

FRDC FINAL REPORT

OPTIMISING WATER QUALITY IN ROCK LOBSTER POST-HARVEST PROCESSES

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*Stephen Battaglione, Jennifer Cobcroft, Mark Powell and
Bradley Crear*



Tasmanian Aquaculture
& Fisheries Institute
University of Tasmania



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1 NON TECHNICAL SUMMARY

2000/252

Optimising water quality in rock lobster post-harvest processes

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OBJECTIVES:

1. The production of a manual on optimising the provision of oxygen during rock lobster post-harvest processes
2. To determine the median lethal concentration (LC50) of ammonia to adult southern and western rock lobsters (stressed and unstressed)
3. To determine the physiological consequences of exposing lobsters to sub-lethal ammonia concentrations, and the consequences of further exposing lobsters to acute post-harvest stressors
4. The production of a manual on ammonia problems during rock lobster post-harvest processes

NON TECHNICAL SUMMARY:**OUTCOMES ACHIEVED TO DATE**

The project provided improved efficiency and increased economic returns to the rock lobster industry, via enhanced management of commercial rock lobster holding and transport systems. Specifically it achieved this outcome by:

1. Determining the appropriate maximum short and long-term safe levels of ammonia in holding and transport systems.
2. Better understanding the mechanisms and effects of ammonia toxicity in rock lobsters.
3. Production of two industry guides suitable for fishermen, transporters and holding facility operators.

Rock lobsters can be exposed to poor water quality during all stages of handling and holding prior to going to market. Poor water quality reduces the time a lobster can be held alive and how many animals can be held in a system and thus may reduce profit. The quality of water can be assessed using many different measurements, with two of the most important being oxygen and ammonia (a form of nitrogen). An earlier FRDC funded study investigated oxygen and how it influenced the holding of rock lobsters. However, prior to the current study there was very limited understanding of the harmful effects of ammonia to rock lobsters. Ammonia can accumulate in holding and transport facilities via natural release of ammonia from lobsters, and from the bacterial decomposition of faeces, excess feed, and dead animals. Ammonia can be harmful to crustaceans in small amounts (or low concentrations) and even fatal if concentrations get too high. The toxicity of ammonia to aquatic animals becomes greater when other factors such as low dissolved oxygen, low salinity, and/or low pH (acidity of the water) also interact. In liquids, total ammonia comprises un-ionised ammonia (NH_3), which is the more toxic component, and ionised ammonia (NH_4^+ ; ammonium) in equilibrium. Lobsters can become stressed (having a higher demand upon their biological systems) during holding and handling but it is uncertain what effect this stress has on the ability of lobsters to tolerate ammonia. This project provided a better understanding of the effect of ammonia and other water quality measurements, on the health of stressed and unstressed lobsters.

Very little research had been done on the effect of ammonia on rock lobsters, and none on Australian species, prior to the current study. The project worked out the amount of ammonia in the water that would kill rock lobsters or reduce the time they can be held and hence their market value. The research was carried out on the two most valuable commercial species of rock lobster in Australia, southern rock lobsters, *Jasus edwardsii*, in Tasmania and western rock lobsters, *Panulirus cygnus*, in Western Australia. In large controlled experiments, rock lobsters were exposed to increasing levels of ammonia. The concentration of ammonia that killed 50% of the lobsters over 96 hours (termed the median lethal concentration or 96 h LC50) was determined for both species. Knowing the toxic level of ammonia for rock lobsters provides a benchmark for commercial operators of holding systems, on boats and in factories, to operate below. Southern rock lobsters at 13°C were found to be more tolerant to total ammonia than western rock lobsters at 18°C. However, when temperature was taken into account, the 96 h LC50 for the highly toxic ionised ammonia for unstressed lobsters are similar for both species: 0.85 to 1.17 mg l⁻¹ NH₃-N for

J. edwardsii; and 0.98 to 1.20 mg l⁻¹ NH₃-N for *P. cygnus*. The results indicate that rock lobsters are able to handle similar levels of ammonia to other crustaceans.

Another part of the study was devoted to gaining a better understanding of the biological processes (internal function) of rock lobsters and how they respond to poor water quality. Information was collected from several experiments to determine how animals dealt with stress and if stress made them less tolerant to ammonia. The rate at which ammonia was released by the animals and blood measurements provided useful ways of measuring the ability of lobsters to tolerate stress. We now know that exposure to even low levels of ammonia results in the potentially harmful accumulation of ammonium within lobsters, but given good water quality, they can rapidly remove it from the blood by increased use of the gills. Fortunately, rock lobsters are physically well equipped to handle poor water quality and can be held live for longer periods of time if operators in industry understand the basics of managing water quality.

