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Importance of balanced Na^+/K^+ ratios for blue swimmer crabs, *Portunus pelagicus*, to cope with elevated ammonia-N and differences between *in vitro* and *in vivo* gill Na^+/K^+-ATPase responses

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ABSTRACT

It is well known that altered Na^+/K^+ ratios of water can reduce the survival and growth of crustaceans due to osmoregulatory disruptions. However, since the mechanisms involved with osmoregulation are also linked with ammonia-N excretion, it is unclear whether altered Na^+/K^+ ratios will also affect the ability of crustaceans to withstand ammonia-N exposure. To investigate this the ammonia-N toxicity to early blue swimmer crab, *Portunus pelagicus*, juveniles was measured at low (18.2), normal (27.3) and high (54.6) Na^+/K^+ ratios with ammonia-N concentrations set at 0, 1.42, 2.85, 4.28, 5.71 and 7.13 mM (20 replicate crabs/treatment). The crabs were exposed to these combinations for 96-h and at the end of the experiment the hemolymph osmolality, hemolymph Na^+, K^+, Ca^{2+} and ammonia-N levels, gill Na^+/K^+-ATPase activity and gill histopathology were measured. Ammonia-N excretion was also measured at 72-h and 96-h. The results revealed that under both low and high Na^+/K^+ ratios the ammonia-N toxicity to *P. pelagicus* juveniles significantly increased. This was likely linked with a significant decrease to gill Na^+/K^+-ATPase activity and ammonia-N excretion rates along with significantly higher hemolymph ammonia-N levels and greater gill histopathological damage. A subsequent *in vitro* experiment measured gill Na^+/K^+-ATPase activity of *P. pelagicus* under various ammonia-N (0, 3.56 and 7.13 mM) and K^+ (25, 50 and 75 mM) combinations. Interestingly, gill Na^+/K^+-ATPase activity of the crabs was not significantly affected by either ammonia-N or K^+ and no significant interactive effect between the two was detected. The toxicity pattern at sub-optimal Na^+/K^+ ratios should be noted by aquaculturists utilizing inland saline water and, furthermore, it is suggested that future *in vitro* studies may also need to consider incorporating *in vivo* tests to evaluate their applicability to living organisms.

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1. Introduction

Interest in the use of inland saline water for aquaculture is growing since in addition to being highly abundant in certain regions around the world it also offers relative isolation to other native species. The latter is particularly important as it can provide bio-security against viruses that have traditionally plagued the aquaculture shrimp industry (Gong et al., 2004; Pan et al., 2006; Prangnell and Fotedar, 2006; Sowers et al., 2006; Tantulo and Fotedar, 2006; Roy et al., 2007; Gullian et al., 2010). While the exact ionic composition of inland saline waters may vary regionally and temporally, they are generally characterized by K^+ deficiencies (Tantulo and Fotedar, 2006) which have been demonstrated to reduce the osmoregulatory abilities of various crustacean species and, in turn, negatively impact their survival and growth (Pan et al., 2006; Prangnell and Fotedar, 2006; Roy et al., 2007; Tantulo and Fotedar, 2006).

In order to reduce osmoregulatory disruptions under K^+ deficient conditions one of the investigated methods was to increase dietary K^+ and lipid supplementation, although this was met with relatively limited success (Gong et al., 2004; Zhu et al., 2006; Roy et al., 2006). On the other hand, more success had been achieved by directly adding K^+ to the water. For example, it was demonstrated that K^+ supplementation to adjust the Na^+/K^+ ratios to approximately 80% to those of natural seawater Na^+/K^+ ratios was sufficient to ensure good survival and growth in various prawn species (Pan et al., 2006; Prangnell and Fotedar, 2006; Tantulo and Fotedar, 2006).

Normal Na^+/K^+ ratios of seawater are between 25 and 30, while deviations of 10 or more in either direction have been shown to significantly disrupt gill Na^+/K^+-ATPase activity in crustaceans (Pan et al., 2006; Sowers et al., 2006). This has significant implications since ouabain-sensitive Na^+/K^+-ATPase regulates monovalent ions (i.e. Na^+, K^+ and Cl^-) which is often required, and indeed significantly increases, when crustaceans are exposed to osmotic stress (Castilho et al., 2001; Genovese et al., 2004; Holliday, 1985; López-Mañanes et al., 2002; Lucu et al., 2008; Piller et al., 1995; Romano and Zeng, 2010a; Torres et al., 2007). Meanwhile, gill Na^+/K^+-ATPase activity is

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also closely associated with, and largely fuels, active ammonia-N excretion (Lucu and Towle, 2003; Freire et al., 2008). According to this process under elevated ammonia-N exposure, NH_4^+ substitutes for K^+ during Na^+/K^+-ATPase activity and NH_4^+ is subsequently transported to the apical membrane of the gills and excreted to the environment (Weihrauch et al., 2004). Since ammonia-N accumulation is a ubiquitous concern in a closed aquaculture setting (Timmons et al., 2002), this process must remain undisrupted to reduce ammonia-N buildup in the hemolymph to thereby minimize their toxic effects. Indeed, exposure to elevated ammonia-N levels has led to increased gill Na^+/K^+-ATPase activity in crustaceans including the saltwater prawn *Penaeus chinensis* (Chen and Nan, 1992), freshwater prawn *Macrobrachium nipponense* (Wang et al., 2003) and *Portunus pelagicus* (Romano and Zeng, 2010a). Similarly, *in vitro* experiments have also shown that elevated ammonia-N increased gill Na^+/K^+-ATPase activity for the freshwater prawn *Macrobrachium olfersii* (Furriel et al., 2004), and the estuarine crabs *Clibanarius vittatus* (Gonçalves et al., 2006), *Callinectes danae* (Masui et al., 2002, 2005, 2009) and *C. ornatus* (Garçon et al., 2007).

