ammonia-N concentration (mg l^{-1}) (± SE) of early *Portunus pelagicus* juveniles after 96-h exposure to different ammonia-N levels (mg l^{-1}) and subjected to salinities of 15 ‰, 30 ‰ and 45 ‰. *

	Salinity		
Ammonia-N (mg l^{-1})	15 ‰	30 ‰	45 ‰
0	^{A} 0.03 ± 0.01 ^{a}	^{A} 0.01 ± 0.01 ^{a}	^{A} 0.01 ± 0.01 ^{a}
20	^{B} 16.3 ± 1.9 ^{a}	^{B} 11.1 ± 1.5 ^{b}	^{B} 10.2 ± 1.2 ^{b}
40	^{C} 34.4 ± 3.1 ^{a}	^{C} 28.8 ± 2.4 ^{ab}	^{C} 27.3 ± 2.9 ^{b}
60	^{D} 58.7 ± 5.6 ^{a}	^{D} 51.3 ± 8.2 ^{ab}	^{D} 47.5 ± 4.2 ^{b}

* Different upper and lower case letters indicate significant differences ($p < 0.05$) within each column and row, respectively.
In all treatments $n = 4$

3.2. Ammonia-N excretion rates

Ammonia-N excretion rates generally reduced as the salinities and/or the ammonia-N levels increased (Table 4). A two-way ANOVA detected significant effects of both ammonia-N and salinity ($p < 0.01$) on ammonia-N excretion. The highest ammonia-N excretion rate was detected for the crabs subjected to the lowest salinity of 15 ‰ without added ammonia-N. Furthermore, as the ammonia-N levels increased, the crabs subjected to a low salinity of 15 ‰ consistently had significantly higher ($p < 0.05$) ammonia-N excretion rates than those exposed to the same ammonia-N level but subjected to higher salinities of 30 ‰ and 45 ‰ (Table 4).

Table 3: The mean ammonia-N excretion rates (μg g^{-1} h^{-1}) (± SE) of early *Portunus pelagicus* juveniles exposed to different ammonia-N levels (mg l^{-1}) and subjected to salinities of 15 ‰, 30 ‰ and 45 ‰ over a 24-h period.*

	Salinity		
Ammonia-N (mg l^{-1})	15 ‰	30 ‰	45 ‰
0	^{A} 265.1 ± 12.3 ^{a}	^{A} 231.2 ± 13.7 ^{ab}	^{A} 201.4 ± 12.1 ^{b}
20	^{A} 232.4 ± 10.2 ^{a}	^{AB} 211.4 ± 12.3 ^{ab}	^{A} 186.2 ± 20.7 ^{b}
40	^{B} 168.9 ± 9.6 ^{a}	^{B} 142.3 ± 10.3 ^{ab}	^{B} 125.4 ± 10.1 ^{b}
60	^{C} 121.2 ± 10.8 ^{a}	^{C} 104.4 ± 9.2 ^{a}	^{C} 81.2 ± 8.3 ^{b}

* Different upper and lower case letters indicate significant differences ($p < 0.05$) within each column and row, respectively.
In all treatments $n = 4$

3.3. Gill Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase activity in response to salinity and ammonia-N were measured from both the anterior and posterior gills. For the crabs from the control, the Na^+/K^+-ATPase activity on the anterior gills showed no significant change ($p > 0.05$) at different salinities and remained significantly lower ($p < 0.01$) than the posterior gills (Fig. 1). In contrast, the Na^+/K^+-ATPase activity of the posterior gills showed a significant salinity dependence ($p < 0.01$); the highest activity was found for the crabs subjected to the lowest salinity of 15 ‰, which was significantly higher ($p < 0.05$) than those subjected to the higher salinities of 30 ‰ and 45 ‰ (Fig. 1).

Compared to the control crabs, the Na^+/K^+-ATPase activity on the anterior gills generally increased significantly ($p < 0.05$) when exposed to increasing ammonia-N levels at all salinities tested. However, the Na^+/K^+-ATPase activity on the posterior gills only significantly increased ($p < 0.05$) with increasing ammonia-N levels at a salinity of 30 ‰ (Fig. 1). No data was obtained for the crabs subjected to a salinity of 15 ‰ at 60 mg l^{-1} ammonia-N since the surviving number of crabs were insufficient to conduct gill Na^+/K^+-ATPase measurements.

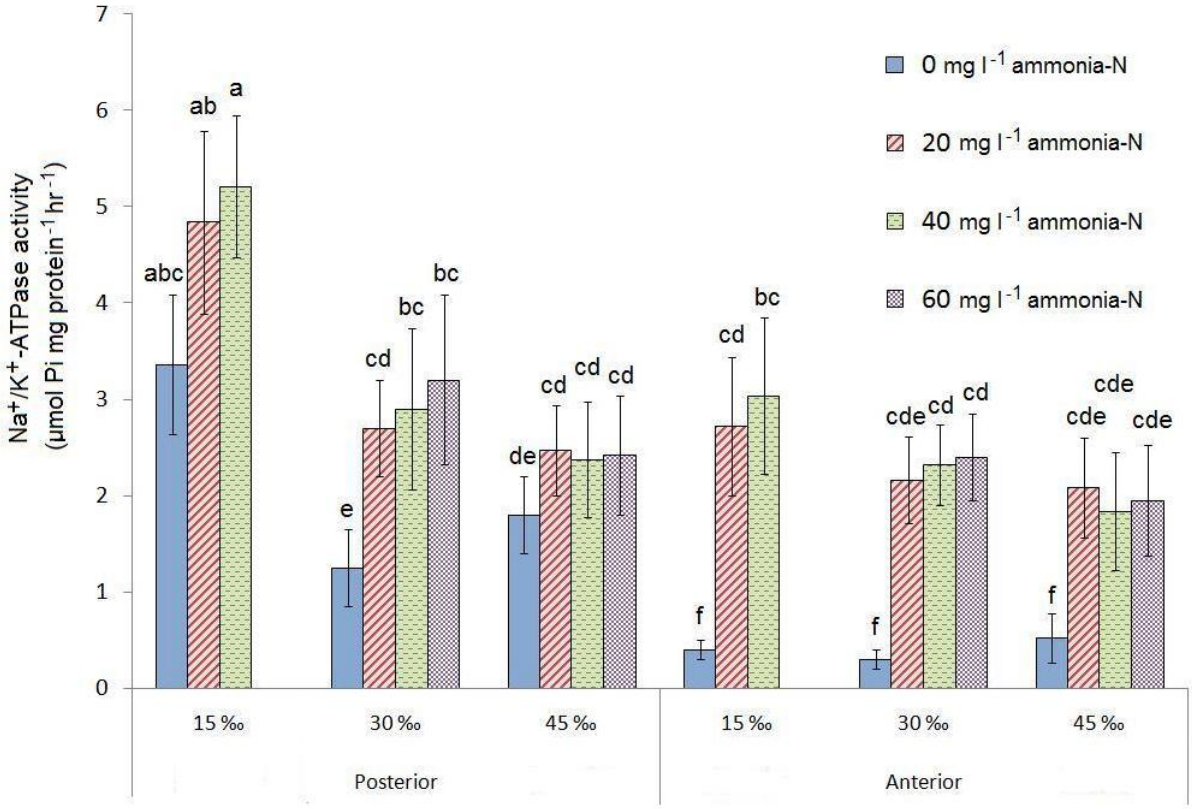

Figure 1: The mean gill Na^+/K^+-ATPase activity (µmol Pi mg $protein^{-1}$ hr^{-1}) (± SE) of the anterior and posterior gills of early *Portunus pelagicus* juveniles after 96-h exposure to different ammonia-N levels (mg l^{-1}) from salinities 15 ‰, 30 ‰ and 45 ‰. Different letters indicate significant differences ($p < 0.05$) between all treatments.

4. Discussion

The current study investigated the osmoregulatory responses and ammonia-N excretion rates of early *P. pelagicus* juveniles and how different ammonia-N and salinity combinations can potentially influence these processes and, in turn, their haemolymph ammonia-N levels. Simultaneously investigating their physiological responses at these environmental conditions was due to their likelihood of both being present in nature. Estuarine systems, which can serve as habitats to early *P. pelagicus* juveniles, can have salinities fluctuate from < 10 ‰ to > 40 ‰ depending on the tides, month and distance from the ocean (de Lestang et al. 2003). Furthermore, not only are the chances to experience elevated ammonia-N levels higher in

estuaries, but sediments with high organic contents, where benthic crustaceans may bury, have been reported to reach as high as 39.2 mg l^{-1} ammonia-N (Weihrauch et al. 1999).

To the best of our knowledge no other investigators have measured the haemolymph ammonia-N levels of crustaceans at different ammonia-N and salinity combinations. However, Lin and Chen (2001) speculated that the significantly higher ammonia-N toxicity of *L. vannamei* at low salinities was the result of increased ammonia-N intake. The results of our current experiment supports this suggestion since at a low salinity of 15 ‰, the haemolymph ammonia-N levels of the crabs exposed to 20, 40 and 60 mg l^{-1} ammonia-N were significantly higher compared to the crabs exposed to the same ammonia-N level at a high salinity of 45 ‰. Furthermore, more mortalities were noticed at a salinity of 15 ‰ which led to reduced samples of these crabs at an ammonia-N of 60 mg l^{-1}.

One of the mechanisms to reduce haemolymph ammonia-N in crustaceans is through active ammonia-N excretion fuelled by gill Na^+/K^+-ATPase activity (Weihrauch et al. 2004). Indeed, this likely explains increased Na^+/K^+-ATPase activity at elevated ammonia-N levels during *in vitro* experiments (using dissected gills) from *Macrobrachium olfersii* (Furriel et al. 2004), *Callinectes danae* (Masui et al. 2002; 2005) and *C. ornatus* (Garçon et al. 2007) as well *in vivo* experiments with *Penaeus chinensis* and *M. nipponense* juveniles (Chen and Nan, 1992; Wang et al. 2003). Similarly, in the current experiment, gill Na^+/K^+-ATPase activity of *P. pelagicus* juveniles generally increased with elevated ammonia-N levels, likely contributing to the haemolymph ammonia-N levels being lower than the experimental test solutions. However, consistent Na^+/K^+-ATPase activity increases at all salinities were only observed on the anterior gills, while Na^+/K^+-ATPase activity in response to ammonia-N significantly increased only on the posterior gills of the crabs at a salinity of 30 ‰. This indicates the anterior gills are likely to be more responsible to ammonia-N excretion, perhaps due to being unburdened by

osmoregulatory requirements. This finding is in agreement with an experiment by Weihrauch et al. (1999) who demonstrated higher Na^+/K^+-ATPase activity and ammonia-N excretion occurred on perfused anterior gills of the crab, *Cancer pagurus*, in response to ammonia-N (Weihrauch et al. 1999). However, in the same study, the difference between ammonia-N excretion on the posterior and anterior gills were negligible for the stronger osmoregulating crabs of *Carcinus maenas* and *Eriocheir sinensis* (Weihrauch et al. 1999). Since both *C. pagurus* and *P. pelagicus* are both weak osmoregulators (Chapter 2; Weihrauch et al. 1999), indicates this characteristic may also contribute to these findings although further research is likely necessary.

Interestingly, ammonia-N excretion at all ammonia-N levels was significantly higher for early *P. pelagicus* juveniles subjected to a salinity of 15 ‰ than those subjected to 45 ‰, although in contrast, the haemolymph ammonia-N levels of the crabs subjected to 15 ‰ were significantly higher at 20, 40 and 60 mg l^{-1} ammonia-N compared to those within the same ammonia-N treatment at 45 ‰. This indicates that additional coping mechanisms are being utilised more heavily at high salinities. Previous investigations may provide an explanation for this finding since changes to metabolic processes from ammontelism to ureotelism at increasing salinities have been demontrated for *Scylla serrata* (Chen and Chia, 1996) and *Marsupenaeus japonicus* (Lee and Chen, 2003), and a similar phenomenon of detoxifying ammonia-N to urea may have occurred in the current study. Certainly further research is required to confirm this. However, since different osmoregulatory strategies are necessary at hypo- and hyper-osmotic conditions it would appear beneficial for crustaceans to prevent excessive Na^+ absorption, in exchange for NH_4^+ excretion, at high salinity conditions.

Despite both gill regions being involved in ammonia-N excretion, gill specialization for respiration and osmoregulation on the anterior and posterior gills, respectively has been

established based on Na^+/K^+-ATPase activity responses to salinity (Péqueux, 1995; Kirschner, 2004; Freire et al. 2008). The results of the current experiment are in agreement with these previous investigations. Anterior gill Na^+/K^+-ATPase activity of early *P. pelagicus* juveniles showed no significant salinity response and remained consistently low whereas the posterior gill Na^+/K^+-ATPase activity of the crabs subjected to a salinity of 15 ‰ after over a week (acclimation period plus 96-h) were 2.6 and 1.8 fold greater than those at 30 ‰ and 45 ‰, respectively. This result of increased gill Na^+/K^+-ATPase activity at low salinities, to increase haemolymph Na^+ and Cl^- uptake, is typical for estuarine and marine crustaceans (Holliday, 1985; Piller et al. 1995; Castilho et al. 2001; López-Mañanes et al. 2002; Genovese et al. 2004; Torres et al. 2007; Lucu et al. 2008). However, gill Na^+/K^+-ATPase activity is unlikely to play a dominant role in hypo-osmoregulation for *P. pelagicus* juveniles since this activity did not significantly change at high salinities, and is a similar finding with other crustacean species including *Neohelice* (*Chasmagnathus*) *granulatus* (Genovese et al. 2004), *Scylla paramamosain* (Chung and Lin, 2006), *Carcinus aestuarii* (Lucu et al. 2008). Interestingly, at the high salinity of 45 ‰, the haemolymph K^+ and Ca^{2+} of *P. pelagicus* juveniles were hyper-regulated, which is in contrast to haemolymph K^+ hypo-regulation of *S. serrata* at hyper-osmotic conditions (Chen and Chia, 1997). Furthermore, there was no significant effect of ammonia-N exposure to the haemolymph osmolality, Na^+, K^+ or Ca^{2+} levels despite a general trend of increased gill Na^+/K^+-ATPase activity at elevated ammonia-N levels. It is unclear as to the exact mechanisms contributing to these results, however, other transport channels (*e.g.* $Na^+/K^+/2Cl^-$, Ca^{2+}-ATPase, K^+-ATPase) and/or the antennal gland may have played a role.

Evidence from the current experiment indicates that *P. pelagicus* juveniles are relatively well adapted to elevated ammonia-N exposure since, regardless of the salinity level or ammonia-N concentration that induced morbidity, ammonia-N excretion and gill Na^+/K^+-

ATPase activity were still functioning while the haemolymph ammonia-N levels remained substantially lower than the experimental test solutions. These findings may be linked with the burying behavior often adopted by *P. pelagicus* in nature, which was suggested to be an important characteristic for the development of an effective mechanism to maintain the haemolymph ammonia-N levels below that of the environment (Weihrauch et al. 2004). However, changes to the external salinity was demonstrated to significantly influence this capacity which likely explains the increased haemolymph ammonia-N of early *P. pelagicus* juveniles at lower salinities and therefore potential ammonia-N toxicity increases. Due to the seemingly contradictory results of increased haemolymph ammonia-N levels but increased ammonia-N excretion of the crabs at low salinities, experimentation on potential detoxification processes, changes to the metabolism and gill permeability are warranted. Such experiments may to help further elucidate the underlying adaptive mechanisms of crustaceans to elevated ammonia-N levels when subjected to different salinity conditions.

Chapter 6

Experimental studies on osmoregulation and ammonia-N excretion of blue swimmer crab, *Portunus pelagicus*, juveniles exposed to ammonia-N and K^+ combinations and differences between *in vitro* and *in vivo* gill Na^+/K^+-ATPase responses

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1. Introduction

As previously mentioned in Chapter 1, the major contributor to the coping responses of aquatic crustaceans during elevated ammonia-N exposure is via active ammonia-N excretion which is fuelled by gill Na^+/K^+-ATPase activity. While this activity has been shown to respond to both salinity and ammonia-N (Chapter 5), results from a succession of *in vitro* experiments have recently revealed that Na^+/K^+-ATPase activity of dissected gills from the freshwater prawn *Macrobrachium olfersii* and the crabs *Clibanarius vittatus*, *Callinectes danae* and *C. ornatus* increased in media containing elevated NH_4^+ and K^+ levels (Furriel et al. 2004; Gonçalves et al. 2006; Garçon et al. 2007; Masui et al. 2002; 2005; 2009).

Although, in the natural environment, elevated ammonia-N in combination with major K^+ fluctuations without salinity changes are unlikely to be encountered by crustaceans (Raveendran and Madany, 1991), such studies may provide important knowledge to help reveal the underlying physiological mechanisms of osmoregulation and ammonia-N excretion in crustaceans. Evidence from these *in vitro* studies indicated that gill Na^+/K^+-ATPase activity increased to accommodate both their osmoregulatory and ammonia-N excretion requirements by either NH_4^+ binding to already available specific sites on the gill (Furriel et al. 2004) or that

new binding sites for NH_4^+ became exposed (Garçon et al. 2007). Although this implies that ammonia-N excretion and ion regulation would be undisrupted in living animals, to date, the implications of these *in vitro* studies have not yet been evaluated.

In order to evaluate these implications on living crustaceans, the present study manipulated K^+ and ammonia-N levels in the seawater to evaluate their effects on the haemolymph osmolality, haemolymph Na^+, K^+, Ca^{2+} and ammonia-N levels of early blue swimmer crab, *Portunus pelagicus*, juveniles. The ammonia-N excretion, gill Na^+/K^+-ATPase activity and gill histopathological changes of the crabs were also measured simultaneously in an attempt to understand the underlying mechanisms. A further *in vitro* experiment was subsequently performed using dissected gills of the same crab species to compare the gill Na^+/K^+-ATPase activity responses, at different K^+ and ammonia-N combinations, with the results of the *in vivo* experiment. Although it has been previously demonstrated that this species are weak osmoregulators (Chapter 2), their haemolymph ammonia-N levels can be regulated substantially lower than the external media (Chapter 4 and 5) likely due to high ammonia-N excretion rates (Chapter 5). These characteristics are likely to be considered desirable when evaluating adaptive responses to ammonia-N exposure and therefore *P. pelagicus* appears to be a good candidate for such studies.

2. Materials and Methods

2.1. In vivo *experiment*

2.1.1. Source of crabs

The crabs were cultured according to the methods described in Chapter 2. The crabs were further cultured until the C7 stage was reached according to the methods described in Chapter 3.