The technical details of the research are complex and difficult to understand. To make the results of this study more accessible two high quality industry guides on oxygen and recirculation systems were produced that incorporated the scientific data generated during the project. The information was provided in a readily digestible form suitable for fishermen, transporters and holding facility operators. During the project, regular visits to commercial operators in Tasmania and Western Australia helped to facilitate the adoption of better holding and handling practises. Results were also communicated through popular articles and conference presentations. Adoption of better handling and holding conditions for rock lobsters has led to improved economic value through increased survival and condition of product to the market, especially in Tasmania. The information from the project has been captured in the new Rock Lobster Industry Code of Practice.

KEYWORDS: Spiny lobster, *Jasus edwardsii*, *Panulirus cygnus*, water quality, oxygen, ammonia, recirculation systems, live holding

2 ACKNOWLEDGMENTS

The project would not have been possible without the collaboration of a large number of rock lobster fishermen in Western Australia and Tasmania. In particular, we thank the Geraldton Fishermen's Co-operative, Western Australia, for the use of facilities and access to *P. cygnus* for experiments. Thanks to Wayne Hosking and Glen Davidson for sharing their knowledge of holding rock lobsters. Many scientists and fishermen provided comments on the two industry guides, in particular, thanks to Patrick Hone, Bruce Phillips, Richard Stevens, Neil Stump, Glen Davidson and Colin Buxton. The staff and colleagues of the Tasmanian Aquaculture and Fisheries Institute are thanked for assistance in obtaining animals, the holding of rock lobsters and involvement in experiments. We were fortunate to have a team of dedicated technicians work on the project. Our sincere thanks to Grant Allen, Alan Beech, John Hardy, Ed Smith, and Craig Thomas for technical assistance. Joan Van Drunen helped immensely in bringing together the oxygen guide and in data collection and analysis. The Fisheries Research and Development Corporation Rock Lobster Post-Harvest Subprogram funded and promoted the research.

3 BACKGROUND

Lobsters are one of the largest commercial fisheries in Australia worth over \$450 million p.a. in 2002-03 (ABARE, 2004). The two most important species are the western rock lobster, *Panulirus cygnus*, and southern rock lobster, *Jasus edwardsii*. Lobsters are caught in baited pots (or traps) that are usually pulled at least once daily and are held for up to two weeks in tanks on board the fishing boat before being landed. Exposure to air, sunlight and handling all contribute to elevated stress levels during this period. After landing, the lobsters may be transported on trucks for a period of several hours before reaching the processing facilities, where they are usually held for a minimum of 2 days. This period of time in tanks helps the lobsters to recover from the stresses of capture and transport, and allows them to purge their gut contents, thus improving their chances of surviving the export process. The design of holding facilities is highly variable and ranges from sea cages to high technology recirculating systems. In an attempt to take advantage of seasonal price fluctuations, some processors are now holding significant quantities of lobsters for extended periods (several weeks/months) prior to export.

Through all stages of post-harvest handling lobsters may be subjected to poor water quality. Poor water quality has a detrimental effect on the quality of lobsters and thus may lead to a reduction in their market value. Two of the most important water quality parameters are oxygen and ammonia. An earlier FRDC funded study (Crear, 1998; Crear and Forteach, 1998) investigated oxygen and its' relationship to the holding of rock lobsters. However, there is very limited understanding of the toxicity of ammonia to lobsters. Ammonia can accumulate in holding and transport facilities via excretion from lobsters as part of the process of catabolism, and from the bacterial decomposition of faeces, excess feed, and dead animals. Ammonia has been shown to be toxic to crustaceans at high concentrations in the water (Tomasso, 1994), and even at low levels can inhibit normal physiological processes (Chen and Lin, 1992). The toxicity of ammonia to aquatic animals is greater when other factors such as low dissolved oxygen levels, low salinity levels, and/or low pH also interact (Wajsbrodt et al., 1989; Chen and Lin, 1992; Russo and Thurston, 1991). Lobsters are generally stressed to some extent during post-harvest procedures but it is uncertain what effect stress has on the ability of lobsters to tolerate ammonia. This project aimed to develop an understanding of the effect of ammonia (in conjunction with stress and other water quality parameters) on the health of lobsters.