Since the physiological mechanisms involved with osmoregulation and ammonia-N excretion are closely associated, a variable reducing the efficiency of one is likely to, in turn, affect the other. Unfortunately, it appears that no studies have yet been conducted to examine the simultaneous exposure of aquatic crustaceans to elevated ammonia-N concentrations at abnormal Na^+/K^+ ratios on their osmoregulatory and ammonia-N excretory abilities. This should have obvious implications for closed aquaculture systems utilizing inland saline water with altered Na^+/K^+ ratios compared to those of natural seawater.

The aim of the current study was to measure the ammonia-N toxicity of blue swimmer crab, *P. pelagicus*, early juveniles at three levels of Na^+/K^+ ratios (low, normal and high) and combined effects on the hemolymph osmolality, Na^+, K^+, Ca^{2+} and ammonia-N levels as well as ammonia-N excretion rates, gill Na^+/K^+-ATPase activity and gill histopathological changes. A further *in vitro* experiment was performed to compare the gill Na^+/K^+-ATPase activity responses with those from the *in vivo* experiment. The blue swimmer crab, *P. pelagicus*, is a good candidate for such investigations since this portunid is commercially important throughout the Indo-Pacific (Romano and Zeng, 2006). Furthermore, although compared to euryhaline crab species, such as mud crab *Scylla* spp., early *P. pelagicus* juveniles are known to be relatively weak osmoregulators (Romano and Zeng, 2007a), they have a strong ability for hemolymph ammonia-N regulation (Romano and Zeng, 2010a,b). These characteristics of *P. pelagicus* make this species desirable for evaluating adaptive responses of crustaceans to elevated ammonia-N exposure.

2. Materials and methods

2.1. In vivo experiment

2.1.1. Source of experimental animals

The *P. pelagicus* crabs used for the *in vivo* experiment were cultured from newly hatched larvae as described in detail by Romano and Zeng (2006). Briefly, mature *P. pelagicus* were caught in estuarine areas in the Townsville, North Queensland, Australia and maintained in a recirculating system. When an egg carrying female was found, the crab was transferred to an indoor hatching tank filled with pristine seawater at a salinity of 32‰. Upon hatching, the newly hatched larvae were transferred to 300-L circular tanks and stocked at a density of 500 individuals l^{-1}. Larvae were initially fed rotifers (*Brachionus* sp.) at a density of 20–50 individuals ml^{-1}. After the larvae molted to the zoea II stage, newly hatched *Artemia* and then enriched *Artemia* were given as the main food source. Throughout the larval culture period of approximately 15 days, the salinity and temperature of the culture water were maintained at 25 ± 1‰ and 29 ± 1 °C, respectively.

Upon larval settling to the first crab stage (C1), the crabs were transferred to outdoor 1000-L recirculating tanks filled with seawater at a salinity of 33 ± 2‰ and a temperature of 28 ± 2 °C for further culture. Each tank contained numerous shelters to reduce cannibalism. Initially the crabs were fed frozen *Artemia* but were soon weaned onto pelleted feeds designed for penaeid shrimp (Ridley). After another 15 days, early juvenile crabs of similar sizes (mean wet weight = 1.59 ± 0.14 g) were selected and transferred indoors for the commencement of the experiment.

2.1.2. Preparation of test solutions

Three treatments of different Na^+/K^+ ratios at 18.2 (low), 27.3 (normal) and 54.6 (high) (or 4.25 mM, 8.50 mM and 12.75 mM total K^+, respectively) were created. The normal treatment had a similar Na^+/K^+ ratio to that of natural seawater at a salinity of 30‰, while the low and high Na^+/K^+ ratio treatments were 4.25 mM K^+ higher and lower than the normal K^+ level, respectively. Based on our previous experiments, the low and high Na^+/K^+ ratios both represent sublethal conditions to early *P. pelagicus* juveniles (Romano and Zeng, 2007b,c). To create these different Na^+/K^+ ratios, natural seawater (UV sterilized and 5 μm filtered) was first diluted 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 natural seawater at a salinity of 30‰ (see Table 1). Therefore, for all of the three Na^+/K^+ ratio conditions, with the exception of K^+, the major ions of the seawater were at identical levels. To create the normal and low Na^+/K^+ ratio treatments, KCl (analytical grade; Sigma) was then added to increase the K^+ concentrations to 8.50 and 12.75 mM, respectively (or Na^+/K^+ ratios of 27.3 and 18.2, 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 was maintained at 8.10 through the addition of sodium hydroxide (NaOH) pellets.

At each Na^+/K^+ ratio, the experimental crabs were subjected to 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 known to induce mortalities, whereas the lower end would elicit adaptive responses (Romano and Zeng, 2007d, 2010b). 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 be combined with each Na^+/K^+ ratio condition.

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 Na^+/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

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