2.1.2. Preparation of test solutions

Three potassium treatments of 4.25, 8.50 and 12.75 mM KCl (or 166.16, 322.23 and 498.49 mg l^{-1} total K^+) were used and designated as low, normal and high K^+, respectively. The normal K^+ treatment had similar K^+ levels to that of natural seawater at a salinity of 30 ‰, while the low and high K^+ treatments were 4.25 mM K^+ lower and higher, respectively than the normal K^+ treatment. Based on our previous experiment, the low and high K^+ treatments both represent sub-lethal concentrations to early *P. pelagicus* juveniles (Romano and Zeng, 2007b). To create these different K^+ levels, natural seawater (UV sterilised and 5 μm filtered) was first reduced from a salinity of 30 ‰ to 15 ‰, using de-chlorinated freshwater, thereby reducing all elements, including K^+ by half (*i.e.* K^+ reduced from 8.50 mM to 4.25 mM). From a salinity of 15 ‰, the Na^+, Cl^-, Mg^{2+}, SO_4^{2-} and Ca^{2+} were added in the forms of NaCl, $MgSO_4$, $MgCl_2$ and $CaCl_2$ (all analytical grade; Sigma) to approximate their original levels found in the natural seawater at a salinity of 30 ‰ (see Table 1). Therefore, for all of the three K^+ treatments, with the exception of K^+, the major ions of the seawater including Na^+, Cl^-, Mg^{2+}, SO_4 and Ca^{2+} were at identical levels. To create the normal and high K^+ treatments, KCl (analytical grade; Sigma) was then added to increase the K^+ concentrations to 8.50 and 12.75 mM, respectively. The water was thoroughly mixed to allow the salts to completely dissolve and left overnight within covered tanks. The following day, a hand-held refractometer (Iwaki, Japan) was used to measure the salinity of all test solutions and a final salinity of 30 ‰ was confirmed. The pH of the test solutions were maintained at 8.10 through the addition of sodium hydroxide (NaOH) pellets.

Table 1: Major ionic composition (mM) of natural seawater at salinities of 15 ‰, 30 ‰ and those of artificial seawater (AFSW) used in the present study. With the exception of the K^+ levels in the various K^+ treatments, the composition of major ions of AFSW was the same as that of 30 ‰ natural seawater.

Ions	15 ‰	30 ‰	AFSW
Cl^-	229.4	458.9	463.6
Na^+	196.8	393.6	393.6
SO_4^{2+}	11.5	23.0	23.0
Mg^{2+}	30.9	61.7	61.7
Ca^{2+}	4.3	8.5	8.5

Each K^+ treatment was then combined with 6 ammonia-N concentrations of 0 (no ammonia-N added), 1.42. 2.85, 4.28, 5.71 and 7.13 mM (or 0, 20, 40, 60, 80 and 100 mg l^{-1}), respectively. The selection of these ammonia-N concentrations was again based on our previous experiment on *P. pelagicus* juveniles with the higher end of the doses to challenge the crabs while the lower end would elicit adaptive responses (Chapter 3, 4 and 5). To create these ammonia-N concentrations, a 10,000 mg l^{-1} ammonia-N stock solution was prepared by dissolving NH_4Cl in 10-L of distilled water according to Chen and Kou (1993). This stock solution was then diluted to obtain the desired ammonia-N concentration to combine with each K^+ treatment.

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2.1.3. Experimental design and set-up

For each K^+/ammonia-N treatment, there were 20 replicate crabs individually kept in 5-L containers filled with 2-L of the desired test solution (total of 360 crabs for 18 treatments). All containers were arranged in a random block design within a freshwater bath kept at a temperature of 28 ± 0.5°C. Each container received a daily 100% water exchange according to the "static renewal method" described by APHA (1989).

2.1.4. Haemolymph osmolality, Na^+, K^+, Ca^{2+} and ammonia-N levels and ammonia-N excretion

The measurements of these variables were performed according to Chapter 5.

2.1.5. Gill histology and gill Na^+/K^+-ATPase activity from the **in vivo** *experiment*

Gill Na^+/K^+-ATPase activity was measured according to the methods described in Chapter 5. Gill histology was performed according to Chapter 3 and histopathological changes at each K^+/ammonia-N treatment were quantified according to Chapter 4. However, since the lamallae collpase was not as prevelant in this experiment, compared with Chapter 4, the number of disrupted pillar cells and epithelial width could be quantified. Pillar cell disruption was identified by the septum (connective tissue spanning across the lamellae) being completely lost, leading to only the nucleus being present and measured by counting their presence within the lamellae. The epithelial width (µm) was measured using a micrometer (1 µm) and defined as being the distance between the outward and inward facing epithelium within the lamellae (*e.g.* for detecting lamellae thickness)

2.2. **In vitro** *experiment*

2.2.1. Source of crabs and **in vitro** *gill Na^+/K^+-ATPase activity*

The crabs used in the *in vitro* experiment were cultured using the same procedures as described for the *in vivo* experiment. However, in order to obtain a sufficient quantity of gill samples, the experimental crabs were larger with a mean weight of 2.14 ± 0.17 g. After a longer period of individually culturing the crabs for 27 days within a recirculating system (salinity of 32 ± 1 ‰), a total of 6 crabs were sacrificed and their posterior gills dissected out. The gills from two crabs were composited to yield 3 replicates, and then snap frozen in liquid

nitrogen and kept in a -80ºC freezer until the assay was conducted, which was within one month.

The centrifuging, preparation of gill homogenates and composition of the solutions were similar to those described above for the *in vivo* experiment. Since this *in vitro* experiment was testing the responses of gill Na^+/K^+-ATPase activity at different K^+ and ammonia-N combinations, three K^+ levels of the assay media were created by adding KCl to achieve 25, 50 and 75 mM K^+, respectively, while maintaining the same levels of NaCl, imidazole-HCl and pH. These K^+ levels were chosen since the medium 50 mM K^+ level was used in the gill Na^+/K^+-ATPase assay (see 2.1.6) for the *in vivo* experiment, which was based on the method described by Holliday (1985). Meanwhile, to create three different ammonia-N concentrations of 0, 3.56 (or 50 mg l^{-1}) and 7.13 (or 100 mg l^{-1}) mM, a 10,000 mg l^{-1} ammonia-N stock solution was created by adding 38.2 g NH_4Cl into 1-L of distilled water. The pH of this stock solution was adjusted to 7.2 (same pH level for the *in vivo* experiment) through the addition of sodium hydroxide pellets. The stock solution was further diluted to 35.6 (or 500 mg l^{-1}) and 71.3 mM (or 1,000 mg l^{-1}) ammonia-N by using distilled water and the pH of 7.2 was maintained.

After the homogenate samples were added to the incubation media of the three K^+ concentrations, 38 µl of either distilled water, 35.6 or 71.3 mM of the ammonia-N stock solutions were added to create a final ammonia-N concentration of 0 mM, 3.56 mM (or 50 mg l^{-1}) and 7.13 mM (or 100 mg l^{-1}), respectively. When this was added to the assay, which contained 67 µl supernatant, 200 µl of the media and 67 µl of the start solution, the final volume was 372 µl. After the media pre-incubated for 10 minutes in a 30ºC water bath, the assay for Na^+/K^+-ATPase activity was run using the same procedure as Chapter 5.

2.3. Data analysis

Prior to conducting any statistical analysis, homogenity of variance was first tested using Levene's test. If homogenity of variance was violated than a log transformation of the data was used. When homogenity of variance was established, a two-way ANOVA was then used to determine any significant ($p < 0.05$) K^+, ammonia-N or interactive effect, which was identified using Tukey's post hoc test, on the haemolymph osmolality, haemolymph Na^+, K^+ and Ca^{2+}, haemolymph ammonia-N levels, ammonia-N excretion and gill histological quantifications from the *in vivo* experiment and gill Na^+/K^+-ATPase activity from both experiments. A one-way ANOVA was then used to determine any significant difference ($p < 0.05$) of the measured variables between treatments using Tukey's post hoc test in both experiments.

3. Results

3.1. Haemolymph osmolality Na^+, K^+, Ca^{2+} and ammonia-N levels

The mean haemolymph osmolality and haemolymph Na^+ and Ca^{2+} levels of *P. pelagicus* juveniles exposed to low (4.25 mM), normal (8.50 mM) and high (12.75 mM) K^+ levels but without added ammonia-N was 982.3, 975.1 and 981.3 mOsm kg^{-1}, 512, 507 and 509 mM Na^+, and 12.2, 12.2 and 12.1 mM Ca^{2+}, respectively (Table 2). No significant K^+ or ammonia-N effect was detected on all these haemolymph parameters (Table 2).

In contrast, a significant ($p < 0.01$) effect of higher external K^+ levels led to higher haemolymph K^+ concentrations of the crabs (Table 2). The haemolymph K^+ levels of the crabs were significantly lower ($p < 0.01$) when exposed to the low external K^+ level of 4.25 mM when compared to those exposed to higher levels of both 8.5 and 12.75 mM K^+. Meanwhile, the haemolymph K^+ levels of the crabs exposed to normal K^+ level of 8.50 mM K^+ had

significantly lower haemolymph K^+ concentrations ($p < 0.01$) than those exposed to the high K^+ level of 12.75 mM (Table 2). However, no significant effect of external ammonia-N level on haemolymph K^+ concentration of the crabs was detected ($p > 0.05$).

Table 2: The mean haemolymph osmolality (mOsm kg^{-1}) and haemolymph Na^+, Ca^{2+} and K^+ levels (mM) of early *Portunus pelagicus* juveniles after 96-h exposure to different ammonia-N and K^+ combinations (n = 10-13).

Osmolality (mOsm kg^{-1}) and ion composition (mM) of artificial seawater at 30 ‰					
Osmolality	Na^+	Ca^{2+}	K^+ (Low)	K^+ (Normal)	K^+ (High)
846	395	8.5	4.25	8.50	12.75
Haemolymph osmolality (mOsm kg^{-1}) and ion composition (mM)					
Treatment		Osmolality	Na^+	Ca^{2+}	K^+
Normal K^+ (8.50 mM)					
0 mM ammonia-N		975.1 ± 4.6 [a]	420.8 ± 6.8 [a]	12.2 ± 0.1 [a]	12.2 ± 0.9 [b]
1.42 mM ammonia-N		969.7 ± 10.3 [a]	419.6 ± 7.4 [a]	12.1 ± 0.6 [a]	11.7 ± 0.4 [b]
2.85 mM ammonia-N		972.8 ± 14.7 [a]	429.3 ± 9.0 [a]	12.3 ± 0.9 [a]	12.0 ± 0.4 [b]
4.28 mM ammonia-N		989.4 ± 12.5 [a]	424.5 ± 6.9 [a]	12.4 ± 0.7 [a]	12.0 ± 0.5 [b]
Low K^+ (4.25 mM)					
0 mM ammonia-N		982.3 ± 13.4 [a]	421.2 ± 8.8 [a]	12.2 ± 0.2 [a]	7.7 ± 0.2 [a]
1.42 mM ammonia-N		974.1 ± 16.8 [a]	418.3 ± 6.3 [a]	12.2 ± 0.4 [a]	7.7 ± 0.4 [a]
2.85 mM ammonia-N		979.6 ± 8.2 [a]	427.6 ± 10.1 [a]	11.9 ± 0.7 [a]	7.2 ± 0.3 [a]
High K^+ (12.75 mM)					
0 mM ammonia-N		981.3 ± 19.8 [a]	413.5 ± 6.9 [a]	12.2 ± 0.6 [a]	15.0 ± 0.3 [c]
1.42 mM ammonia-N		972.3 ± 7.8 [a]	428.3 ± 8.2 [a]	12.0 ± 0.4 [a]	16.6 ± 0.4 [c]
2.85 mM ammonia-N		964.3 ± 14.9 [a]	410.7 ± 11.3 [a]	12.3 ± 0.3 [a]	16.3 ± 0.6 [c]

* Different superscripted letters indicate significant differences ($p < 0.05$) within each column.

The haemolymph ammonia-N levels of the crabs exposed to low, normal and high K^+ levels but without added ammonia-N (0 mM ammonia-N) were 0.05, 0.07 and 0.06 mM ammonia-N, respectively, and were not significantly different from each other ($p > 0.05$) (Table 3). Similarly, for the crabs exposed to 1.42 mM (or 50 mg l^{-1}) ammonia-N at different K^+ levels, the haemolymph ammonia-N levels were not significantly different ($p > 0.05$).

However, at a higher ammonia-N concentration of 2.85 mM (or 100 mg l^{-1}), crabs exposed to both low and high K^+ had significantly higher haemolymph ammonia-N levels ($p < 0.05$) than those exposed to the normal K^+ level (Table 3). While haemolymph ammonia-N levels of the crabs were significantly affected by both K^+ and ammonia-N ($p < 0.05$) levels, no significant interaction between the two ($p > 0.05$) was detected.

Table 3: The mean haemolymph ammonia-N levels (± SE) (mM) of early *Portunus pelagicus* juveniles after 96-h exposure to 8.50 (normal), 4.25 (low) and 12.75 (high) mM K^+ levels at different ammonia-N concentrations (mM) ($n = 3$).

	Ammonia-N concentration			
Treatment	0 mM	1.42 mM	2.85 mM	4.28 mM
Normal K^+ (8.50 mM)	^{A} 0.028 ± 0.01 ^{a}	^{A} 0.60 ± 0.12 ^{b}	^{A} 1.76 ± 0.40 ^{c}	3.44 ± 0.52 ^{d}
Low K^+ (4.25 mM)	^{A} 0.035 ± 0.01 ^{a}	^{A} 0.82 ± 0.11 ^{b}	^{B} 2.55 ± 0.41 ^{c}	n/a
High K^+ (12.75 mM)	^{A} 0.028 ± 0.01 ^{a}	^{A} 0.66 ± 0.18 ^{b}	^{B} 2.24 ± 0.34 ^{c}	n/a

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) within each ammonia-N and K^+ treatment, respectively. "n/a" indicates no data available due to total mortality of crabs under the treatment.

3.2. Ammonia-N excretion

The ammonia-N excretion rates of the crabs exposed to different K^+ levels, but with no added ammonia-N, were not significantly different ($p > 0.05$) (Table 4). However, when the crabs were exposed to 1.42 and 2.85 mM ammonia-N, significantly reduced mean excretion rates ($p < 0.05$) were detected at low K^+ than at normal K^+. Furthermore, at 2.85 mM ammonia-N, the crabs had significantly lower ($p < 0.05$) mean excretion rates at high K+ than at normal K^+. Meanwhile, within the same K^+ level, increased ammonia-N levels led to significantly reduced mean ammonia-N excretion rates ($p < 0.05$) at all K^+ levels tested (Table 4).

Table 4: The mean ammonia-N excretion rates (μg^{-1} g^{-1} hr^{-1}) (± SE) of early *Portunus pelagicus* juveniles after 96-h exposure to 8.50 (normal), 4.25 (low), and 12.75 (high) mM K^+ at different ammonia-N concentrations (mM) (n = 3).

	Ammonia-N concentration			
Treatment	0 mM	1.42 mM	2.85 mM	4.28 mM
Normal K^+ (8.50 mM)	^{A} 207.5 ± 18.1 ^{a}	^{A} 178.2 ± 10.9 ^{b}	^{A} 131.9 ± 15.8 ^{c}	88.3 ± 15.2 ^{d}
Low K^+ (4.25 mM)	^{A} 218.3 ± 15.2 ^{a}	^{B} 134.1 ± 14.8 ^{b}	^{B} 64.2 ± 19.2 ^{c}	n/a
High K^+ (12.75 mM)	^{A} 223.5 ± 13.8 ^{a}	^{AB} 169.3 ± 12.4 ^{b}	^{B} 81.1 ± 17.7 ^{c}	n/a

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) within each ammonia-N and K^+ treatment, respectively. "n/a" indicates no data available due to total mortality of crabs under the treatment.

3.3. Gill Na^+/K^+-ATPase activity from in vivo *and* in vitro *experiments*

At normal K^+, increasing ammonia-N levels led to an increase in gill Na^+/K^+-ATPase activity for the crabs which became significantly higher ($p < 0.05$) at 2.85 and 4.25 mM ammonia-N than those in the control treatment with no ammonia-N added (Fig. 1). Conversely, at both low and high K^+ levels, significantly higher gill Na^+/K^+-ATPase activities were detected ($p < 0.05$) for crabs subjected to no added ammonia-N than those exposed to higher ammonia-N levels of 1.42 to 4.28 mM (Fig. 1). Meanwhile, the gill Na^+/K^+-ATPase activity of the crabs exposed to low K^+ and 2.85 mM ammonia-N was significantly lower ($p < 0.05$) than those exposed to normal K^+ and at 2.85 and 4.28 mM ammonia-N as well as the crabs exposed to high K^+ at 1.42 and 2.85 mM ammonia-N. No significant difference were detected between gill Na^+/K^+-ATPase activity of the crabs exposed to 4.85 mM ammonia-N at both normal and high K^+ levels (Fig. 1).

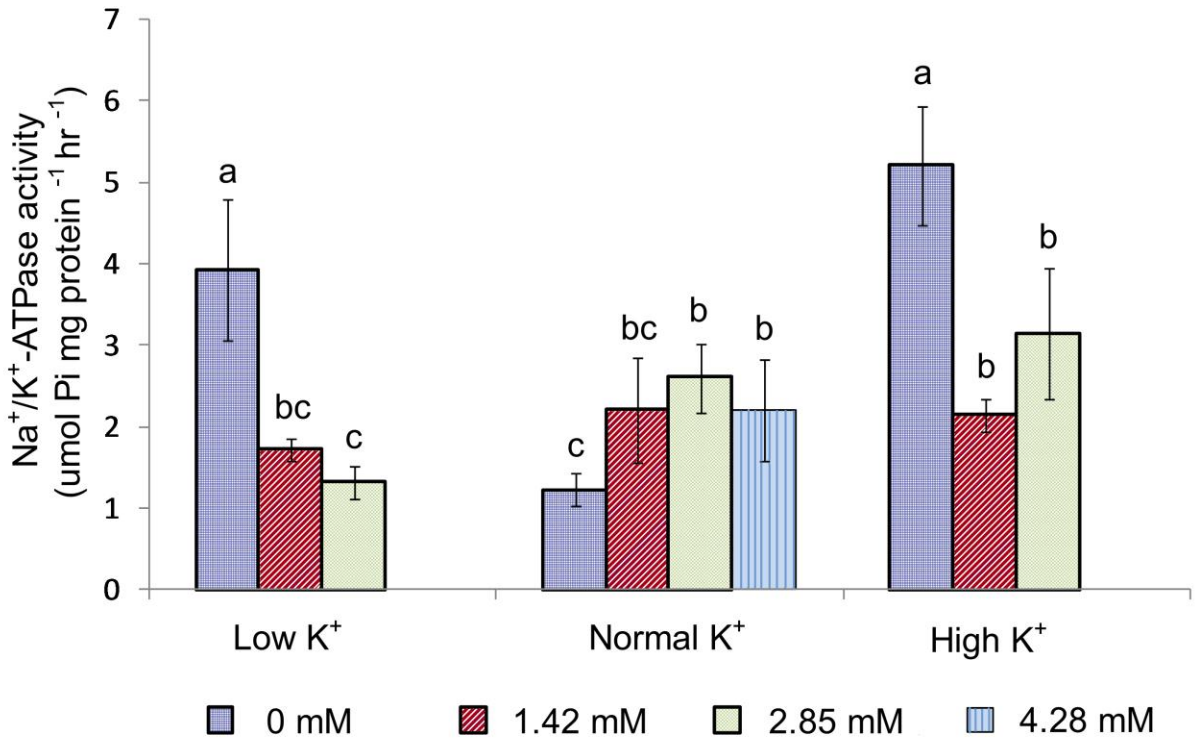

Figure 1: The mean Na^+/K^+-ATPase activity (µmol Pi mg $protein^{-1}$ hr^{-1} ± SE) of the posterior gills from early *Portunus pelagicus* juveniles after 96-h exposure to various ammonia-N and K^+ combinations in the *in vivo* experiment.

The gill Na^+/K^+-ATPase activity of the control treatment with no added ammonia-N and using 50 mM KCl (the same concentration used for gill Na^+/K^+-ATPase activity assay for the *in vivo* experiment) showed similar Na^+/K^+-ATPase activity rates of 1.68 µmol Pi hr^{-1} mg $protein^{-1}$ compared to 1.25 µmol Pi hr^{-1} mg $protein^{-1}$ in the *in vivo* experiment. However, in contrast to the *in vivo* experiment, when ammonia-N and K^+ levels were altered, no significant ammonia-N, K^+ or their interactive effect on gill Na^+/K^+-ATPase activity was detected in the *in vitro* experiment ($p > 0.05$) (Fig. 2).