4 NEED

There are concerns from industry that poor water quality may be having an effect on the health of post-capture rock lobsters and hence the financial return to processors and fishers. This project addresses the issue of water quality management in three ways:

1. Determining the level of ammonia in holding and transport systems that has no detrimental effect on rock lobsters. This will contribute to the understanding of optimal system design.
2. Understanding the mechanism of ammonia toxicity on rock lobsters. This will enable industry to develop methods to counteract the harmful effects of ammonia build up in holding systems.
3. Providing information to the rock lobster industry, in an appropriate form, to ensure uptake of the results of research on optimising water quality, specifically in the area of oxygen and ammonia.

5 OBJECTIVES

Objectives were unchanged from those proposed in the application.

1. The production of a manual on optimising the provision of oxygen during rock lobster post-harvest processes
2. To determine the median lethal concentration (LC50) of ammonia to adult southern and western rock lobsters (stressed and unstressed)
3. To determine the physiological consequences of exposing lobsters to sub-lethal ammonia concentrations, and the consequences of further exposing lobsters to acute post-harvest stressors
4. The production of a manual on ammonia problems during rock lobster post-harvest processes

6 CHAPTER 1. MEDIAN LETHAL AMMONIA CONCENTRATIONS

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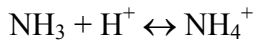
6.1 Abstract

Static-renewal bioassays were undertaken to determine the acute toxicity of ammonia to the southern rock lobster, *Jasus edwardsii*, and the western rock lobster, *Panulirus cygnus* under conditions similar to those encountered in industry holding tanks. Lobsters were held in non-stressed (non-handled) or stressed (handled) conditions and were exposed to total ammonia nitrogen concentrations between 0 and 135 mg l⁻¹. Southern rock lobsters held at temperatures of 13°C were found to be more tolerant to ammonia than western rock lobsters held at 19°C. The 96 hour median lethal concentration (96 h LC50) was 83 mg l⁻¹ NH₄-N for southern rock lobsters, and 39 mg l⁻¹ NH₄-N for non-stressed western rock lobsters. Stressed southern and western rock lobsters held as a group had similar ammonia tolerances to non-stressed lobsters when held as a group. However, lobsters held as a group displayed increased aggression with increasing ammonia concentrations, which contributed to mortality. Individually-held lobsters tolerated higher levels of ammonia. Individual non-stressed, western rock lobsters, had a higher 96 h LC50 of 61 mg l⁻¹ NH₄-N than their counterparts held as a group, 39 mg l⁻¹ NH₄-N. An important consideration in determining the toxicity of ammonia to animals is the pH of the water. The higher the pH the greater the proportion of the highly toxic un-ionised ammonia (NH₃) in solution. The 96 h LC50 values adjusted for the pH drop in the static renewal treatments (pH 7.7 - 7.9) in relation to the normal pH of seawater (pH 8.1 - 8.2), when held as a group were; 21 to 37 mg l⁻¹ NH₄-N for *J. edwardsii* and 14 to 22 mg l⁻¹ NH₄-N for *P. cygnus*. “Safe” levels for long-term exposure to ammonia were calculated by multiplying the LC50 values by a commonly utilised ‘application factor’ of 0.1. In seawater with a pH of 8.2, safe exposures were 2.1 to 3.7 mg l⁻¹ NH₄-N for *J. edwardsii* and 1.4 to 2.2 mg l⁻¹ NH₄-N for *P. cygnus*. Results from this study will assist in the management of rock lobster holding facilities.

6.2 Introduction

Lobsters are one of the largest commercial fisheries in Australia worth over \$450 million p.a. in 2002-03 (ABARE, 2004). The two most important species are the western rock lobster, *Panulirus cygnus*, and southern rock lobster, *Jasus edwardsii*. Both species are often held alive in tanks prior to processing or shipment to markets. Throughout all of the stages of post-harvest handling they may be subjected to poor water quality. Understanding the effects of particular components of water quality is required to provide appropriate conditions for transport and holding (Crear and Allen, 2002; Crear and Forteach, 2002). Poor water quality has a detrimental effect on the quality of lobsters and may lead to decreased revenue, either through the complete loss of the lobsters or a reduction in market value. One of the most important water quality parameters affecting lobsters is ammonia. Ammonia results from the normal metabolic processes of lobsters and from the bacterial decomposition of faeces, bait and dead animals (Claybrook, 1983). Peak ammonia levels in holding systems

usually occur shortly after a fresh consignment of lobsters is put in the tanks, usually after being kept in air for a number of hours (Crear et al., 2003). This peak in ammonia is caused by the storage of waste products in the bodies of lobsters that are rapidly excreted when they are put into holding tanks (Crear and Forteach, 2002). Lobsters are ammonotelic and the majority of ammonia is excreted through the gills (Regnault, 1987). At high concentrations, ammonia has been shown to be toxic to crustaceans (Tomasso, 1994), and at low concentrations it can inhibit normal physiological processes (Chen and Lin, 1992). In solution, total ammonia comprises un-ionised ammonia (NH_3) and ionised ammonia (NH_4^+ ; ammonium) in equilibrium:



where total ammonia nitrogen (TAN) is the weight of nitrogen in both forms
(= $\text{NH}_3 + \text{NH}_4^+$)

The NH_3 is the highly toxic component and the equilibrium is influenced heavily by pH, but also temperature, salinity and pressure (see next chapter for further details). There are tables available to calculate the amount of un-ionised ammonia for a range of conditions (Trussell, 1972; Forteach et al., 1993). The toxicity of ammonia to aquatic animals is generally greater when other factors such as low dissolved oxygen, pH and salinity levels interact (Wajsbrodt et al., 1989; Chen and Lin, 1992; Russo and Thurston, 1991). Much of what is known about ammonia toxicity in crustaceans has come from studies on prawns (for a review see Allan, 1992), but there is limited understanding of ammonia toxicity in lobsters. Indeed, the only comparable study, with a large decapod crustacean, is for the clawed lobster *Hormarus americanus* (Young-Lai et al. 1991). Crear and Forteach (2002) studied the excretion of ammonia in *J. edwardsii*, and *P. cygnus* in relation to temperature, body weight, emersion, daily rhythm and feeding. Temperature and body weight were found to strongly influence the excretion of ammonia but feeding had the largest effect.

Tests of acute lethality are often used as an initial benchmark and to compare the relative tolerance of different species (Sprague, 1990). A common end point is an accurate estimate of the concentration of a toxicant (in this case ammonia) that would kill half of a typical group of lobsters. For example, the 96 h LC50 is the median lethal concentration of ammonia that will on average kill 50% of lobsters over 96 h. An important consideration in determining acute lethality, beyond the chemistry of the water as discussed above, is the health and stress of the lobsters. Lobsters are stressed during post-harvest procedures, such as grading and tank transfer, but the effect of stress on the ability of lobsters to tolerate ammonia has until recently received little study (Spanoghe, 1997). Lobster haemolymph is a solution carrying inorganic ions and gases, products of digestion, and the substrates and wastes of metabolism; amines, hormones, proteins, peptides and haemocytes (Paterson and Spanoghe, 1997). Haemolymph components, typically pH, lactate, glucose and other ions provide important information on the stress and physiological condition of lobsters (Taylor et al., 1997; Paterson and Spanoghe 1997).

Our study establishes for the first time the acute ammonia toxicity for adult *J. edwardsii* and *P. cygnus* under conditions similar to those experienced in holding tanks. We determine and compare: i) the 96 h LC50 for both species, ii) the 96 h LC50 for handled (“stressed”) and non-handled (“unstressed”) animals of both species, iii) the tolerance to ammonia of *P. cygnus* held individually, and iv) oxygen consumption and ammonia excretion rates, haemolymph pH, glucose and lactate as indicators of stress.

6.3 Materials and Methods

6.3.1 General methods

Static-renewal bioassays (Sprague 1990) were undertaken to determine the acute toxicity (96 h LC₅₀ value) of ammonia to *J. edwardsii* and *P. cygnus*. Experiments with *J. edwardsii* and with *P. cygnus* were conducted at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (MRL), and Geraldton Fishermen's Co-operative (GFC) live holding facility in Geraldton, Western Australia, respectively. Following preliminary trials, nominal ammonia concentrations of 0, 40, 60, 90 and 135 mg l⁻¹ NH₄-N as well as a flow-through water control treatment without additional ammonia were used in the experiments. The temperature was maintained at 13°C for *J. edwardsii* and 19-20°C for *P. cygnus*. The stocking densities of lobsters within the tanks were similar to densities used in industry holding tanks (80 kg t⁻¹ for *J. edwardsii* and 70 kg t⁻¹ for *P. cygnus*).