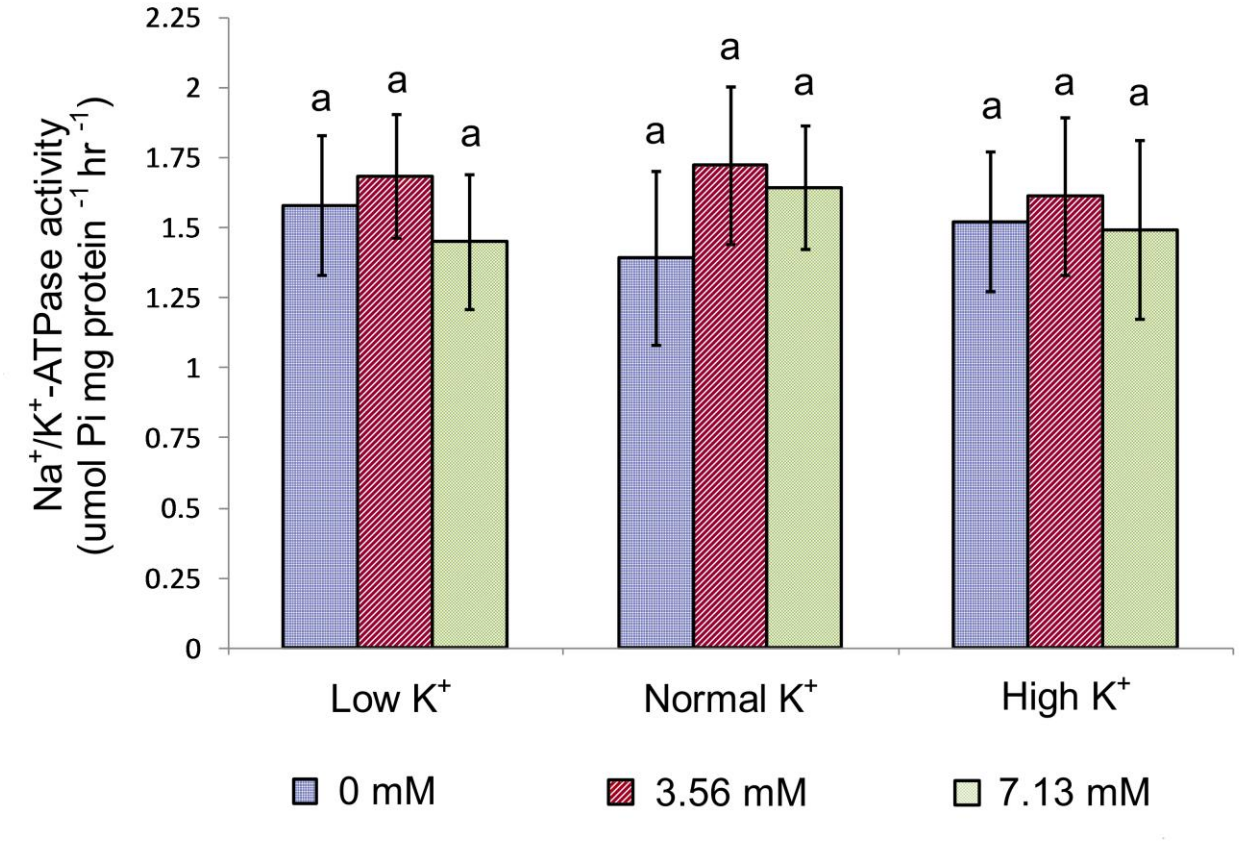

Figure 2: The mean Na^+/K^+-ATPase activity (µmol Pi mg $protein^{-1}$ hr^{-1} ± SE) of the posterior gills from early *Portunus pelagicus* juveniles subjected to different ammonia-N and K^+ levels in the *in vitro* experiment.

3.4. Gill histology

At the normal K^+ level, the gills of the control crabs exposed to normal K^+ and with no added ammonia-N showed normal lamellae structure, prevalence of intact pillar cells (PC) with the septum connecting each other, and only a few haemocytes (HAE) within the gill lamellae (Fig. 3A). However, an increase in ammonia-N concentration to 1.42 and 2.85 mM led to a significant increase ($p < 0.01$) in the number of haemocytes present, occurrence of disrupted pillar cells (DPC) within the gill lamellae and epithelial width. Meanwhile, the lamellae size significantly decreased ($p < 0.05$) when the crabs were exposed to 2.85 mM ammonia-N as compared to those exposed to 0 and 1.42 mM ammonia-N (Fig. 3B; Table 5).

Similarly, at low and high K^+ levels, there were also general trends of increased gill histopathological changes as the ammonia-N levels increased, albeit often a reversed tendency to what was observed at the normal K^+ level was found (Table 5; Figs. 3C, D, E and F). For example, at low K^+, lamellae size more than doubled as ammonia-N increased from 0 to 1.42 mM, and nearly doubled again as ammonia-N increased to 2.85 mM, which is in contrast to the shrinking trend observed at normal K^+ with increasing ammonia-N levels (Table 5; Figs. 3C, D, E and F). As the ammonia-N level increased from 0 to 1.42 mM, all gill parameters measured was significantly higher ($p < 0.05$) at high K^+ than at normal and low K^+, the only exception was disrupted pillar cells which was not significantly different to that at low K^+. A further increase of ammonia-N to 2.85 mM led to all measured gill morphological parameters at both low and high K^+ to become significantly different ($p < 0.05$) than those of the normal K^+ while no significant difference was detected between low and high K^+ treatments ($p > 0.05$) (Table 5; Fig. 3).

On the other hand, a comparison of gill morphology of the crabs subjected to the same ammonia-N level but had different K^+ levels revealed that at low K^+, the gill lamellae significantly shrank ($p < 0.01$) while at high K^+, significantly swelled ($p < 0.05$) when compared to those at normal K^+ (Table 5). Furthermore, generally, more severe histopathological damage were observed at both low and high K^+ than at normal K^+ (Figs. 3C, D, E and F). At low K^+, the number of haemocytes present in the lamellae, disrupted pillar cells and epithelial width all significantly increased ($p < 0.05$) while at high K^+, similar trends of increased disrupted pillar cells and epithelial width were observed. However, the number of haemocytes present was not significantly different ($p > 0.05$) from the crabs at normal K^+ (Table 5).

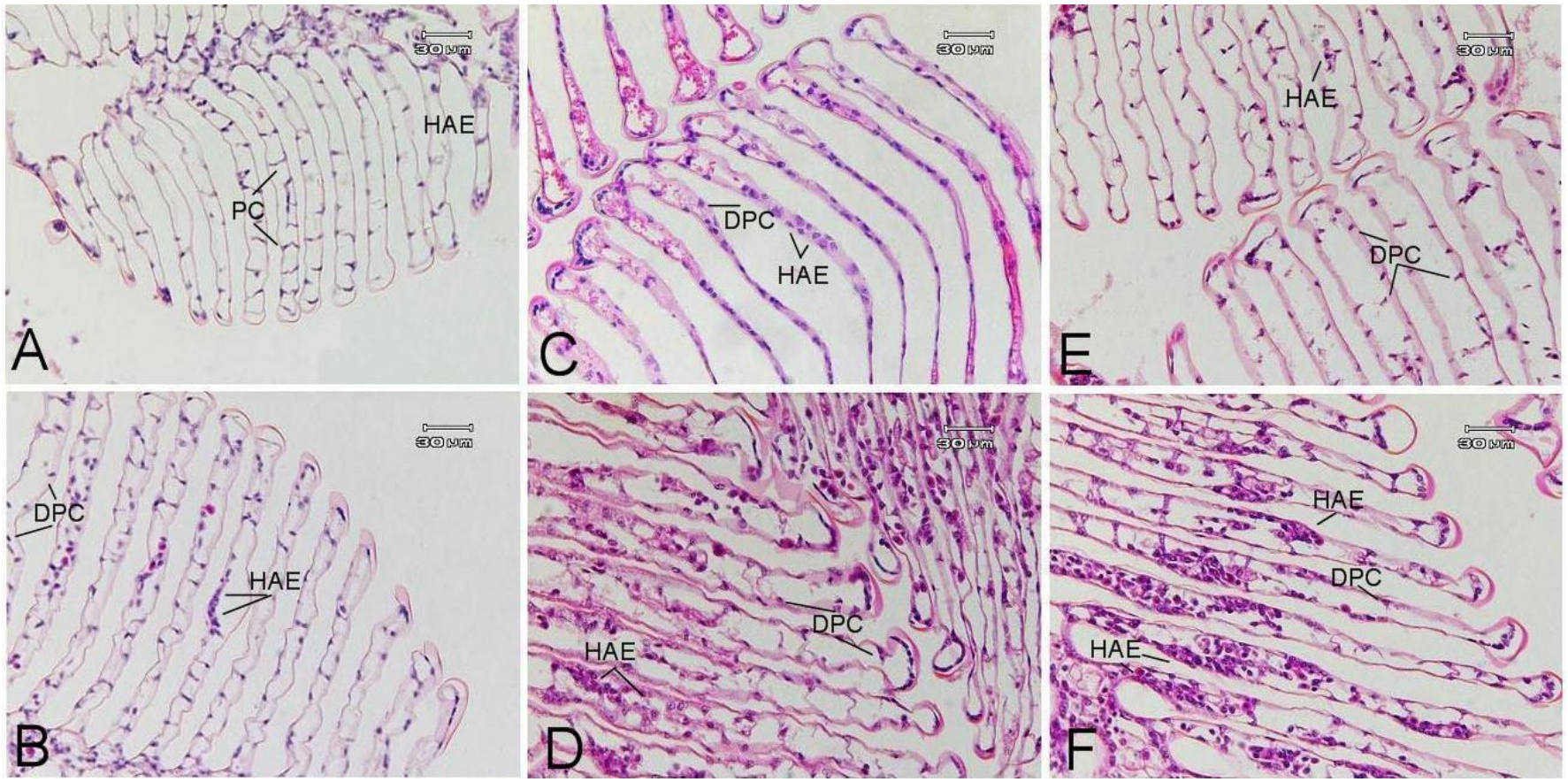

Figure 3: The histological changes in the anterior gills of early *Portunus pelagicus* juveniles exposed to normal (8.50 mM, the K^+ level in 30 ‰ natural seawater) (A & D), low (4.25 mM) (B & E) and high (12.75 mM) K^+ levels (C & F) in combination with 0 mM (no added ammonia-N) (A, B & C) and 2.85 mM ammonia-N (D, E & F). Note the increased prevalence of distorted pillar cells (DPC) and presence of haemocytes (HAE) along with higher degrees of lamellae distortion when crabs were subjected to either low or high K^+ as compared to those under normal K^+ level. Magnification × 20; scale bars = 30 µm.

Table 5: The mean (± SE) lamellae diameter (µm), epithelial thickness (µm) and the number of haemocytes and disrupted pillar cells within the lamellae of posterior gills of early *Portunus pelagicus* juveniles after 96-h exposure to different ammonia-N and K^+ combinations (n = 4).

	8.50 mM K^+			4.25 mM K^+			12.75 mM K^+		
Treatment	0 mM	1.42 mM	2.85 mM	0 mM	1.42 mM	2.85 mM	0 mM	1.42 mM	2.85 mM
Lamellae size (µm)	15.3 ± 0.4 ^{c}	11.9 ± 0.3 ^{bc}	9.0 ± 0.6 ^{b}	4.8 ± 0.4 ^{a}	11.5 ± 0.8 ^{bc}	21.0 ± 0.9 ^{d}	28.1 ± 0.6 ^{d}	23.7 ± 0.5 ^{d}	26.8 ± 0.5 ^{d}
Epithelial width (µm)	1.64 ± 0.09 ^{a}	3.72 ± 0.20 ^{b}	4.65 ± 0.34 ^{b}	4.14 ± 0.41^{b}	4.24 ± 0.49 ^{b}	6.44 ± 0.62 ^{c}	8.58 ± 0.34 ^{d}	6.62 ± 0.36 ^{c}	6.41 ± 0.39 ^{c}
Presence of haemocytes	1.17 ± 0.17 ^{a}	3.82 ± 0.29^{cd}	5.20 ± 0.48 ^{d}	2.78 ± 0.39^{bc}	4.58 ± 0.54 ^{d}	6.79 ± 0.42 ^{e}	1.69 ± 0.24^{ab}	6.72 ± 0.43 ^{e}	6.93 ± 0.45 ^{e}
Disrupted pillar cells	0.72 ± 0.14 ^{a}	2.86 ± 0.28 ^{b}	3.24 ± 0.26 ^{b}	5.10 ± 0.45 ^{c}	5.03 ± 0.30 ^{c}	5.07 ± 0.34 ^{c}	5.48 ± 0.29 ^{c}	5.24 ± 0.32 ^{c}	5.31 ± 0.35 ^{c}

* Different superscripted letters indicate significant differences ($p < 0.05$) within each row.

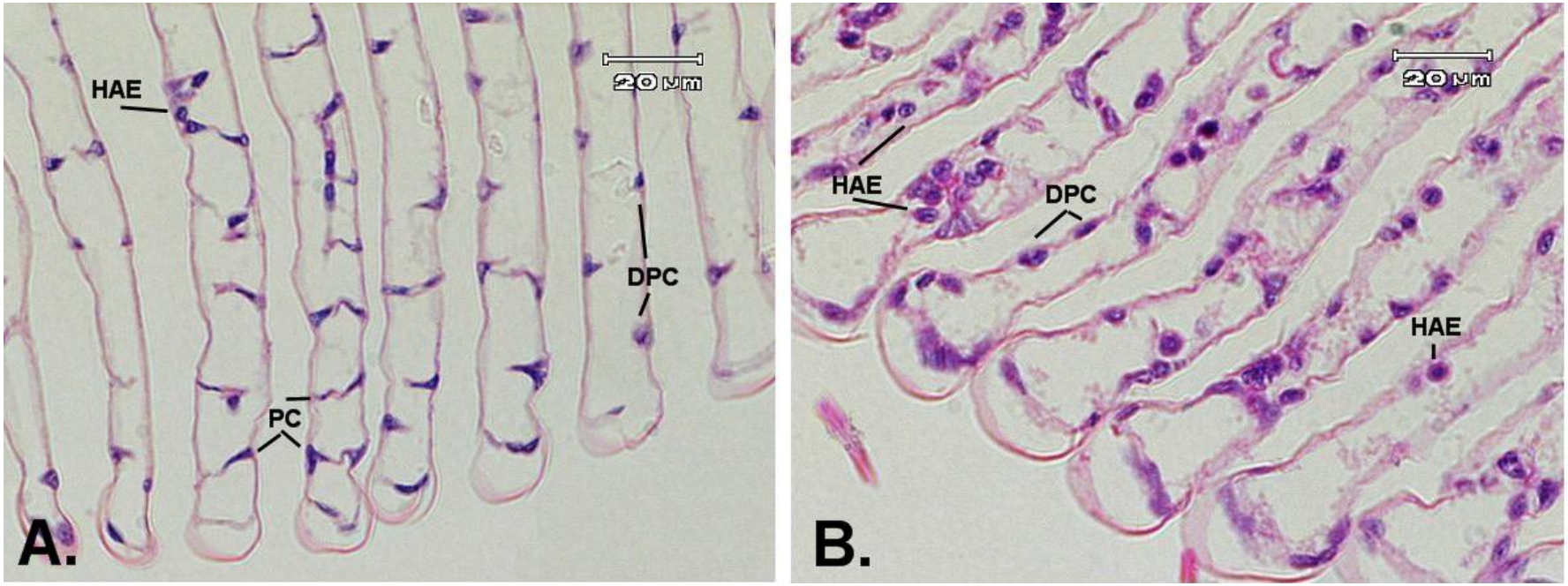

Figure 4: Closer magnification (× 40) of the anterior gills from early *Portunus pelagicus* juveniles in the control at medium K^+ (A) and those exposed to 2.85 mM ammonia-N (40 mg l^{-1}) at low K^+ (B). Note the increased prevalence of disrupted pillar cells (DP), presence of haemocyes (HAE), increased epithelial thickness and greater degree of lamellae distortion of the gills exposed to 2.85 mM ammonia-N at low K^+ after 96-h. Scale bars = 20 µm.

4. Discussion

In the past, studies have been conducted to measure gill Na^+/K^+-ATPase activity and ammonia-N excretion rates of crustaceans when exposed to elevated ammonia-N concentrations (Chen and Nan, 1992; Wang et al. 2004) and evaluating variations in these responses based on species-specific osmoregulatory abilities and gill permeabilities from different crab species (Weihrauch et al. 1999). More recently, a succession of investigations has been carried out to quantity gill Na^+/K^+-ATPase activity and their kinetic properties from dissected gills of aquatic crustaceans at different NH_4^+ and K^+ concentrations (Furriel et al. 2004; Gonçalves et al. 2006; Garçon et al. 2007; Masui et al. 2002; 2005; 2009). These studies have provided invaluable information that help better our understanding regarding the mechanisms involved in crustacean ammonia-N and ion regulation. However, it appears that, so far, no studies have yet been conducted to test the adaptive responses of crustaceans as whole organisms when directly exposed to different ammonia-N and K^+ combinations of the water or simultaneously compared gill Na^+/K^+-ATPase activity rates under both *in vivo* and *in vitro* conditions. Furthermore, while the ion constituents of *in vitro* conditions are often artificial, the environment of aquatic crustaceans may also be artificially manipulated, and for longer periods. In such cases, the gills, which are the main boundary structures of living aquatic crustaceans (Péqueux, 1995), are in constant contact with the external medium and could therefore provide a complimentary approach to *in vitro* experiments for understanding haemolymph ammonia-N and ion regulation of crustaceans.

Results from previous *in vitro* experiments demonstrated that elevated K^+, ammonia-N and their combination stimulated gill Na^+/K^+-ATPase activity of the freshwater prawn, *Macrobrachium olfersii* (Furriel et al., 2004), the hermit crab, *Clibanarius vittatus* (Gonçalves et al. 2006) and the blue crabs *Callinectes ornatus* (Garçon et al. 2007) and *C. danae* (Masui et

al. 2002; 2005; 2009). Since it is well known that NH_4^+ can substitute for K^+ during Na^+/K^+-ATPase activity (Weihrauch et al. 2004), evidence from these experiments with *M. olfersii*, *C. vittatus, C. ornatus* and *C. danae* indicated that, within media containing elevated NH_4^+ and K^+ levels, specific binding sites to these ions became available and/or new binding sites became exposed via gill conformational changes (Masui et al. 2002; Furriel et al. 2004; Gonçalves et al. 2006; Garçon et al. 2007). In contrast, Pan et al. (2006) performed an *in vivo* experiment showing that increased K^+ (or reduced Na^+/K^+ ratios) of artificial seawater significantly reduced gill Na^+/K^+-ATPase activity of postlarvae peneaid shrimp *Marsupenaeus japonicus* between 12 to 72-h of exposure.