6.3.2 Experimental animals

Intermoult *J. edwardsii* for Experiment 1 were obtained from a commercial live lobster holding facility and transported in oxygenated seawater to the MRL. They were allowed to recover and purge overnight in flow-through tanks. Lobsters for the stressed trial, Experiment 2, were obtained from a scientific research cruise. They were placed into holding tanks at the MRL with flow-through seawater for several days to allow them to recover prior to commencing the experiment. Lobsters were unfed for the duration of the experiments.

Intermoult *P. cygnus* for Experiments 3-5 were obtained from stock held at the GFC live holding facility in Geraldton. Lobsters were starved for 36 h prior to the experiments and were unfed for the duration of the experiments.

6.3.3 Experimental system

The static-renewal bioassay systems used for the experiments with *J. edwardsii* consisted of eighteen 70 l experimental tanks, which were placed in nine 350 l tanks as water baths. For *P. cygnus*, eighteen 40 l experimental tanks were used. Aeration to each tank was provided by two airstones, maintaining an oxygen saturation level of greater than 70%. Eight lobsters were placed into each treatment tank and acclimated to the experimental system for 36 h prior to the start of each experiment. During acclimation flow-through water was maintained to each experimental tank. At the start of the experiment the tanks were drained and refilled with seawater of the appropriate ammonia treatment concentration from stock ammonia solutions, made up using reagent grade ammonium chloride (Sigma). Water in the experimental tanks was renewed daily by draining the tanks and refilling with water from stock tanks containing the treatment solutions.

6.3.4 Experimental procedure

The start of each treatment was staggered by 15 minutes so that there was sufficient time for sampling. All treatments were run in triplicate and 8 animals were held in each replicate tank. Lobsters were observed and behaviour and survival noted at 1, 3, 6, 12, 24, 36, 48, 60, 72, 84 and 96 h. A lobster was recorded as a mortality when there was no observable movement of its scaphognathites. Dead lobsters were removed from the tanks. At each observation time the temperature, pH and oxygen level (mg l⁻¹ and % saturation) in the tanks were measured. Prior to each 24 h water exchange a water sample was collected from each treatment reservoir for ammonia analysis.

6.3.5 *Ammonia excretion and oxygen consumption*

At the completion of the 96 h toxicity Experiments 1, 2, 3, and 5, the standard and maximum oxygen consumption rates were determined for those treatments where sufficient lobsters survived. At the end of the 96 h toxicity trials standard rates of oxygen consumption were determined by measuring the initial and final dissolved oxygen content in the tank over approximately 2 h for *J. edwardsii* and 30 min for *P. cygnus*. No aeration was added to the tanks during that time (water oxygen levels did not fall below 70% saturation whilst undertaking the oxygen consumption tests). Control tanks without lobsters were used to correct for the diffusion of oxygen across the air/water interface (De la Gándara et al., 2002) and to correct for microbial oxygen consumption. The water was then re-aerated and haemolymph samples were taken (pre-stress data). After the haemolymph samples were taken the lobsters were handled for a further 15 minutes and maximum rates of oxygen consumption were measured over a 20-25 min period. Additional lobster haemolymph samples were taken after the maximum oxygen consumption was determined (post-stress data). Ammonia excretion rates were determined in Experiments 1 and 5. After completing the oxygen consumption tests and the haemolymph sampling the water was replaced with water containing no ammonia. Ammonia excretion rates were determined by taking water samples at the start and end of a 1 h period. The water continued to be aerated during this period. Ammonia was analysed by the phenol-hypochlorite method of Solorzano (1969).

6.3.6 *Haemolymph sampling and analysis*

Prior to the start of the toxicity trial, haemolymph samples were taken from 9 lobsters that were not used in the subsequent experiment, to provide initial haemolymph data. As indicated above, haemolymph samples were taken after the 96 h toxicity trial and measurement of basal oxygen consumption, then again following assessment of maximum oxygen consumption. A prebranchial haemolymph sample (1.5 to 2.0 ml) was withdrawn from the infrabranchial sinus via an arthroal membrane at the base of a walking leg, using an ice chilled 3 ml syringe with a 21 gauge needle. A maximum of three lobsters per replicate tank were sampled. The haemolymph samples were placed in an Eppendorf tube and sampled as follows:

1. A 500 μ l sample was added to 4.5ml of distilled H₂O for later ammonia analysis.
2. The pH was measured.
3. A 500 μ l sample was added to 1000 μ l 0.6M perchloric acid (PCA) in an Eppendorf tube and vortexed.
4. All samples were frozen for later analysis

Haemolymph was analysed for total ammonia nitrogen concentration to determine the effect of external ammonia concentration on haemolymph content, glucose and lactate as indicators of stress, and refractive index N_D and delta ion (difference in osmolality between the haemolymph and treatment water).