In the current study, elevated K^+ levels significantly increased gill Na^+/K^+-ATPase activity of early *P. pelagicus* juveniles, however, when ammonia-N was present, gill Na^+/K^+-ATPase activity significantly decreased indicating a species-specific response. However, despite the presence of ammonia-N depressing gill Na^+/K^+-ATPase activity of the crabs at high K^+, compared to those without added ammonia-N, this still remained relatively high and was not significantly different to those at normal K^+. While gill Na^+/K^+-ATPase is known to be one of the main driving forces for excreting ammonia-N across a gradient (Weihrauch et al. 2004), the ammonia-N excretion rates significantly decreased and the haemolymph ammonia-N levels significantly increased for *P. pelagicus* juveniles when simultaneously exposed to elevated ammonia-N and K^+, compared to those at normal K^+ of the seawater. Although our previous studies demonstrated the high adaptability of *P. pelagicus* juveniles to elevated ammonia-N (Romano and Zeng, 2007c, 2009, 2010) and suggested that this was linked to their ability to regulate haemolymph ammonia-N levels substantially below the environment likely via increased gill Na^+/K^+-ATPase activity (Romano and Zeng, 2009, 2010), the results from the

current study revealed that K^+ imbalances in the seawater significantly diminished such an ability.

A reduction in the adaptability of *P. pelagicus* to both elevated ammonia-N and K^+ exposure may be linked with reduced ammonia-N excretion efficiency due to these ions competing during Na^+/K^+-ATPase activity. If such a competition does exist, it would imply that the gills of *P. pelagicus* possess non-specific binding sites for NH_4^+ and K^+ or that new binding sites for these ions were either not available or were insufficiently exposed. The results of our *in vitro* experiment appears to support such a possibility. When the assay was run at various ammonia-N and K^+ combinations, no significant difference was detected on the gill Na^+/K^+-ATPase activity among any treatment. While our preliminary *in vitro* experiment would require confirmation by further research utilising a broader K^+ range and evaluating the kinetic properties of gill Na^+/K^+-ATPase activity, the fact that no significant ammonia-N, K^+ or interactive effect was observed on gill Na^+/K^+-ATPase activity clearly shows inconsistent results with those of the *in vivo* experiment. A possible explanation may have included adverse physiological changes during the *in vivo* experiment, such as disruptions to their haemolymph chemistry (Racotta and Hernández-Herrera, 2000) and/or cardiac function (Schmitt and Uglow, 1997; Martinez-Vea et al. 1999) prior to their sampling. Certainly, these factors are likely to impact living organisms, particularly since *P. pelagicus* juveniles are known to be highly vulnerable to elevated K^+ exposure (Romano and Zeng. 2007b).

While K^+ deficiencies of seawater have been shown to negatively impact the survival and growth of crustaceans (Pan et al. 2006; Prangnell and Fotedar, 2006; Romano and Zeng, 2007d; 2009b), to our knowledge, no prior studies have investigated the adaptive responses of live aquatic animals to elevated ammonia-N exposure at K^+ deficient conditions. The results of the current experiment show that the crabs subjected to these conditions resulted in

significantly lower ammonia-N excretion rates and gill Na^+/K^+-ATPase activity while the haemolymph ammonia-N levels significantly increased. Again, the results confirm a reduced ability of *P. pelagicus* juveniles to cope with elevated ammonia-N exposure when K^+ levels are unbalanced in seawater. This result is consistent with a previous investigation measuring ammonia-N excretion from the serosal side to the mucosal side of turtle bladders, which revealed that K^+ deficiencies on the mucosal side reduced ammonia-N transport (Talor et al. 1987). Although the exact mechanisms leading to these are not yet clear, evidence suggests that, for the K^+ level used in the current *in vivo* experiment, K^+ was not a limiting factor since gill Na^+/K^+-ATPase activity was more than 3 fold higher at low K^+, than at normal K^+, when no ammonia-N was added. Interestingly, this latter result is again different to a previous investigation on *M. japonicus* in which, under similar conditions of no added ammonia-N at K^+ deficient (or high Na^+/K^+ ratios) conditions, a significant decrease to gill Na^+/K^+-ATPase activity was detected (Pan et al. 2006).

Finally, for the crabs exposed to the same ammonia-N level, both low and high K^+ caused significantly more gill damage. It is, however, unclear whether such damage may hinder/ prevent necessary gill conformational changes to allow additional specific K^+ and NH_4^+ sites to emerge in living animals and this interesting topic clearly warrants further research.

5. Conclusions

While it is known that early *P. pelagicus* juveniles exhibit a high adaptability to elevated ammonia-N exposure (Romano and Zeng, 2007c; 2009; 2010), the current study revealed that altered K^+ levels, in relation to that of natural seawater, significantly diminished such an ability. This was reflected by reduced ammonia-N excretion rates and gill Na^+/K^+-ATPase activity, increased haemolymph ammonia-N levels and more severe gill damage. The

discrepant gill Na^+/K^+-ATPase results between the *in vivo* and *in vitro* experiments when K^+ and ammonia-N were manipulated in the culture water and during the Na^+/K^+-ATPase assay, respectively, may have included combined ammonia-N and K^+ stress leading to disruptions to other physiological processes and gill damage over time in living organisms. Clearly more research is required to identify the exact cause(s). It is nevertheless suggested that future *in vitro* studies on ammonia-N excretion and osmoregulatory changes of crustaceans under different ammonia-N and K^+ levels may need to consider incorporating *in vivo* studies to test their applicability on living organisms.

Chapter 7

The roles of dietary highly unsaturated fatty acids and phospholipids on improvement of survival, growth and haemolymph ion maintenance of early *Portunus pelagicus* juveniles

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1. Introduction

Blue swimmer crab, *Portunus pelagicus,* early juveniles are relatively weak hypo-and hyper-osmoregulators (Chapter 2). This can be viewed as a significant limiting factor to the aquaculture potential of this commercially important species because it may limit site selection and their productivity when subjected to salinity fluctuations (Hurtado et al. 2006), such as many tropical regions with high seasonal rainfalls or droughts (Cowan et al. 1999; Wahab et al. 2003). For example, prawn pond farms in the Australian north Queensland region commonly experience salinities as high as 45 ‰ during the dry season whereas, during the rainy season, salinities can rapidly decrease to salinities as low as 13 ‰ (Romano, pers. obser.). Since it has been demonstrated in Chapter 2 that the survival and growth of *P. pelagicus* juveniles is highly salinity dependent, mitigating this potential limitation through diets specifically designed to improve their performance under hypo-and hyper-osmotic conditions clearly will benefit the industry.

Highly unsaturated fatty acids (HUFA) and phospholipids (PL) are important essential nutrients to marine crustaceans (Sheen and Wu, 2002; Coutteau et al. 1997). HUFA have many important functions for marine species, which include being an integral component of cell membranes and a precursor for eicosanoids necessary for molting (Hazel, 1984; Pruitt, 1990;

Sheen and Wu, 2002). PL also have many roles since, in addition to being important components to cell membranes, it also facilitates lipid transport within the haemolymph (via lipoproteins), provides an important energy source and aids hepatopancreatic lipid storage (Coutteau et al. 1997; Roy et al. 2006). These two important lipid components often need to be supplemented in the diets of marine crustaceans to ensure optimal survival, development and growth since marine crustaceans commonly exhibit either an inability to synthesise both HUFA and PL *de novo* or to a limited degree insufficient to meet their metabolic needs (Kanazawa et al. 1979; Kanazawa et al. 1985). While it is known that optimal dietary supplemental levels for both HUFA and PL are often species-specific (Deering et al. 1997; Merican and Shim, 1997; Sheen and Wu, 2002), salinity changes in the culture water may further alter dietary HUFA and PL requirements of a particular crustacean (Hurtado et al. 2006).

For the Pacific white shrimp, *Litopenaeus vannamei*, it has been previously demonstrated that at a high salinity of 50 ‰, higher dietary HUFA levels were required to reach comparable growth rates as those cultured at a normal salinity of 30 ‰ (Hurtado et al. 2006). However, interestingly, higher dietary HUFA did not improve their growth at a low salinity of 5 ‰ (Hurtado et al. 2006). In spite of their improved growth at hyper-osmotic conditions, a similar experiment reported that diets containing either high or low HUFA had no significant effect on the haemolymph osmolality of *L. vannamei* cultured under either high or low salinities (Hurtado et al. 2007). In contrast to this finding, high dietary HUFA supplementation has been reported to significantly increase the haemolymph osmolality of the blue shrimp, *Peneaus stylirostris*, when exposed to a low salinity of 10 ‰ (Chim et al. 2001). While these differences may be attributed to species-specific osmoregulatory abilities, dietary HUFA supplementation appears to represent a potential venue for improving crustacean productivity in aquaculture when cultured under sub-optimal salinity conditions.

Although investigations on the long term effects of dietary PL supplementation on osmotic stress of crustaceans are lacking, interestingly, it has been previously demonstrated that higher dietary PL supplementation can substantially improve the ability of crustaceans and fish to withstand sudden salinity stress tests (SSST) (Camara et al. 1997; Kontara et al. 1997; Tago et al. 1999; Wu et al. 2007). However, it should be noted that clear distinctions exist between the two since SSST are not designed to predict long term performance of the animal under the tested salinities, but rather their overall strength or quality (reviewed by Palacios and Racotta, 2007).

A dietary approach to improve the productivity of cultured crustaceans at osmotically stressful conditions appears to have potential, hence, two separate experiments were designed and performed to determine if dietary HUFA and PL supplementation can improve the survival and growth of early *P. pelagicus* juveniles when cultured under hypo-and hyper-osmotic conditions and if any such improvements are linked with enhanced haemolymph ion maintenance. Furthermore, these experiments will also help determine whether optimal dietary HUFA and PL levels for *P. pelagicus* juveniles may vary depending on the culture salinities.

2. Materials and Methods

2.1. Source of crabs and acclimation

The crabs used in both experiments were cultured from newly hatched larvae as described in Chapter 2. Two separate batches of larvae hatched from two different females were cultured for the fish oil inclusion level and PL experiments, respectively. To acclimatise the newly settled first stage crabs (C1) from the larval cultures for the subsequent fish oil inclusion level and PL experiments, the following procedures were adopted.

Upon the appearance of newly settled C1 crabs in the larval culture tanks, healthy crabs with full appendages were transferred to 3 separate 300-L tanks filled with seawater of the same salinity and temperature as in the larviculture tanks, *i.e.* 25 ‰ and 28°C, respectively. Subsequently, over the course of 48-h, the salinity in the three tanks were adjusted to become 14, 30 and 42 ‰, respectively with a salinity adjustment rate of -2 or +2 ‰ per 5-h through the addition of either de-chlorinated freshwater or concentrated brine. During the 48-h acclimation period, the juvenile crabs were fed to satiation on a combination of frozen enriched *Artemia* and pelleted food (Ridley) designed for the black tiger prawn, *Penaeus monodon*, with uneaten food and faeces removed daily. After the 48-h acclimation period, healthy and intact crabs that had just molted to the C2 stage were transferred randomly from the acclimation tanks to their respective salinity treatments for the commencement of the experiments.

2.2. Preparation of salinity treatments

Three salinities of 14, 30 and 42 ‰, representing low, normal and high salinities, were set up for both the fish oil inclusion level and PL experiments. These salinities were chosen since it has been shown in the previous salinity experiment (Chapter 2) that both the high and low salinities of 14 and 42 ‰ represent sublethal but osmotically stressful conditions for early *P. pelagicus* juveniles, which were sufficient to significantly reduce their survival and growth while not causing a total mortality. The natural seawater (5 μm filtered and UV sterilised), with an original salinity of 36 ‰, and ammonia, nitrite and nitrate levels all < 0.01 mg l^{-1}, was used as the source of seawater. To reduce the salinity to 14 and 30 ‰, de-chlorinated freshwater was added, while to increase the salinity, the source seawater was evaporated until it reached 42 ‰. The pH of the water was adjusted to 8.1 by adding NaOH pellets when required to ensure all salinity treatments had a similar pH throughout the experiment.

2.3. Diet preparation

For both fish oil inclusion level and PL experiments, five iso-nitrogenous and iso-lipidic semi-purified diets were formulated with five different levels of fish oil inclusion (0, 3, 6, 9 and 12 % diet weight) or PL (0, 4, 8, 12 and 16 % diet weight), respectively. Both the fish oil (Table 1) and PL (Table 2) used for supplementing the experimental diets were sourced from Sigma Aldrich Pty. Ltd and the formulation of the other diet ingredients was based on those used in similar diet experiments for juveniles (Sheen, 2000) and megalopae (Holme et al. 2007) of the mud crab, *Scylla serrata*, with slight modifications.

For both experiments, prior to diet preparation, the fishmeal was first pulverized and sieved through a 300 µm mesh before being added to a chloroform/methanol solution (2:1, v:v) (Folch et al. 1957) and mixed vigorously on a magnetic stirrer to remove inherited fats. The solution was then filtered with the aid of a vacuum pump and this de-fatting process was repeated three times to ensure the maximum removal of inherited lipids in the fishmeal.

For the fish oil inclusion level experiment, to ensure iso-lipidic levels in each diet, macadamia oil was used to balance the differences of fish oil added to each diet. The choice of macadamia oil as the balancing oil was based on the fact that macadamia oil has a negligible amount of *n*-3 HUFA which is lower than most other commercially available oils. For the PL experiment, soybean oil was used to ensure each diet was also iso-lipidic, and the use of soybean oil is based on the fact it contains high PUFA level, therefore can prevent PUFA discrepancies between diets.

In the subsequent diet preparation, all dry and wet ingredients of the diets were first mixed well in separate mixing bowls before the two were combined together and thoroughly blended in an electric mixer. Agar, which was used as the binder, was then dissolved in 100 ml of distilled water heated to $\geq 80^{o}$C. As the agar solution turned clear, the heat was turned off

and the solution was stirred at a low speed with a magnetic stirrer until the temperature dropped to approximately 40^{o}C. The stirrer was then turned to a high speed and the prior mentioned diet mixture was slowly poured into the agar solution to finalise the diet formulation process. The diet mixture quickly became gelatinised when the temperature reduced further to 37^{o}C (Sheen, 2000). The gelatinised diet was then cut into small pieces of approximately 2 mm^{3} and stored at -20^{o}C until used.

Table 1: Ingredients of experimental diets (% diet dry weight) used for the fish oil inclusion level experiment.

	Fish oil inclusion level				
Ingredient	0 %	3 %	6 %	9 %	12 %
De-fatted fish meal [a]	50	50	50	50	50
Fish oil [b1]	0	3	6	9	12
Macadamia oil [c]	12	9	6	3	0
Cholesterol [b2]	1	1	1	1	1
Lecithin [b3]	6	6	6	6	6
Vitamin mix [d1]	4	4	4	4	4
Mineral mix [d2]	4	4	4	4	4
Choline [b4]	1	1	1	1	1
DCP [b5]	0.6	0.6	0.6	0.6	0.6
Agar [b6]	12	12	12	12	12
Starch [b7]	5	5	5	5	5
Cellulose [b8]	1.4	1.4	1.4	1.4	1.4

[a] Skretting Tasmania
[b] Sigma Aldrich Co; [1] from menhaden F8020 [2] 98% powder C8667 [3] 30% Phosphatidylcholine P3644 [4] 98% powder C7527 [5] dibasic calcium phosphate C4131 [6] A7002 [7] S4126 (corn) [8] alpha C8002
c Macadamia (Aust) Ltd.
[d] Rabar Pty Ltd 1 ZZ600 DPI, each 1 kg contains: vitamin A 2 miu, vitamin D3 0.8 miu, vitamine E 40 g, vitamin K 2.02 g, inositol 50 g, vitamin B3 30.40 g, vitamin B5 9.18 g, vitamin B9 2.65 g, vitamin B2 4.48 g, vitamin B12 0.004 g, biotin 0.1 g, vitamin B6 4g, vitamin B1 3.4 g, vitamin C 44.4 g, para amino benzoic acid 20 g, tixosil 5 g, antioxidant 30 g [2] ZZ605 DO DPI, each 1 kg 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 mg, aluminum 100 mg, chromium 100 mg.

Table 2: Ingredients of experimental diets (% diet dry weight) used for the PL experiment.

	PL level				
Ingredient	0 %	4 %	8 %	12 %	16 %
De-fatted fish meal [a]	50	50	50	50	50
Total lipids (1:1; fish:soybean)	4	4	4	4	4
Phospholipids [c]	0	4	8	12	16
Soybean oil [c1]	16	12	8	4	0
Cholesterol [b2]	1	1	1	1	1
Vitamin mix [d1]	4	4	4	4	4
Mineral mix [d2]	4	4	4	4	4
Choline [b4]	1	1	1	1	1
DCP [b5]	0.6	0.6	0.6	0.6	0.6
Agar [b6]	12	12	12	12	12
Starch [b7]	5	5	5	5	5
Cellulose [b8]	2.4	2.4	2.4	2.4	2.4

[a] Skretting Tasmania
[b] Sigma Aldrich Co; [1] from menhaden F8020 [2] 98% powder C8667 [3] 30% Phosphatidylcholine P3644 [4] 98% powder C7527 [5] dibasic calcium phosphate C4131 [6] A7002 [7] S4126 (corn) [8] alpha C8002
c [1] CSD Grains PTY LTD
[d] Rabar Pty Ltd 1 ZZ600 DPI, each 1 kg contains: vitamin A 2 miu, vitamin D3 0.8 miu, vitamine E 40 g, vitamin K 2.02 g, inositol 50 g, vitamin B3 30.40 g, vitamin B5 9.18 g, vitamin B9 2.65 g, vitamin B2 4.48 g, vitamin B12 0.004 g, biotin 0.1 g, vitamin B6 4g, vitamin B1 3.4 g, vitamin C 44.4 g, para amino benzoic acid 20 g, tixosil 5 g, antioxidant 30 g [2] ZZ605 DO DPI, each 1 kg 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 mg, aluminum 100 mg, chromium 100 mg.

2.4. Dietary fatty acid analysis

All diets used for fish oil inclusion level experiment were analysed to obtain their actual fatty acid (FA) contents (mg g^{-1}). This was done by first extracting the lipid from the diets using chloroform/methanol according to Folch et al. (1957). The lipid extract was then further broken down to fatty acid methyl esters (FAME) using 14% boron trifluoride-methanol and analyzed in a gas chromatograph on a 30 m × 0.25 mm fused silica capillary column with a 0.25 µm coating (Agilent Technologies, USA). Starting at 140^{o}C, the column temperature was gradually increased to 210^{o}C at 3^{o}C per minute. The FA were identified by comparing their retention time with reference FAME from Sigma Aldrich Co, USA. FA quantification were

determined by comparing the peak response with heneicosanoic acid and are presented in Table 3.

Table 3: Analysed fatty acid composition of the diets used for fish oil inclusion level experiment (mg g^{-1}).

Fatty acid	Dietary fish oil inclusion level (% diet dry weight)				
	0 %	3 %	6 %	9 %	12 %
∑HUFA	0.2	9.4	17.3	26.5	37.3
∑SFA	34.8	39.2	39.1	41.9	46.7
∑MUFA	109.5	93.3	70.4	52.0	34.6
∑PUFA	40.1	43.2	41.3	42.7	43.4
∑*n*-3	4.3	14.7	23	30.7	44.7
∑*n*-6	36	38	35.6	36.5	36
14	0.8	2.7	4.3	6.0	8.4
15	-	0.3	0.4	0.6	0.9
16	20.6	23.8	24.5	26.7	29.9
16:1*n*-7	18.7	17.6	14.9	13.2	12.2
17	-	0.3	0.5	0.7	0.9
18	7.0	7.0	6.3	6.1	6.0
18:1*n*-9	81.2	66.5	47.0	30.6	14.2
18:1*n*-7	5.5	5.4	4.9	4.7	4.7
18:2*n*-6	36.0	37.7	34.7	34.8	33.8
18:3*n*-3	4.1	4.6	4.6	4.9	5.3
18:4*n*-3	-	1.0	1.8	2.7	3.9
20	4.3	3.5	2.3	1.2	0.3
20:1*n*-11	-	-	0.2	0.3	0.4
20:1*n*-9	3.5	3.2	2.6	2.3	2.0
20:1*n*-7	0.2	0.2	0.3	0.3	0.4
20:2*n*-6	-	-	0.2	0.3	0.4
20:4*n*-6	-	0.3	0.5	0.8	1.1
20:3*n*-3	-	-	-	0.2	0.3
20:5*n*-3	-	3.9	7.0	10.7	15.2
22	1.4	1.1	0.8	0.6	0.3
22:1*n*-9	0.4	0.4	0.3	0.3	0.2
22:4*n*-6	-	-	-	0.2	0.2
22:5*n*-6	-	-	0.2	0.4	0.5
24	0.7	0.5	-	-	-
22:5*n*-3	-	0.8	1.8	2.4	3.2
22:6*n*-3	0.2	4.4	7.8	11.8	16.8
24:1*n*-9	-	-	0.2	0.3	0.5

*∑ include minor fatty acids that are not shown in the table

2.5. Experimental design and set-up

Two separate fish oil inclusion level and PL experiments were run with *P. pelagicus* cultured under 3 salinity conditions (14 ‰, 30 ‰ and 42 ‰) while fed diets containing 5 levels of HUFA or PL (Table 1 and 2), respectively. Due to cannibalistic nature of the crabs, in both experiments, all experimental crabs were maintained individually in round containers of 1-L capacity to avoid cannibalism that could bias the data. Each container was filled with 0.6-L of water with desired salinity and all culture units were arranged in a random block design. For each treatment with different diet and salinity combination, there were three replicates with 7 crabs per replicate. A total of 315 culture units were hence set up for both HUFA and PL experiments and in both cases, experiments was run for 20 days. Throughout the duration of the experiments, each morning, mortalities and molts were checked and recorded, followed by a 100 % water exchange for each container and then the crabs were fed to satiation with one of the designated diets. When any new molts were observed, the carapace lengths of the newly molted crabs were measured after the carapaces had hardened and absolute increase in carapace length was determined by subtracting the new carapace length with the old one. The carapace length was defined as the distance from the longest rostral spike to the abdomen and measured using a digital caliper (0.01 mm) (Mitutoyo, Japan). For the PL experiment, the initial wet weights of the crabs were measured and then again on day 20 for all crabs that were at least 3-days post-molt.

Each day the water temperature and salinity from one replicate of each treatment were measured. Each week the ammonia-N and dissolved oxygen (DO) levels of two replicates from each treatment were measured using an Aquarium Pharmaceuticals ammonia kit and a digital probe (TPS, WP82, Australia), respectively. Throughout the experiments, the water

temperature was maintained at 26 ± 2°C for both experiments and the ammonia-N levels remained < 0.5 mg l^{-1} and the DO always remained > 6.0 mg l^{-1}.

At the end of the experiments on day 20, the carapace length of all surviving crabs were measured. The specific growth rates (SGR) over the 20 day culture period were calculated using the following formula:

$$SGR = (\ln W_f - \ln W_0)/t \times 100$$

Where W_f is the mean carapace length of surviving crabs at the end of the experiments; W_0 is the mean carapace length of crabs at the beginning of the experiments; t is the days of culture.

2.6. Haemolymph sampling

At the ends of both the dietary fish oil inclusion and PL experiments, after the measurement of carapace length, surviving crabs at the intermolt stage (n = 5 to 12) were sampled for haemolymph Na^+, K^+ and Ca^{2+} level measurement. The haemolymph was withdrawn by a syringe inserted through the proximal anthrodial membrane at the base of the second walking leg of the experimental crabs. The haemolymph (20 µl) obtained was immediately diluted with 2 ml of distilled water and the samples were subsequently analysed on a flame photometer (Sherwood 410, Cambridge, UK) for total Na^+, K^+ and Ca^{2+} levels

2.7. Data analysis

The survival, intermolt period, absolute carapace length increases, specific growth rates (SGR) of carapace length/wet weights and the haemolymph ions were analysed using a two-way ANOVA to determine if any salinity, dietary or their interactive effect exists. Prior to analysis, homogeneity of variance was first established using Leven's test. If homogeneity of

variance was violated, a log transformation of the data was performed. If any significant differences were detected ($p < 0.05$), differences among treatments were identified using Tukey's HSD post-hoc test (Zar, 1999). To determine significant differences of survival, growth, development and haemolymph ions for each diet or salinity treatment, a one-way ANOVA was used and significant differences were identified using Duncan's multiple range test (Duncan, 1955). All statistical analysis was performed using the SPSS statistical package, version 16.0.

3. Results

3.1. Survival

3.1.1. Fish oil inclusion level experiment

At all salinity conditions tested, the diet containing deficiencies in fish oil (containing only 0.02 % HUFA) led to the lowest survival rates of the crabs after 20 days of culture (Table 4a). However, no significant dietary HUFA effect on survival was detected ($p > 0.05$) in either the low or normal salinity treatments of 14 ‰ and 30 ‰, respectively (Table 4a). In contrast, in the high salinity treatment of 42 ‰, the crabs fed the diet deficient in fish oil had a significantly lower ($p < 0.05$) survival rate than all other dietary treatments within the same salinity, as well as those fed the same deficient fish oil diets but subjected at salinities of 14 and 30 ‰ (Table 4a). However, fish oil inclusion levels of 3 % (0.94 % HUFA) led to no significant survival difference ($p > 0.05$) for the crabs fed higher fish oil inclusion levels at a salinity of 42 ‰ or between those fed the same diets but at different salinities (Table 4a). A two-way ANOVA detected a significant salinity ($p < 0.05$), dietary HUFA effect ($p < 0.01$), as well as an interactive effect ($p < 0.05$) between the salinity and dietary HUFA level on crab survival (Table 4b).

Table 4a: The mean surival rates (± SE) of early *Portunus pelagicus* juveniles fed diets with different fish oil inclusion levels at salinities of 14, 30 and 42 ‰ for 20 days.

Treatment	Dietary fish oil inclusion level (analysed HUFA level) (% diet dry weight)				
	0 % (0.02 %)	3 % (0.94 %)	6 % (1.73 %)	9 % (2.65 %)	12 % (3.73 %)
Salinity					
14 ‰ salinity	^{A} 75.6 ± 4.6 ^{a}	^{A} 85.3 ± 8.3 ^{a}	^{A} 85.3 ± 8.3 ^{a}	^{A} 85.3 ± 8.3 ^{a}	^{A} 90.0 ± 5.0 ^{a}
30 ‰ salinity	^{A} 75.6 ± 4.6 ^{a}	^{A} 80.3 ± 4.6 ^{a}	^{A} 85.3 ± 8.3 ^{a}	^{A} 90.0 ± 5.0 ^{a}	^{A} 80.3 ± 4.6 ^{a}
42 ‰ salinity	^{B} 42.6 ± 8.3 ^{b}	^{A} 75.5 ± 4.6 ^{a}	^{A} 80.3 ± 4.6 ^{a}	^{A} 85.3 ± 8.3 ^{a}	^{A} 90.0 ± 5.0 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) among treatments within a same fish oil inclusion level or salinity, respectively.

Table 4b: Results of 2-way ANOVA analysis of salinity, dietary HUFA level and their interactive effects on the survival of early *Portunus pelagicus* juveniles.

Source of variance	Df	Sum of squares	Mean square	F	P
Salinity	2	761.91	380.95	3.91	0.031
HUFA	4	3043.33	760.83	7.82	0.001
Salinity × HUFA	8	1843.20	230.40	2.37	0.041
Error	30	2916.66	97.22		

3.1.2. PL experiment

For the PL experiment, crabs cultured at a high salinity of 42 ‰ and fed the diets without PL supplementation (0%) and supplemented with the lowest level of PL (4%) had significantly lower ($p < 0.05$) survival rates than those fed the diets with higher PL supplementations of 12 and 16 % at 42 ‰. Similarity, for the crabs cultured at a low salinity of 14 ‰, those fed the diet without PL supplementation had significantly lower ($p < 0.05$) survival rates than all other diet treatments with dietary PL supplementations within a salinity of 14‰ (Table 5a). However, for the crabs cultured at a normal salinity of 30 ‰, survival remained consistently high of between 95 - 100 % for all treatments, including the one without dietary

PL supplementation, and no significant difference ($p < 0.05$) in survival was detected among any of them (Table 5a).

Crabs fed 0 and 4 % PL supplementation and cultured at salinities of 14 and 42 ‰ had significantly lower survival rates ($p < 0.05$) than those fed the same diets but cultured at 30 ‰ (Table 5a). However, when the dietary PL supplementation increased to 8 % and above, no significant differences were detected between salinities. A two-way ANOVA detected significant effects of salinity ($p < 0.01$) as well as dietary PL supplementation level ($p < 0.05$) on crab survival but not interactive effect of the two ($p > 0.05$) (Table 5b).

Table 5a: The mean survival rates (± SE) of early *Portunus pelagicus* juveniles fed different dietary PL levels at salinities of 14, 30 and 42 ‰ after 20 days.

Treatment	PL level				
	0 %	4 %	8 %	12 %	16 %
Salinity					
14 ‰ salinity	^{B} 63.3 ± 4.6 ^{b}	^{B} 75.6 ± 4.6 ^{ab}	^{A} 80.3 ± 4.6 ^{ab}	^{A} 90.0 ± 5.0 ^{a}	^{A} 95.0 ± 5.0 ^{a}
30 ‰ salinity	^{A} 95.0 ± 5.0 ^{a}	^{A} 95.0 ± 5.0 ^{a}	^{A} 100 ^{a}	^{A} 100 ^{a}	^{A} 95.0 ± 5.0 ^{a}
42 ‰ salinity	^{B} 75.6 ± 4.6 ^{b}	^{B} 75.6 ± 4.6 ^{b}	^{A} 85.3 ± 8.3 ^{a}	^{A} 85.3 ± 8.3 ^{a}	^{A} 85.3. ± 8.3 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) among treatments within a same dietary PL level or salinity, respectively.

Table 5b: Results of 2-way ANOVA analysis of salinity, dietary PL level and their interactive effects on the survival of early *Portunus pelagicus* juveniles.

Source of variance	Df	Sum of squares	Mean square	F	P
Salinity	2	2954.53	1477.26	21.25	0.001
PL	4	841.77	210.44	3.02	0.033
Salinity × PL	8	1030.35	128.79	1.85	0.106
Error	30	2085.33	69.51		

3.2. Development and growth

3.2.1. Fish oil inclusion level experiment

In the fish oil inclusion level experiment, the absolute carapace length increase for the crabs at a low salinity of 14 ‰ was only significantly lower ($p < 0.05$) for those fed diets containing deficient fish oil inclusion levels of 0 % (containing 0.02 % dietary HUFA) at the C2 to C3 stages, compared to those being fed a 9 % fish oil inclusion level (2.65 % HUFA), while at the C3 to C4 stages, those being fed 0 % fish oil inclusion had significantly lower ($p < 0.05$) growth rates compared to the crabs fed 12 % fish oil inclusion levels (3.73 % HUFA). However, by the C4 to C5 stage more significant differences were detected since the crabs fed 0 % fish oil inclusion had significantly lower absolute carapace length increase compared to those fed 6, 9 and 12 % (Table 6). At a salinity of 30 ‰, the only significant difference in absolute carapace length increase were the crabs fed 0 % fish oil inclusion level compared with those fed 9 and 12 % fish oil inclusion levels at the C3 to C4 stages. For the crabs cultured at a high salinity of 42 ‰, those fed 0 % fish oil inclusion levels had significantly lower absolute carapace length increases at the C2 to C3 stages compared to those fed 12 % fish oil inclusion levels, while at the C3 to C4 stages, those fed 0 and 3 % fish oil inclusion level had significantly lower absolute carapace length increases than the higher fish oil inclusion levels of 6, 9 and 12 % (Table 6).

Comparing the absolute carapace length increase of the crabs fed the same fish oil inclusion levels but at different salinities showed no significant growth rate differences between salinities of 14 and 30 ‰ at any of the molt stages (Table 6). However the absolute carapace length increases were significantly lower ($p < 0.05$) for those fed 0 % fish oil inclusion level at a high salinity of 42 ‰ compared to those at 30 ‰ during the first molt stage of C2 to C3 and second molt stage of C3 to C4. While at the C4 to C5 stage, an insufficient amount of crabs fed

0 % fish oil inclusion level at a salinity of 42 ‰ prevented statistical analysis, those fed 3, 6 and 9 % fish oil inclusion levels had significantly lower ($p < 0.05$) absolute carapace length increases compared to those fed the same fish oil inclusion levels at a salinity of 30 ‰ (Table 6). However, those fed the highest fish oil inclusion level of 12 % resulted in no significant absolute carapace length differences ($p > 0.05$) between any of the salinity or crab molt stages (Table 6).

Table 6: The absolute carapace length increase (mm) (± SE) at each molt of early *Portunus pelagicus* juveniles cultured at salinities of 14‰, 30‰ and 42 ‰ and fed diets with different fish oil inclusion levels.

Salinity	Dietary fish oil inclusion level (analysed HUFA level) (% diet dry weight)				
	0 % (0.02 %)	3 % (0.94 %)	6 % (1.73 %)	9 % (2.65 %)	12 % (3.73 %)
	From C2 to C3				
14 ‰	^{AB} 0.42 ± 0.35 ^{b}	^{A} 0.46 ± 0.03 ^{ab}	^{A} 0.49 ± 0.03 ^{ab}	^{A} 0.53 ± 0.05 ^{a}	^{A} 0.51 ± 0.03 ^{ab}
30 ‰	^{A} 0.51 ± 0.05 ^{a}	^{A} 0.53 ± 0.03 ^{a}	^{A} 0.52 ± 0.02 ^{a}	^{A} 0.51 ± 0.03 ^{a}	^{A} 0.56 ± 0.02 ^{a}
42 ‰	^{B} 0.36 ± 0.06 ^{b}	^{A} 0.46 ± 0.04 ^{ab}	^{A} 0.47 ± 0.05 ^{ab}	^{A} 0.48 ± 0.07 ^{ab}	^{A} 0.52 ± 0.02 ^{a}
	From C3 to C4				
14 ‰	^{AB} 0.52 ± 0.05 ^{b}	^{AB}0.58 ± 0.08 ^{ab}	^{A} 0.64 ± 0.07 ^{ab}	^{A} 0.67 ± 0.09 ^{ab}	^{A} 0.69 ± 0.05 ^{a}
30 ‰	^{A} 0.57 ± 0.08 ^{b}	^{A} 0.66 ± 0.09 ^{ab}	^{A} 0.69 ± 0.08 ^{ab}	^{A} 0.68 ± 0.03 ^{a}	^{A} 0.67 ± 0.04 ^{ab}
42 ‰	^{B} 0.43 ± 0.05 ^{b}	^{B} 0.44 ± 0.06 ^{b}	^{A} 0.58 ± 0.08 ^{a}	^{A} 0.66 ± 0.05 ^{a}	^{A} 0.59 ± 0.09 ^{a}
	From C4 to C5				
14 ‰	^{A} 0.56 ± 0.10 ^{b}	^{AB} 0.66 ± 0.08 ^{ab}	^{AB} 0.74 ± 0.08 ^{a}	^{AB} 0.78 ± 0.12 ^{a}	^{A} 0.81 ± 0.13 ^{a}
30 ‰	^{A} 0.62 ± 0.08 ^{a}	^{A} 0.68 ± 0.04 ^{a}	^{A} 0.81 ± 0.05 ^{a}	^{A} 0.86 ± 0.12 ^{a}	^{A} 0.70 ± 0.07 ^{a}
42 ‰	0.46 **	^{B} 0.58 ± 0.08 ^{a}	^{B} 0.67 ± 0.06 ^{a}	^{B} 0.68 ± 0.09 ^{a}	^{A} 0.71 ± 0.07 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) among treatments within a same dietary fish oil inclusion level or salinity, respectively, at each juvenile stage.
** Not enough crabs to calculate SE.

Generally, the crabs fed deficient fish oil inclusion diets in all salinities tested had consistently longer mean developmental times than those fed higher fish oil inclusion diets of 3, 6, 9 and 12 % (Table 7). The only exception to this trend were between the crabs fed 0 %

and 12 % fish oil inclusion levels at a salinity of 30 ‰, since these were not significantly different from each other ($p > 0.05$) (Table 7).

For the crabs fed the same fish oil inclusion levels, no significant differences ($p > 0.05$) were detected between salinities of 14 and 30 ‰. With the exception of the crabs fed 12 % fish oil inclusion level at the salinity of 30 ‰, those cultured at a high salinity of 42 ‰ consistently had significantly longer ($p < 0.05$) developmental times at all fish oil inclusion levels compared to those fed the same fish oil inclusion levels at salinities of 30 and 14 ‰ (Table 7).

Table 7: The mean development time (days) (± SE) from the C2 to C4 molt stage of early *Portunus pelagicus* juveniles of 14‰, 30‰ and 42 ‰ and fed diets with different fish oil inclusion levels.

Salinity	Dietary fish oil inclusion level (analysed HUFA level) (% diet dry weight)				
	0 % (0.02 %)	3 % (0.94 %)	6 % (1.73 %)	9 % (2.65 %)	12 % (3.73 %)
14 ‰	^{A} 14.50 ± 0.84 ^{b}	^{A} 12.73 ± 0.38 ^{a}	^{A} 11.71 ± 0.35 ^{a}	^{A} 12.08 ± 0.42 ^{a}	^{A} 12.34 ± 0.51 ^{a}
30 ‰	^{A} 13.97 ± 0.62 ^{b}	^{A} 12.66 ± 0.52 ^{a}	^{A} 11.78 ± 0.37 ^{a}	^{A} 12.26 ± 0.54 ^{a}	^{AB}13.40 ± 0.48 ^{ab}
42 ‰	^{B} 16.01 ± 0.22 ^{b}	^{B} 14.85 ± 0.27 ^{a}	^{B} 15.10 ± 0.15 ^{a}	^{B} 14.59 ± 0.48 ^{a}	^{B} 14.47 ± 0.32 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) among treatments within a same dietary fish oil inclusion level or salinity, respectively, at each juvenile stage.

For the SGR as carapace length (CL), the crabs fed 0 % fish oil inclusion levels were often significantly lower at all salinities. For example, at the low and normal salinity of 14 ‰ and 30 ‰, respectively, the crabs fed 0 % fish oil inclusion levels had significantly lower ($p < 0.05$) SGR as CL than those fed the higher fish oil inclusion levels. Meanwhile, those cultured at a high salinity of 42 ‰ and fed 0 % fish inclusion oil level had significantly lower SGR than those fed 9 and 12 % (Table 8). However, the degree of differences changed with salinity since those fed 3 % fish oil inclusion levels at 14 ‰ and 42 ‰ were significantly different ($p < 0.05$) from those fed 9 % and 12 % fish oil inclusion levels, respectively, whereas those fed 3 % fish

oil inclusion levels at a salinity of 30 ‰ were not significantly different ($p > 0.05$) than the higher fish oil inclusion diets (Table 8).

The SGR as CL of the crabs cultured at salinities of 14 and 30 ‰ were not significantly different ($p > 0.05$) at all the fish oil inclusion levels (Table 8). At 42 ‰, those fed 0, 3, 6 and 9 % fish oil inclusion levels had significantly lower SGR than those at salinities of 14 and 30 ‰. However, when the crabs were fed the highest fish oil inclusion of 12 %, no significant differences ($p > 0.05$) were detected between salinities (Table 8).

Table 8: The specific growth rates (SGR) of the carapace length of early *Portunus pelagicus* cultured at salinities of 14, 30 and 42 ‰ and fed diets with different fish oil inclusion levels.

Salinity	Dietary fish oil inclusion level (analysed HUFA level) (% diet dry weight)				
	0 %(0.02 %)	3 % (0.94 %)	6 % (1.73 %)	9 % (2.65 %)	12 % (3.73 %)
14 ‰	^{A} 2.11 ± 0.17 ^{c}	^{A} 2.65 ± 0.08 ^{b}	^{A}2.82 ± 0.10 ^{ab}	^{A} 3.07 ± 0.08 ^{a}	^{A}3.05 ± 0.11 ^{ab}
30 ‰	^{A} 2.17 ± 0.07 ^{b}	^{A} 2.78 ± 0.06 ^{a}	^{A} 3.11 ± 0.12 ^{a}	^{A} 3.06 ± 0.07 ^{a}	^{A} 2.98 ± 0.15 ^{a}
42 ‰	^{B} 1.83 ± 0.15 ^{c}	^{B} 2.08 ± 0.10 ^{bc}	^{B}2.26 ± 0.09^{bc}	^{B}2.41 ± 0.08 ^{ab}	^{A} 2.75 ± 0.09 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) among treatments within a same dietary fish oil inclusion level or salinity, respectively, at each juvenile stage.
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A two-way ANOVA detected both a significant fish oil inclusion level and salinity effect ($p < 0.05$) on the absolute carapace length increase, developmental time and SGR of carapace length of the crabs, however, no significant interaction ($p > 0.05$) between the two were detected.

3.2.2. PL experiment

In the PL experiment, no immediate effect was detected on the growth rates (from C2 to C3 molt stages) between each dietary PL treatment within all salinities (Table 9). However, by the second molt (from C3 to C4 molt stages) and third molt (from C4 to C5 molt stages) the

absolute carapace length increase was significantly less ($p < 0.05$) for the crabs fed dietary PL levels of 0 and 4 % at a salinity of 42 ‰, when compared to those fed 12 and 16 % PL diets. Furthermore, for crabs in salinities of 14 ‰, the absolute carapace length increase was significantly less ($p < 0.05$) for crabs fed diets 0 and 4 % for the last molt stage of C4 to C5 when compared to those fed 8 to 16 % dietary PL levels while those at the last molt at a salinity of 30 ‰ on those fed 0 % PL supplementation were significantly less ($p < 0.05$) than those fed 8, 12 and 16 % PL supplementation (Table 9).

For the crabs fed the same dietary PL supplementation of 0 and 4 % resulted in significantly lower ($p < 0.05$) absolute carapace length increases for those cultured at 14 and 42 ‰ from the C2 to C3 stages compared to those fed the same diets at a salinity of 30 ‰ (Table 9). By the next molt stage of C3 to C4, the crabs cultured at salinities of 14 and 42 ‰ and fed 0 % PL supplementation had significantly lower ($p < 0.05$) absolute carapace length increases compared to those fed 0 % PL at 30 ‰. At the last molt stage of C4 to C5, the only significant difference ($p < 0.05$) between salinities were with those fed 8 % PL supplementation at salinities of 42 and 30 ‰. However, when crabs were fed the highest PL supplementation tested of 16 % PL, no significant absolute carapace length increases were detected between any salinities or different molt stages ($p > 0.05$) (Table 9).

Table 9: The mean absolute carapace length increase (mm) (± SE) of early *Portunus pelagicus* juveniles cultured at salinities of 14, 30 and 42 ‰ and fed diets containing different PL levels.

Salinity	PL level (% diet dry weight)				
	0 %	4 %	8 %	12 %	16 %
	From C2 to C3				
14 ‰	^{B} 0.62 ± 0.05 ^{a}	^{B} 0.69 ± 0.04 ^{a}	^{A} 0.66 ± 0.03 ^{a}	^{A} 0.70 ± 0.03 ^{a}	^{A} 0.70 ± 0.05 ^{a}
30 ‰	^{A} 0.70 ± 0.05 ^{a}	^{A} 0.71 ± 0.04 ^{a}	^{A} 0.65 ± 0.05 ^{a}	^{AB} 0.68 ± 0.03^{a}	^{A} 0.76 ± 0.06 ^{a}
42 ‰	^{B} 0.62 ± 0.05 ^{a}	^{B} 0.61 ± 0.07 ^{a}	^{A} 0.67 ± 0.04 ^{a}	^{B} 0.64 ± 0.03 ^{a}	^{A} 0.67 ± 0.05 ^{a}
	From C3 to C4				
14 ‰	^{B} 0.55 ± 0.05 ^{a}	^{AB} 0.61 ± 0.04^{a}	^{AB} 0.68 ± 0.07^{a}	^{A} 0.67 ± 0.04 ^{a}	^{A} 0.65 ± 0.05 ^{a}
30 ‰	^{A} 0.68 ± 0.04 ^{a}	^{A} 0.67 ± 0.06 ^{a}	^{A} 0.71 ± 0.05 ^{a}	^{A} 0.69 ± 0.05 ^{a}	^{A} 0.69 ± 0.07 ^{a}
42 ‰	^{B} 0.56 ± 0.03 ^{b}	^{B} 0.59 ± 0.04 ^{b}	^{B} 0.64 ± 0.03 ^{ab}	^{A} 0.67 ± 0.05 ^{a}	^{A} 0.70 ± 0.07 ^{a}
	From C4 to C5				
14 ‰	^{A} 0.59 ± 0.04 ^{b}	^{A} 0.61 ± 0.06 ^{b}	^{A} 0.76 ± 0.05 ^{a}	^{A} 0.74 ± 0.03 ^{a}	^{A} 0.73 ± 0.04 ^{a}
30 ‰	^{A} 0.61 ± 0.08 ^{b}	^{A} 0.69 ± 0.05^{ab}	^{AB} 0.73 ± 0.02^{a}	^{A} 0.74 ± 0.03 ^{a}	^{A} 0.71 ± 0.04 ^{a}
42 ‰	^{A} 0.59 ± 0.04 ^{b}	^{A} 0.61 ± 0.02 ^{b}	^{B} 0.67 ± 0.06 ^{ab}	^{A} 0.73 ± 0.05 ^{a}	^{A} 0.71 ± 0.04 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) within each dietary PL and salinity treatment, respectively at each juvenile stage.

Crabs fed 0 % PL supplementation had significantly longer ($p < 0.05$) mean developmental times at all salinities compared to those fed higher PL supplementation. (Table 10). Crabs cultured at salinities of 14 and 30 ‰ and fed 0 % PL supplementation had significantly longer developmental times compared to those fed 8, 12 and 16 % (Table 10). At a high salinity of 42 ‰, the crabs fed 0 % PL supplementation had significantly longer ($p < 0.05$) developmental times than those fed the higher PL diets, meanwhile, those fed 4 % PL supplementation had significantly longer ($p < 0.05$) developmental times than the crabs being fed 8 %, 12 % and 16 % PL diets at a salinity of 42 ‰ (Table 10).

Comparing the developmental time of the crabs fed the same diets but at different salinities, the crabs cultured at a high salinity of 42 ‰ consistently had longer developmental times at each dietary PL supplementation compared to those at salinities of 14 and 30 ‰ (Table

10). Further, the crabs fed 0 % PL supplementation were significantly longer ($p < 0.05$) at a salinity of 14 ‰ than those at 30 ‰ (Table 10).

Table 10: The mean developmental time (days) (± SE) of early *Portunus pelagicus* juveniles cultured at salinities of 14, 30 and 42 ‰ and fed diets containing different PL levels.

Salinity	PL level (% diet dry weight)				
	0 %	4 %	8 %	12 %	16 %
14 ‰	^{B} 14.72 ± 0.28 ^{b}	^{A} 13.76 ± 0.19 ^{ab}	^{A} 12.75 ± 0.18 ^{a}	^{A} 12.91 ± 0.15 ^{a}	^{A} 12.79 ± 0.12 ^{a}
30 ‰	^{A} 13.92 ± 0.22 ^{b}	^{A} 13.27 ± 0.27 ^{ab}	^{A} 12.49 ± 0.21 ^{a}	^{A} 12.72 ± 0.24 ^{a}	^{A} 12.80 ± 0.11 ^{a}
42 ‰	^{C} 16.94 ± 0.26 ^{c}	^{B} 15.46 ± 0.31 ^{b}	^{B} 14.91 ± 0.13 ^{a}	^{B} 14.87 ± 0.12 ^{a}	^{B} 14.89 ± 0.19 ^{a}

* Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) within each dietary PL and salinity treatment, respectively.

For the mean specific growth rates there was a clear trend of an increase to crab growth with increasing dietary PL supplementation level at all salinities and the differences were often significant ($p < 0.05$) (Table 11). For example, both the SGR as CL were significantly increased ($p < 0.05$) for the crabs fed 8 %, 12 % and 16 % PL supplementation at 14 and 30 ‰ compared to those being fed 0 % PL supplementation. Meanwhile those fed 12 and 16 % PL supplementation at a salinity of 42 ‰ had significantly higher ($p < 0.05$) SGR as CL than those being fed 0 % PL diets (Table 11). Further, the crabs being fed 12 % and 16 % PL supplementation at salinities of 30 and 42 ‰ had significantly higher ($p < 0.05$) SGR as wet weights (WW) than those fed 0 % PL diets, while at a salinity of 14 ‰, crabs fed 8 %, 12 % and 16 % PL supplementation had significantly higher ($p < 0.05$) SGR as WW than those fed 0 % PL supplementation (Table 11).

The mean SGR as CL and WW were significantly lower ($p < 0.05$) for the crabs cultured at a high salinity of 42 ‰ at all dietary PL levels compared to those cultured at

salinities of 14 and 30 ‰ (Table 11). Meanwhile the SGR as CL or WW were not significantly different ($p > 0.05$) between salinities of 14 and 30 ‰ at all dietary PL levels (Table 11).

Table 11: The mean specific growth rates of carapace length (SGR CL) (mm) (± SE) and specific growth rates wet weight (SGR WW) (g) (± SE) of early *Portunus pelagicus* juveniles cultured at salinities of 14, 30 and 42 ‰ and fed diets containing different PL levels after 20 days.

	PL level (% diet dry weight)				
Salinity					
SGR CL	0 %	4 %	8 %	12 %	16 %
14 ‰ salinity	^{AB} 1.80 ± 0.09 ^{d}	^{A} 1.94 ± 0.06 ^{cd}	^{A} 2.08 ± 0.09 ^{bc}	^{A} 2.23 ± 0.06 ^{ab}	^{A} 2.38 ± 0.07 ^{a}
30 ‰ salinity	^{A} 1.93 ± 0.05 ^{d}	^{A} 1.97 ± 0.09 ^{cd}	^{A} 2.15 ± 0.05 ^{bc}	^{A} 2.33 ± 0.06 ^{ab}	^{A} 2.44 ± 0.04 ^{a}
42 ‰ salinity	^{B} 1.50 ± 0.10 ^{c}	^{B} 1.55 ± 0.05 ^{bc}	^{B} 1.59 ± 0.08 ^{bc}	^{B} 1.79 ± 0.09 ^{ab}	^{B} 1.92 ± 0.06 ^{a}
SGR WW					
14 ‰ salinity	^{AB} 5.07 ± 0.15 ^{d}	^{A} 5.74 ± 0.16 ^{cd}	^{AB} 5.72 ± 0.26 ^{bc}	^{AB} 6.17 ± 0.21 ^{ab}	^{A} 6.68 ± 0.19 ^{a}
30 ‰ salinity	^{A} 5.45 ± 0.26 ^{c}	^{A} 5.63 ± 0.29 ^{c}	^{A} 6.00 ± 0.11 ^{bc}	^{A} 6.59 ± 0.21 ^{ab}	^{A} 6.75 ± 0.19 ^{a}
42 ‰ salinity	^{B} 4.66 ± 0.17 ^{b}	^{B} 4.94 ± 0.15 ^{b}	^{B} 5.04 ± 0.22 ^{b}	^{B} 5.75 ± 0.12 ^{a}	^{B} 5.58 ± 0.13 ^{a}

*Different uppercase and lowercase letters indicate significant differences ($p < 0.05$) within each dietary PL and salinity treatment, respectively.

3.3. Haemolymph ion composition

3.3.1. Dietary fish oil inclusion experiment

In the fish oil inclusion experiment, at the low salinity of 14 ‰, crabs fed the diet deficient of fish oil (containing only 0.02 % HUFA) had significantly lower ($p < 0.05$) haemolymph Na^+ levels than all other diet treatments with fish oil inclusion level higher than 3% (0.94 % HUFA and higher) while no significant difference ($p > 0.05$) was detected for the haemolymph K^+ and Ca^{2+} levels among all diet treatments (Table 12a). At the high salinity of 42 ‰, the crabs fed the fish oil deficient diet had significantly higher ($p < 0.05$) Na^+ levels as well as significantly lower ($p < 0.05$) K^+ levels when compared to those crabs fed the diets with fish oil inclusion level higher than 6% (1.73 % HUFA and higher). Interestingly, at the normal

salinity of 30 ‰, no significant difference ($p > 0.05$) in the haemolymph ion level was detected among any diet treatments (Table 12a).

Between each salinity level, the haemolymph Na^+, K^+ and Ca^{2+} levels of the crabs were all significantly different ($p < 0.01$), regardless of the dietary treatment. The haemolymph ions were lowest at the low salinity of 14 ‰ and the highest at a high salinity of 42 ‰ (Table 12a).

Table 12a: The mean haemolymph Na^+, K^+ and Ca^{2+} levels (mmol l^{-1}) (± SE) of early *Portunus pelagicus* juveniles fed different levels of fish oil inclusion at salinities 14, 30 and 42 ‰ after 20 days. The haemolymph ions were significantly different ($p < 0.01$) at each salinity treatment.

14 ‰ salinity	Haemolymph ionic composition (mmol l^{-1}) of crabs at 14 ‰		
HUFA diets	Sodium	Potassium	Calcium
0 % (0.02 %)	224.7 ± 4.6 ^{b}	6.4 ± 0.2 ^{a}	7.4 ± 0.1 ^{a}
3 % (0.94 %)	249.4 ± 6.9 ^{a}	6.8 ± 0.2 ^{a}	7.5 ± 0.2 ^{a}
6 % (1.73 %)	252.6 ± 1.8 ^{a}	7.4 ± 0.2 ^{a}	7.2 ± 0.2 ^{a}
9 % (2.65 %)	257.8 ± 2.1 ^{a}	7.2 ± 0.3 ^{a}	7.4 ± 0.1 ^{a}
12 % (3.73 %)	255.5 ± 1.2 ^{a}	7.2 ± 0.3 ^{a}	7.5 ± 0.2 ^{a}
30 ‰ salinity	Haemolymph ion composition (mmol l^{-1}) of crabs at 30 ‰		
HUFA diets	Sodium	Potassium	Calcium
0 % (0.02 %)	484.2 ± 3.2 ^{a}	10.0 ± 0.3 ^{a}	10.5 ± 0.2 ^{a}
3 % (0.94 %)	495.7 ± 2.4 ^{a}	10.4 ± 0.5 ^{a}	10.6 ± 0.3 ^{a}
6 % (1.73 %)	502.3 ± 1.4 ^{a}	10.4 ± 0.2 ^{a}	10.3 ± 0.3 ^{a}
9 % (2.65 %)	505.0 ± 4.3 ^{a}	10.6 ± 0.6 ^{a}	10.3 ± 0.2 ^{a}
12 % (3.73 %)	498.4 ± 3.4 ^{a}	10.2 ± 0.4 ^{a}	10.5 ± 0.4 ^{a}
42 ‰ salinity	Haemolymph ion composition (mmol l^{-1}) of crabs at 42 ‰		
HUFA diets	Sodium	Potassium	Calcium
0 % (0.02 %)	560.1 ± 1.3 ^{b}	12.0 ± 0.3 ^{b}	15.2 ± 0.4 ^{a}
3 % (0.94 %)	553.6 ± 1.7 ^{ab}	13.9 ± 0.2 ^{ab}	15.8 ± 0.6 ^{a}
6 % (1.73 %)	547.3 ± 2.2 ^{a}	15.8 ± 0.4 ^{a}	15.5 ± 0.6 ^{a}
9 % (2.65 %)	549.7 ± 1.5 ^{a}	15.5 ± 0.6 ^{a}	15.8 ± 0.4 ^{a}
12 % (3.73 %)	545.5 ± 2.9 ^{a}	16.2 ± 0.5 ^{a}	15.4 ± 0.5 ^{a}

* Different letters indicate significant differences ($p < 0.05$) within each fish oil inclusion treatments.
**The Na^+ level at salinities 14, 30 and 42 ‰ was 183.5, 390 and 563 mmol l^{-1}, respectively, the K^+ level at salinities 14, 30 and 42 ‰ were 3.9, 8.5 and 12.0 mmol l^{-1}, respectively and the Ca^{2+} levels at a salinities of 14, 30 and 42 ‰ were 3.9, 8.5 and 12.0 mmol l^{-1}, respectively.

A two-way ANOVA detected both a significant effect of the levels of fish oil inclusion and salinity ($p < 0.01$) as well as a significant interaction between these on the haemolymph Na^+ ($p < 0.01$) and K^+ ($p < 0.05$) levels, but not on haemolymph Ca^{2+} levels ($p > 0.05$) (Table 12b).

Table 12b: Analysis of salinity, dietary fish oil inclusion levels and their interactions on the haemolymph Na^+, K^+ and Ca^{2+} levels of early *Portunus pelagicus* juveniles when cultured at salinities of 14, 30 and 42 ‰ and fed diets containing different fish oil inclusion levels.

2-way ANOVA of salinity, dietary fish oil inclusion and their interaction on haemolymph Na^+					
Source of variance	Df	Sum of	Mean square	F	P
Salinity	2	1305499.38	652749.69	1.100 E4	0.001
HUFA	4	1272.72	318.18	5.36	0.001
Salinity × HUFA	8	3558.08	444.76	7.49	0.001
Error	60	3560.40	59.34		
2-way ANOVA of salinity, dietary fish oil inclusion and their interaction on haemolymph K^+					
Source of variance	Df	Sum of	Mean square	F	P
Salinity	2	230.82	115.41	123.65	0.001
HUFA	4	23.54	5.88	6.30	0.001
Salinity × HUFA	8	16.37	2.04	2.19	0.041
Error	60	56.00	0.93		
2-way ANOVA of salinity, dietary fish oil inclusion and their interaction on haemolymph Ca^{2+}					
Source of variance	Df	Sum of	Mean square	F	P
Salinity	2	458.42	229.21	181.91	0.001
HUFA	4	3.65	0.91	0.72	0.578
Salinity × HUFA	8	5.70	0.71	0.56	0.801
Error	60	75.60	1.26		

3.3.2. PL experiment

In the PL experiment, no significant difference ($p > 0.05$) was found on the haemolymph Na^+, K^+ or Ca^{2+} levels of early *P. pelagicus* juveniles when cultured at the same salinity but fed diets with different PL levels (Table 13). However, there was a significant

salinity effect ($p < 0.01$) on the haemolymph Na^+, K^+ or Ca^{2+} levels with the crabs cultured at the low salinity having significantly lower ion concentrations ($p < 0.01$) when compared to those cultured at the normal and the high salinities of 30 and 42 ‰, respectively. Meanwhile, the crabs cultured at 42 ‰ had significantly higher ($p < 0.05$) haemolymph Na^+, K^+ and Ca^{2+} levels ($p < 0.01$) than those cultured at the normal and the low salinities of 30 and 14 ‰, respectively (Table 13). A two-way ANOVA detected no significant interactive effect between dietary PL level and salinity ($p>0.05$).

Table 13: The mean haemolymph Na^+, K^+ and Ca^{2+} levels (mmol l^{-1}) (± SE) of early *Portunus pelagicus* juveniles fed different levels of dietary PL supplementation at salinities 14, 30 and 42 ‰ after 20 days. The haemolymph ions were significantly different ($p < 0.01$) between each salinity treatment **

14 ‰ salinity	Haemolymph ionic composition (mmol l^{-1}) of crabs at 14 ‰		
PL diets	Sodium	Potassium	Calcium
0 %	259.2 ± 5.6 [a]	7.5 ± 0.3 [a]	7.3 ± 0.1 [a]
4 %	259.1 ± 4.8 [a]	7.3 ± 0.2 [a]	7.4 ± 0.2 [a]
8 %	254.7 ± 3.5 [a]	7.4 ± 0.3 [a]	7.2 ± 0.2 [a]
12 %	261.2 ± 3.3 [a]	7.3 ± 0.2 [a]	7.5 ± 0.1 [a]
16 %	258.2 ± 2.4 [a]	7.4 ± 0.3 [a]	7.4 ± 0.2 [a]
30 ‰ salinity	Haemolymph ion composition (mmol l^{-1}) of crabs at 30 ‰		
PL diets	Sodium	Potassium	Calcium
0 %	499.2 ± 3.6 [a]	10.1 ± 0.4 [a]	10.2 ± 0.4 [a]
4 %	497.2 ± 2.7 [a]	10.3 ± 0.7 [a]	10.5 ± 0.2 [a]
8 %	495.8 ± 3.6 [a]	10.5 ± 0.2 [a]	10.2 ± 0.3 [a]
12 %	500.0 ± 4.3 [a]	10.4 ± 0.4 [a]	10.5 ± 0.4 [a]
16 %	501.7 ± 3.8 [a]	10.3 ± 0.5 [a]	10.6 ± 0.5 [a]
42 ‰ salinity	Haemolymph ion composition (mmol l^{-1}) of crabs at 42 ‰		
PL diets	Sodium	Potassium	Calcium
0 %	552.6 ± 2.3 [a]	15.8 ± 0.4 [a]	15.6 ± 0.2 [a]
4 %	548.6 ± 2.8 [a]	16.1 ± 0.3 [a]	15.7 ± 0.5 [a]
8 %	549.8 ± 2.3 [a]	15.9 ± 0.5 [a]	15.4 ± 0.3 [a]
12 %	547.5 ± 3.0 [a]	15.7 ± 0.6 [a]	15.3 ± 0.8 [a]
16 %	545.5 ± 1.9 [a]	16.2 ± 0.3 [a]	15.9 ± 0.7 [a]

* Different letters indicate significant differences ($p < 0.05$) within each PL treatment.
**The Na^+ level at salinities 14, 30 and 42 ‰ was 183.5, 390 and 563 mmol l^{-1}, respectively, the K^+ level at salinities 14, 30 and 42 ‰ were 3.9, 8.5 and 12.0 mmol l^{-1}, respectively and the Ca^{2+} levels at a salinities of 14, 30 and 42 ‰ were 3.9, 8.5 and 12.0 mmol l^{-1}, respectively.

4. Discussion

The results of the current study demonstrate that early *P. pelagicus* juveniles have a limited ability to maintain haemolymph ions independent of the external salinities. This finding is in agreement with Chapters 2, 5 and 6 demonstrating that *P. pelagicus* juveniles are relatively weak hyper-and hypo-osmoregulators as well as weak hyper-and hypo-ionoregulators

over a wide range of salinities. While this characteristic can be viewed as a significant disadvantage to their culture due to potentially wide salinity fluctuations in estuaries and open aquaculture systems, particularly in tropical regions, the current study revealed that at high salinities the survival and maintenance of haemolymph ions of early *P. pelagicus* juveniles can be improved through increased dietary HUFA supplementation in the form of fish oil inclusion. Meanwhile, the survival of the crabs can be improved at both low and high salinities by proper dietary PL supplementation. Furthermore, the optimal dietary HUFA requirements for the crabs at low and high salinities were also revealed to be greater, than at normal salinities, which was likely linked with higher demands for dietary HUFA levels for improving haemolymph ion maintenance.

4.1. Dietary HUFA requirements at different salinities

Over 20 days of culture, crabs fed the lowest fish oil inclusion diets of 0 % (0.02 % HUFA), led to the lowest survival in the high, low and normal salinities, however, this was only significant at a high salinity of 42 ‰. Furthermore, the crabs fed diets with deficient fish oil inclusion levels had also significantly lower growth and development rates at all salinities tested. These findings are in agreement with other reports that demonstrated dietary HUFA supplementation is necessary to ensure optimal survival and growth of crustaceans (Deering et al. 1997; Merican and Shim, 1997) and the results of the current experiment also appear to indicate an inability of early *P. pelagicus* juveniles to either synthesise HUFA or in sufficient quantities to meet their metabolic needs. Based on previous studies, decreased survival and growth of crustaceans when fed dietary HUFA deficient diets are likely linked to reduced lipid digestibility, membrane synthesis and/or production of molting hormone eicosanoids (Glencross et al. 2002; Sheen and Wu, 2002).

Interestingly, at a salinity of 30 ‰, an increase from the lowest dietary HUFA level of 0.02 % to the second lowest levels used of 0.94 % was sufficient to cause the SGR to be similar to those fed the diets with higher HUFA levels. However, for a similar result at low and high salinities, higher dietary HUFA levels to 2.65 % and 2.65-3.73 %, respectively were required to increase the SGR of the crabs at comparable levels of those cultured at 30 ‰. These results clearly demonstrate that the dietary HUFA requirement of early *P. pelagicus* juveniles is salinity dependent and their growth can be significantly improved with higher dietary HUFA supplementation when they are under salinity stress, particularly at high salinities.

Similarly in a previous study, when diets with two dietary HUFA supplementation levels (2.9 % and 34.0 %, % based on total fatty acids in diets) were fed to the Pacific White shrimp *L. vannamei* juveniles cultured at low (5 ‰) and high (50 ‰) salinities over a 21 days period, the high HUFA diet improved their growth at the high salinity which was comparable to those cultured at the normal salinity of 30 ‰ (Hurtado et al. 2006). However, in the same study, Hurtado et al. (2006) detected no significant growth improvement at low salinities. Since the growth benefits of *P. pelagicus* juveniles were clear and significant when fed higher dietary HUFA levels at a low salinity of 14 ‰ it may be possible that this discrepancy between the two studies may be due to species-specific differences or the substantially lower salinity condition used for *L. vannamei*. Another possibility may be linked with the limited but extreme dietary HUFA range, of 2.9 and 34.0 %, which caused detrimental effects to *L. vannamei*, was also observed with *Penaeus monodon* (Merican and Shim, 1997).

A contributor for significantly improved growth of *P. pelagicus* juveniles fed diets with higher HUFA levels at osmotically stressful conditions may be due to improved haemolymph ion maintenance since a significant dietary HUFA level, salinity and interactive effect on the haemolymph Na^+ and K^+ levels was detected. The crabs fed HUFA deficient diets at a low

salinity of 14 ‰ had significantly lower haemolymph Na^+ levels compared to those fed diets containing 0.94-3.73 % HUFA and, similarly at a high salinity of 42 ‰, crabs fed diets containing 0.02 % HUFA had significantly increased and decreased haemolymph Na^+ and K^+ levels, respectively compared to those fed 1.73-3.73 % HUFA diets. These findings indicate the different physiological responses to compensate passive salt loss and influx at hypo- and hyper-osmotic conditions, respectively, and may be explained by dietary HUFA directly or indirectly increasing a compensatory process (*e.g.* increased active ion transport), limiting process (*i.e.* decreased gill permeability) or both (Palacios et al. 2004a; Palacios and Racotta, 2007) to improve haemolymph ion maintenance.

An improvement to osmoregulation was also reported for *Penaeus stylirostris* with dietary HUFA manipulation since Chim et al. (2001) showed that after 28 days of feeding on diets with low and high dietary HUFA supplementation, followed by a 4 day exposure to a hypo-osmotic condition of 10 ‰, the haemolymph osmolality was significantly higher in the high HUFA treatment. In contrast, Hurtado et al. (2007) reported that low and high HUFA supplementation did not change the osmoregulatory capacity of *L. vannamei* juveniles when cultured at either low or high salinities after 21 days. However, the same author showed in a similar study that the growth of *L. vannamei* was significantly improved by using high HUFA supplementation when cultured at high salinities (Hurtado et al. 2006). Subsequently, Hurtado et al. (2007) suggested that the shrimps fed the low and high HUFA diets probably relied more on a compensatory and limiting process for haemolymph ion maintenance, respectively, since less energy would be required for a limiting process thus explaining significantly improved growth of the prawns fed the high HUFA diets. However, it is yet unclear as to the exact mechanism(s) involved in improving growth of *L. vannamei* at high salinities since, in addition to no significant dietary HUFA effect on haemolymph osmolality, no significant dietary HUFA

effect on gill water content, gill Na^+/K^+-ATPase activity or any change to haemolymph free amino acids were detected in their experiment (Hurtado et al. 2007). Nevertheless, these findings indicate that haemolymph osmolality measurements should not be used as the sole criteria or predictor for the optimal HUFA level for crustaceans cultured under osmotically stressful conditions.

Interestingly there are indications that the maintenance of haemolymph ion levels for early *P. pelagicus* juveniles are prioritised before growth. For example, the crabs cultured at both low and high salinities of 14 and 42 ‰, respectively, those fed the diets containing relatively lower HUFA levels of 0.94 % to1.73 % had significantly lower SGR compared to the crabs fed diets with higher HUFA contents. However, as previously stated, the haemolymph ion levels of the crabs significantly changed at these respective dietary HUFA treatments. These findings indicate that such a prioritisation for ion regulation when dietary HUFA is in limited supply is probably linked with an attempt to prevent the mortality of the animal, due to disrupted osmoregulation, at extreme salinity conditions.

4.2. Dietary PL

In the PL experiment, the crabs fed the diets with no PL supplementation had significantly decreased survival rates at both low and high salinities than at a salinity of 30 ‰. Based on limited investigations on the PL requirements for crustaceans, dietary PL deficiencies have been reported to cause significantly reduced survival on *Penaeus japonicus* larvae (Kanazawa et al. 1985; Teshima et al. 1986b) and, in contrast, no significant difference in the survival or growth of *L. vannamei* post-larvae were observed when fed PL deficient diets (Coutteau et al. 1996; González-Félix et al. 2002). Coutteau et al. (1996) suggested this was the result of earlier staged crustaceans requiring higher amounts of dietary PL to support their

relatively rapid growth, compared with their older stages. This suggestion was further supported in a later study when it was revealed that higher PL levels were necessary for *P. japonicus* larvae compared to their respective post-larval stages (Camara et al. 1997). In the current study, the high survival of early *P. pelagicus* juveniles at a salinity of 30 ‰, even when deficient dietary PL was provided, may be attributed to the use of juveniles rather than larvae. However, when cultured at osmotically stressful conditions the survival rates of the crabs fed PL deficient diets significantly decreased. Obviously, this indicates higher PL requirements at both low and high salinity conditions for these crabs and our results appear to be the first to demonstrate this pattern.

Interestingly, in contrast to the HUFA experiment, higher dietary PL levels were limited in overcoming the negative growth effects of early *P. pelagicus* juveniles when cultured at a high salinity of 42 ‰. Furthermore, the optimal dietary PL levels for their growth appear similar in each salinity treatment since the highest PL level of 16 % provided the best growth at all salinities. Generally, increased dietary PL has been shown to improve the growth of crustaceans, to a certain point (Kanazawa et al. 1985; Coutteau et al. 1996). In contrast, this is the first experiment demonstrating that a comparable growth benefit at different salinities when the crabs were fed a wide range of PL levels.

Furthermore, in the current experiment, dietary PL level was demonstrated to be non-essential for the haemolymph ion maintenance of early *P. pelagicus* juveniles at different salinities. Since it was demonstrated that HUFA is an essential nutrient to aid ion regulation of *P. pelagicus* at both low and high salinities, the presence of HUFA at the same level within all PL diets may in fact explain the similar haemolymph ions of the crabs within each salinity and dietary PL treatment. It is unclear if this result is species-specific, or linked with their weak osmoregulatory abilities (Chapter 2), and more research is warranted in this field.

5. Conclusions

The results of the current experiment demonstrate that both dietary HUFA and PL are essential for optimal survival and growth of early *P. pelagicus* juveniles. However, at osmotically stressful conditions, their requirement for dietary HUFA for optimal growth and haemolymph ion maintenance was higher, while dietary PL was necessary for their survival. It is therefore recommended that these nutrients should be present at appropriate levels to optimise the production of *P. pelagicus*, particularly when cultured under osmotically stressful conditions for prolonged periods.

Chapter 8

General discussion and conclusions

Throughout this thesis osmoregulation and the coping processes of *P. pelagicus* juveniles to elevated ammonia-N exposure were investigated since these are essential physiological processes for the survival and well being of crustaceans (Péqueux, 1995; Lucu and Towle, 2003; Weihrauch et al. 2004; Freire et al. 2008). Furthermore, these processes were examined both alone and in conjunction with each other due to their physiological link as well as these abiotic factors co-existing in both nature and aquaculture settings (Weihrauch et al. 2004). However, with the exception of strict osmoconformers, it is necessary to emphasise that although the degree of osmoregulation and ammonia-N excretion of crustaceans is greatly dependent on the environment they inhabit, the species concerned as well as their ecological behavior, osmoregulation is a continual process for all aquatic crustaceans (Péqueux, 1995). Thus, the information provided within this thesis will have broad implications for osmoregulating crustaceans as well as the majority of crustaceans that utilise active ammonia-N excretion as a coping mechanism during elevated ammonia-N exposure.

1.1. Salinity

Salinities in aquatic systems range from being stable (*e.g.* open ocean) to highly variable with rapid changes (*e.g.* estuaries). Despite the continual salinity challenges that estuaries present, these environments are known to be important ecosystems for the development of *P. pelagicus* juveniles (Kangas, 2000). However, during the rainy season their

mass emigration to the ocean has often been observed in Australia (Kangas, 2000). The exact cause for this migration was unclear at the time, however, evidence for the cause that likely triggers this phenomenon was presented in Chapter 2. By measuring the survival, growth and osmoregulatory abilities of early *P. pelagicus* juveniles at different salinities it was determined that this crab species are relatively weak osmoregulators, and consequently, prolonged periods of low salinities could severely impact their survival and well being (Chapter 2). Therefore this low adaptability of *P. pelagicus* juveniles at hypo-osmotic conditions appears to be a major limitation in their ecological distribution. Moreover, these findings will likely provide important information for their aquaculture and fisheries management while also being a crucial first step for implementing future experimental designs to understand their responses.

One of the most important factors influencing the iono-osmoregulatory abilities of crustaceans are gill Na^+/K^+-ATPase activity rates, since this enzyme is responsible for monovalent ion (*i.e.* Na^+, K^+ and Cl^-) regulation (Lucu and Towle, 2003; Freire et al. 2008). Results from Chapters 5 and 6 revealed that early *P. pelagicus* juveniles had relatively low gill Na^+/K^+-ATPase activity rates over a broad salinity range and, consequently, led to our suggestion that this is a major contributor to their weak iono-osmoregulatory abilities. Obviously this characteristic can be viewed as a significant limiting factor for their aquaculture in systems with fluctuating salinities or those with sub-optimal salinities for prolonged periods, however, in Chapter 7 methods were investigated to mitigate this potential problem. Results from Chapter 7 provided evidence that it was feasible to enhance the haemolymph ion maintenance of *P. pelagicus* along with improving their survival and growth when subjected to sub-optimal salinities through incresed dietary HUFA and PL supplementation. Indeed, dietary HUFA was deemed essential for haemolymph ion maintaience at sub-optimal salinties which had productivity implications (Chapter 7). The mechanisms underlying these findings at the

present can only largely be explained by speculation and further investigations on the roles of gill Na^+/K^+-ATPase activity (*e.g.* compensatory process) or gill permeability changes (*e.g.* limiting process) associated with these nutrient manipulations may prove fertile.

1.2. Elevated ammonia-N exposure

Another important part of this thesis was to investigate the coping processes of *P. pelagicus* juveniles during elevated ammonia-N exposure due to their link with osmoregulation as well as the relevance of this pollutant in both aquaculture systems and nature (*i.e.* either during their burying or in ecosystems receiving excessive anthropogenic discharges).

In contrast to their low adaptability to salinity challenge, early *P. pelagicus* juveniles had a high ammonia-N tolerance which increased through juvenile development (Chapter 3). To investigate this pattern, histopathological changes to the gills were observed since these structures of crustaceans are responsible for many crucial physiological processes related to coping mechanisms during ammonia-N exposure (Péqueux, 1995). It was revealed that less severe gill damage was detected at the older stages, which directly coincided with increased ammonia-N tolerance which illustrates the importance of gills during elevated ammonia-N exposure (Chapter 3). Interestingly, in Chapter 4, ammonia-N induced gill damage was shown to be reversible, (if the crabs were returned to pristine seawater for sufficient time) and the cause for gill healing of *P. pelagicus* was suggested to be facilitated by significant haemocyte increases within the gill lamellae and haemolymph during elevated ammonia-N exposure (Chapter 4). This response of haemocyte increases was in direct contrast with what had been previously reported with other crustaceans (Jiang et al. 2004; Liu and Chen, 2004; Hong et al. 2007; Verghese et al. 2007; Mugnier et al. 2008; Rodríguez-Ramos et al. 2008). Moreover, gill damage had no significant affect on the haemolymph osmolality, ions or pH in the surviving

crabs while the haemolymph ammonia-N levels remained substantially lower than their environment indicating that ammonia-N toxicity may be related to decreased gas exchange rather than interrupted osmoregulation. Again, this finding of no significant ammonia-N effect on osmoregulation or haemolymph pH was also in contrast with those reported from other crustaceans (Young-Lai et al. 1991; Chen and Chen, 1996; Harris et al. 2001; Cheng and Chen, 2002; Romano and Zeng, 2007a). The cause for this, as well as their ability to maintain haemolymph ammonia-N levels lower than the environment, is likely explained by ammonia-N not disrupting gill Na^+/K^+-ATPase activity or ammonia-N excretion (Chapters 5 and 6) despite their weak iono-osmoregulatory abilities (Chapters 2, 5, 6 and 7). Although increased gill Na^+/K^+-ATPase activity during elevated ammonia-N levels is not a unique response for crustaceans (Chen and Nan, 1992; Furriel et al. 2004; Masui et al. 2002; 2005; Wang et al. 2004; Garçon et al. 2007), the fact that this process remained uninterrupted was a novel finding likely contributing to their high ammonia-N tolerance.

1.3. Combined ammonia-N, salinity and K^+ exposure

Chapters 5 and 6 provided more comprehensive investigations by measuring additional physiological parameters of *P. pelagicus* juveniles in a more varied range of environments which included different ammonia-N, salinity and K^+ combinations. Through these experiments it was revealed that although early *P. pelagicus* juveniles were highly adaptive to elevated ammonia-N exposure, salinity and K^+ changes significantly altered these abilities.

For the first time the results from Chapter 5 showed that low salinities increased ammonia-N excretion rates in *P. pelagicus* juveniles, although simultaneously, caused increased haemolymph ammonia-N levels. These seemingly contradictory findings were significant for two reasons. Firstly, the results indicate that ammonia-N excretion is unlikely to

be the sole mechanism for reducing haemolymph ammonia-N levels in *P. pelagicus* juveniles as well as the degree of responsibility changing at different salinities and these findings may also apply to other crustaceans. Other potential mechanisms to explain such a finding may include ammonia-N detoxification or gill permeability changes (Chen and Chia, 1996; Weihrauch et al. 1999; Lee and Chen, 2003), however, since no experiments have investigated these responses at different salinity/ammonia-N combinations on the same species obviously highlights further areas that remain to be explored. Secondly, since haemolymph ammonia-N levels are directly linked with ammonia-N toxicity (Weihrauch et al. 2004), results from Chapter 5 identified the cause for the general phenomenon of low salinities increasing ammonia-N toxicity to many crustaceans species (Chen and Lin, 1991; Chen and Lin, 1992; Lin and Chen, 2001; Kir and Kumlu, 2006; Li et al. 2007) which previously was only speculation.

While many mechanisms explaining both the weak osmoregulatory abilities and high ammonia-N tolerance of *P. pelagicus* juveniles had been answered, the effects of K^+ needed investigating due to this major ion being directly associated with gill Na^+/K^+-ATPase activity and ammonia-N excretion (Lucu and Towle, 2003; Weihrauch et al. 2004). Furthermore, *in vitro* experiments demonstrated that K^+ and ammonia-N increases within test media of dissected gills increased gill Na^+/K^+-ATPase leading to the suggestion that osmoregulation and ammonia-N excretion would remain undistrupted (Furriel et al. 2004; Masui et al. 2002; 2005; Garçon et al. 2007). However, subjecting living crustaceans to different seawater K^+ and ammonia-N combinations (*i.e. in vivo* experiments) had not yet been investigated.

Interestingly, the *in vitro* results from Chapter 6 showed that K^+ and ammonia-N manipulations of the test media had no significant effect on the gill Na^+/K^+-ATPase activity rates of *P. pelagicus*. In contrast, when the crabs were subjected to K^+ imbalances (either high

or low K^+), related to normal ratios in seawater, the ammonia-N excretion rates of *P. pelagicus* juveniles and their ability to reduce haemolymph ammonia-N levels were significantly diminished (Chapter 6). At elevated K^+ levels, this may have been due to competition between K^+ and NH_4^+ during ammonia-N excretion via Na^+/K^+-ATPase activity, while the causes for a similar result when seawater K^+ was low is still unknown and clearly warrants further investigations. Nevertheless, the fact that *in vitro* and *in vivo* studies were not representative of each other was both novel and significant since this illustrates that *in vitro* studies conducted in the future (*e.g.* Furriel et al. 2004; Masui et al. 2002; 2005; Garçon et al. 2007) may first need confirmation with *in vivo* investigations. This should therefore pave the way for more accurate and reliable physiological studies when attempting to characterise the functions of crustacean gill Na^+/K^+-ATPase activity on ammonia-N excretion and ion regulation.

1.4. Link between osmoregulation and ammonia-N exposure

In a review paper on crustacean ammonia-N excretion, Weihrauch et al. (2004) presented evidence that crustaceans with weak osmoregulatory abilities typically have a higher gill permeability to NH_4^+, and therefore, would rely more heavily on active ammonia-N excretion to counter greater NH_4^+ influxes into their haemolymph. These authors further suggested that, as a result of high organic contents within sediments along with the continual excretion of their metabolic by-products, benthic crustaceans that bury for prolonged periods will likely encounter elevated levels of localised ammonia-N. Consequently, crustaceans that exhibit this ecological behavior developed an efficient ammonia-N excretion mechanism (Weihrauch et al. 2004). Since it was demonstrated that early *P. pelagicus* juveniles are relatively weak osmo-ionoregulators (Chapters 2, 5, 6 and 7) and are known to bury for prolonged periods in nature (Kangas, 2000) these characteristics may help explain many of the

findings within this thesis including: relatively high ammonia-N tolerance (Chapter 3); undisrupted haemolymph osmolality, ions and pH while the haemolymph ammonia-N remained lower than the environment when exposed to elevated ammonia-N (Chapter 4); relatively high and uninterrupted ammonia-N excretion rates (Chapters 5 and 6) and gill Na^+/K^+-ATPase activity increasing at higher ammonia-N levels (Chapters 5 and 6). Although the results presented in some of these Chapters were able to explain how ammonia-N excretion and osmoregulation were maintained and why *P. pelagicus* were highly adaptive to elevated ammonia-N exposure, future work should now be focused on gill NH_4^+ permeabilities, their relationship to osmoregulatory abilities at different salinities as well as quantifying the involvement of gill Na^+/K^+-ATPase activity in ammonia-N excretion versus other potential channels (*e.g.* V-type H^+ ATPase activity). These will all help further our understanding of these crucial but interlinked and complex processes.

In conclusion, using *P. pelagicus* as the experimental animal, the various investigations performed within this thesis have significantly enhanced our understanding of the link between crustacean osmoregulation and ammonia-N excretion while also offering a more accurate method for accomplishing this task. These experiments also furthered our knowledge on the basic biology of *P. pelagicus*, such as their optimal salinity range, salinity-dependent nutrient requirements, ammonia-N tolerance and their associated coping mechanisms under different environmental conditions. Indeed, prior to the above experiments, very limited information in this area existed regarding this highly important commercial species to both aquaculture and fisheries. Therefore this thesis will likely aid in the further expansion of the *P. pelagicus* aquaculture industry as well as highlighting future research directions in order to further our understanding of many crucial physiological functions in aquatic crustaceans.

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Appendix 1:

Standard Operating Procedure for Na^+/K^+-ATPase activity (modified from Holliday (1985)

First step: Homogenising the Gills

The first step is homogenising the gills. The idea is to first break up the cells (using sodium deoxycholate and centrifuging), remove excess debris and preserving the gill contents for the assay in homogenising buffer (HB).

1. First step is to make the HB at a concentration of 0.25 M sucrose, 6 mM EDTA, 50 mM Tris and 0.1 % (w/v) of sodium deoxycholate. The addition of sodium deoxycholoate will turn the solution a milky color (because the sodium deoxycholate will not dissolve at a low pH, due to the addition of EDTA) – but once the buffer pH is increased to 7.2, throughout additions of NaOH solution, the solution will become clear.

To determine the mass of chemicals required to obtain this concentration (based on the molarity given on the containers of the chemicals) use the following formula...

M × Volume × FW (formula weight) = amount (in grams) necessary

So to make a solution with 50 mM Tris in 100 ml...

0.05 M × 0.1 liters × FW = answer

To determine the mass of chemicals required to obtain a given percentage (w/v) the following formula is used...

Weight to measure = (% desired / 100) × solution volume desired

In 100 ml the amount of chemical added are...

Sucrose	8.55 g
EDTA	0.223 g
Tris	0.788 g
Sodium deoxycholate	0.1 g

This should be done between 2-7 days prior to its use. Label this and put in a refrigerator.

When the experiment is complete on the animal...

Equipment needed: HB, glass pipettes and bulb, micropipettes, beakers, centrifuge tubes, digital scale, ice and ice box, small vials and mortar, centrifuge (supra and ultra centrifuge) and rotor (the rotor needs to be placed in a refrigerator overnight).

2. Dissect out the gills, put them in a labelled cryogenic vial and snap freeze them in liquid nitrogen. Often gills will have to be composited to increase their individual weights. Furthermore, because the assay will need to be done in duplicate, and its good to have a back up, make sure there are enough vials.

3. When preparing to homogenise the gills, thaw them out on ice. Then weigh them and add the HB at a ratio of 1:9 (w/v). For example if the gills weigh 2 grams, then the following formula is used...

$1 / 9 \times 2$ grams of gills / X (which is the volume of HB required) -- Cross multiply

X = 18 ml of HB required.

4. Homogenize the gills with twisting strokes on ice (ideally should be consistent amounts, but more important to make sure they are all homogenized).

5. Remove homogenised gills with a glass pipette and put in a labelled centrifuge tube on ice.

6. Prepare the centrifuge to be run at 4°C (preparing this, for the centrifuge to be cold, should be done at least 30 min. prior).

7. Piror to centrifuging, weigh homogenised gills (in the centrifuge tubes + cap) on a digital scale. Record total weight. In order to balance the centrifuge properly, add HB until equal weights are achieved (will normally be drops).

8. First spin on the supracentrifuge will be at 12,000 rpm for 30 minutes.

9. When the centrifuge is finished place centrifuge tubes back on ice. Remove the supernatant with a glass pipette (leave the pellet behind which should be a lot) and the supernatant in new labelled centrifuge tubes on ice.

10. Weigh the homogenised gills (in the centrifuge tubes + cap) on a digital scale. Record total weight. Add HB if needed to achieve equal weights for the ultracentrifuge (again should be just drops).

11. Prepare the ultracentrifuge to be run at 4°C (should be done at least 30 min. prior).

12. Centrifuge again (on the ultracentrifuge) at 26,925 rpm (on the SW 41) or 89,516 RCF for 1 hour.

13. Remove the supernatant with pipette and store in vials within a -80°C freezer. If more than 0.5 ml is obtained than can be distributed to many vials. If a pellet is formed, add 1:5 (w/v) HB (weight based on previous measurements), re-suspend the pellet and similarly stored as the supernatant (but labelled as the pellet).

Second step: Performing the assay -- modified according to Holliday (1985)

This end-point assay is designed to colorimetrically measure liberated inorganic phosphate from the gills (or any organ) in the presence of K^+ and ATP, which will therefore measure Na^+/K^+-ATPase activity. This is done by running two assays from the same sample (with K^+ and no ouabain, known as medium #1) and the other (without K^+ and with ouabain known as medium #2). The reason is that ouabain inhibits Na^+/K^+-ATPase activity. The results from medium #2 are then subtracted from medium #1 to yield the inorganic phosphate reading and is expressed as Pi (inorganic liberated phosphate) hr^{-1} mg $protein^{-1}$.

Before the assay can be run (and a description on how to do this) a number of solutions need to be made first and each be placed in a refrigerator

1. Two different incubation solutions (for running the assay)

2. One start reaction solution

3. One stop reaction solution

4. Phosphate standards (at least 5)

Equipment needed (in addition to above solutions): micropipettes, glass pipettes and bulbs, spectrometer, beakers (for incubation) and warm water bath (at 30°C).

Solutions A & B: Two different incubation solutions

The formula weights (FW) of the required chemicals (note: FW may vary depending on their purity and therefore always check the label).

Chemical	Formula weights
NaCl	58.44
KCl	74.55
Imidazole-HCl	104.54
Ouabain octahydrate	728.77

Concentration

1. 167 mM NaCl, 50 mM KCl, 33 mM imidazole-HCl at pH 7.2 (with K^+ and no ouabain which is the reaction solution).

2. 217 mM NaCl, 1.67 mM ouabain, 33 mM imidazole-HCl at pH 7.2 (without K^+ and with ouabain so no phosphate is released via Na^+/K^+-ATPase activity).

Incubation solution #1: Solution with K^+ but no ouabain

Volume (ml)	NaCl (g)	KCl (g)	Imidazole-HCl (g)
10	0.0976	0.0373	0.0345

Incubation solution #2: Solution without K^+ but with ouabain

Volume (ml)	NaCl	Ouabain octahydrate (g)	Imidazole-HCl (g)
10	0.1268	0.0122	0.0345

Solution C – Start reaction solution: One start reaction is needed and is added to both assay media.

Chemical	Formula Weight
Na_2ATP	551.1
$MgCl_2$ hexahydrate	203.31

Concentration

25 mM Na_2ATP, 50 mM $MgCl_2$ and NaOH solution until the solution reaches a pH of 7.2.

Volume (ml)	Na_2ATP (g)	$MgCl_2$ (g)
10	0.1378	0.1017

Solution D – Stop reaction solution -- "Bonting's reagent". This solution will both stop the reaction as well as develop the color (based on the concentration of liberated phosphate).

Chemical	Formula weight
Ammonium molybdate	1235.86
Iron (II) sulphate heptahydrate	278.01

Concentration

560 mM H_2SO_4, 8.1 mM ammonium molybdate, 176 mM $FeSO_4$

Determining how much of H_2SO_4 is required is different because this is a solution rather than a solid. First need to know what the molarity of H_2SO_4 is which is 18.4 M (If, for example, normality is required, than M just has to be multiplied by 2).

So, by using the above concentration as an example in 10 ml, the following formula is used...

$$C_A \times V_A = C_B \times V_B$$

$$18.4\ M \times V_B = 0.560\ M \times 10\ ml$$

$$V_B = 0.3043\ ml\ (or\ 314\ \mu l)$$

CAUTION: Because is H_2SO_4 an acid, this must be added to water (never water to acid). This should added very slowly using a glass pipette, while wearing gloves (and a coat) and under a fume hood. Lower the fume hood as low as possible to minimise any chance of splashing.

Volume (ml)	H_2SO_4 (ml)	Ammonium molybdate (g)	Iron (II) sulphate heptahydrate (g)
20	0.6086 (or 609 µl)	0.2002	0.9786
200	6.08	2.002	9.786

Solution E – Phosphate Standard

The first step is to "range find" the samples. However, based on haemolymph ions often being in the $mmol^{-1}$ range, this is a good place to start. Note: Despite Na^+/K^+-ATPase activity often being in the $\mu mol\ l^{-1}$ range, this value is *subtracted* from the two media.

To determine the final standard concentration of standards below, the following formula is used.

Volume of standard / Total volume of solution = X

X × concentration of standard = concentration of the diluted standard

For example, if 0.01 ml of 100 $\mu mol\ l^{-1}$ is added to a total volume of 2 ml (1.5 ml "Bonting's reagent" and 0.49 ml of phosphate free water), then...

0.01 ml / 2 ml = 0.005

0.005 × 100 µmol/l = 0.5 $\mu mol\ l^{-1}$

The concentration of the phosphate standard (from Sigma) is 1000 $mg\ l^{-1}$ PO_4^{3-}. Or 1 $g\ l^{-1}$ PO_4^{3-}.

The FW of PO_4^{3-} is 94.9697.

This will equal 0.01053 moles l^{-1} or 10.53 $mmol\ l^{-1}$ or 10,530 $\mu mol\ l^{-1}$.

The following tables are based on no dilutions of the standard, a 1:10 dilution of standard and a 1:100 dilution of standard – to have a final volume of 2 ml.

No dilutions = 10,530 µmol l^{-1} starting concentration: For a final volume of 2 ml.

Concentration of solution	Volume of solution (1053 µmol/l)	Volume of "Bonting's reagent"	Phosphate free water	Final volume
1579.5 µmol l^{-1}	0.30 ml (or 300 µl)	1.5 ml	0.20 ml	2 ml
789.8 µmol l^{-1}	0.15 ml (or 150 µl)	1.5 ml	0.35 ml	2 ml
368.5 µmol l^{-1}	0.07 ml (or 75 µl)	1.5 ml	0.43 ml	2 ml
157.9 µmol l^{-1}	0.03 ml (or 30 µl)	1.5 ml	0.47 ml	2 ml
52.6 µmol l^{-1}	0.01 ml (or 10 µl)	1.5 ml	0.49 ml	2 ml
0 µmol l^{-1} (blank)	0	1.5 ml	0.50 ml	2 ml

For a 1:10 dilution = 1053 µmol l^{-1} starting concentration: For a final volume of 2 ml.

Concentration of solution	Volume of solution (1053 µmol/l)	Volume of "Bonting's reagent"	Phosphate free water	Final volume
157.95 µmol l^{-1}	0.30 ml (or 300 µl)	1.5 ml	0.20 ml	2 ml
78.98 µmol l^{-1}	0.15 ml (or 150 µl)	1.5 ml	0.35 ml	2 ml
36.85 µmol l^{-1}	0.07 ml (or 75 µl)	1.5 ml	0.43 ml	2 ml
15.79 µmol l^{-1}	0.03 ml (or 30 µl)	1.5 ml	0.47 ml	2 ml
5.26 µmol l^{-1}	0.01 ml (or 10 µl)	1.5 ml	0.49 ml	2 ml
0 µmol l^{-1} (blank)	0	1.5 ml	0.50 ml	2 ml

For a 1:100 dilution = 105.3 µmol l^{-1} starting concentration: For a final volume of 2ml

Concentration of solution	Volume of solution (1053 µmol/l)	Volume of "Bonting's reagent"	Phosphate free water	Final volume
15.79 µmol l^{-1}	0.30 ml (or 300 µl)	1.5 ml	0.20 ml	2 ml
7.89 µmol l^{-1}	0.15 ml (or 150 µl)	1.5 ml	0.35 ml	2 ml
3.68 µmol l^{-1}	0.07 ml (or 75 µl)	1.5 ml	0.43 ml	2 ml
1.58 µmol l^{-1}	0.03 ml (or 30 µl)	1.5 ml	0.47 ml	2 ml
0.53 µmol l^{-1}	0.01 ml (or 10 µl)	1.5 ml	0.49 ml	2 ml
0 µmol l^{-1} (blank)	0	1.5 ml	0.50 ml	2 ml

Once the solutions are made, then it's ready for running the assay

Brief overview: The idea is to measure liberated phosphate from ATPase activity (due to the composition of the solutions, it will measure Na^+/K^+-ATPase activity). Because the gill homogenates will inherently contain phosphate, two medums are used which will be subtracted from the other. The first solution will allow Na^+/K^+-ATPase activity to occur, while the other solution will not (since ouabain is present and K^+ is absent) – therefore two assays, from the same sample, will be run.

Equipment needed: Micropipettes, water bath, beakers, spectrometer (and cuvettes), stop watch, good reading book.

Note: know what the final volume needs to be for proper readings on the spectrometer. Higher or lower volumes can be made by adjusting the volume of added stop reaction (must be consistent with all assays).

Step 1: Thaw out the vials on ice. Do a 5 point phosphate curve of Solution E (following the above tables: which is the standard + "Bonting's reagent" + phosphate-free water). Once phosphate curve is done than do step 2.

Step 2: Put 200 µl of each of the two incubation solutions (Solutions A and B) in two separate beakers and place them in a water bath at 30°C.

Step 3: Add 67 µl of the homogenised gills into each of the two beakers containing the two different incubation solutions.

Step 4: Pre-incubate this for 10 minutes at 30°C.

Step 5: Start reaction by adding 67 µl of the start reaction solution (Na_2ATP and $MgCl_2$ or Solution C). Let this reaction continue (in the water bath) for 30 minutes. **Note:** for weak osmoregulators the water temperature or incubation time can be increased to cause stronger reaction (which is considered desirable for more accurate results).

Step 6: After 15 minutes, stop reaction by adding 1.5 ml of ice-cold stop solution ("Bonting's" reagent or Solution D). Incubate at room temperature for 20 minutes for color formation.

Step 7. Put these solutions in the two cuvettes in the spectrometer for the absorbance reading at 720 nm (to colorimetrically measure the reduced phosphomolybdate complex).

Step 8: Measure the protein content. Can be done using according to a BCA protein assay and absorbance read on a spectrometer.