6.3.7 *Experiment 1 – ‘unstressed’ *Jasus edwardsii* held as a group*

The aim of Experiment 1 was to determine the LC50 of ‘unstressed’ *J. edwardsii* over 96 h when held at commercially simulated stocking densities. The actual ammonia treatment concentrations were 0 (flow-through control), 2.1, 40.5, 55.6, 81.8 and 121.6 mg l⁻¹ NH₄-N. The initial mean weight of individual lobsters was 703 g and in each 70 l holding tank the

total biomass was 5.636 ± 0.036 kg, mean \pm SE, which is equivalent to a stocking density of $81 \text{ kg } 1000 \text{ l}^{-1}$.

6.3.8 Experiment 2 – ‘stressed’ *Jasus edwardsii* held as a group

The aim of Experiment 2 was to determine the LC50 of ‘stressed’ *J. edwardsii* over 96 h when held at commercially simulated stocking densities. The lobsters were handled prior to and during the experiment to simulate industry practices and processes that may induce stress. This involved maintaining lobsters in air-chilled to 11°C for 3 h to simulate road transport prior to placing them in the experimental treatments. During simulated transport, the carry baskets were shuffled and jolted every 30 min to mimic transport and handling. At the end of each 24 h period each experimental tank was drained and the lobsters left out of water for 15 min during which time the lobsters were each handled to simulate an inspection.

The actual ammonia treatment concentrations were 0.8 (flow-through control), 0.8, 39.5, 59.6, 82.4 and $120.8 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$. The initial mean weight of lobsters was 576 g and in each 70 l experiment tank the total biomass was 4.605 ± 0.042 kg, mean \pm SE, which is equivalent to a stocking density of $66 \text{ kg } 1000 \text{ l}^{-1}$. Ammonia excretion was not measured in this experiment.

6.3.9 Experiment 3 – ‘unstressed’ *Panulirus cygnus* held as a group

The aim of Experiment 3 was to determine the LC50 of ‘unstressed’ *P. cygnus* over 96 h when held at commercially simulated stocking densities. The actual ammonia treatment concentrations were 0.2 (flow-through control), 0.4, 32.9, 60.1, 81.7 and $124.0 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$. The initial mean weight of lobsters was 445 g and in each 40 l experiment tank the total biomass was 3.564 ± 0.026 kg, mean \pm SE, which is equivalent to a stocking density of $89 \text{ kg } 1000 \text{ l}^{-1}$.

6.3.10 Experiment 4 – ‘stressed’ *Panulirus cygnus* held as a group

The aim of Experiment 4 was to determine the LC50 of ‘stressed’ *P. cygnus* over 96 h when held at commercially simulated stocking densities. The lobsters used were handled prior to and during the experiment to simulate ‘normal’ industry practices. Initially, the lobsters were left out of water for 0.5 h at ambient temperature in the experimental room. The tanks were moved and jolted to mimic processor handling then transferred to an 11°C cool room with seawater sprays for 3 h. During this time, the tanks were moved and jolted every hour to simulate road transport. The tanks were then transferred back to the experimental room. At the end of every 24 h period, each tank was drained and the lobsters were left out of water for 15 min during which time the lobsters were handled individually to simulate an inspection.

The initial mean weight of lobsters was 446 g and in each 40 l experiment tank the total biomass was 3.571 ± 0.024 kg, mean \pm SE, which is equivalent to a stocking density of $89 \text{ kg } 1000 \text{ l}^{-1}$. The actual ammonia treatment concentrations were 0.2 (flow-through control), 0.5, 33.4, 59.7, 82.0 and $128.3 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$.

6.3.11 Experiment 5 – ‘unstressed’ *Panulirus cygnus* held individually

The aim of Experiment 5 was to determine the LC50 of ‘unstressed’ *P. cygnus* over 96 h when held individually. Lobsters were placed in individual mesh containers within the experiment tanks to prevent physical contact between the animals. The mesh containers were made of plastic mesh with square holes of approximately 10 mm width. All other

procedures were as for Experiment 3. The initial mean weight of lobsters was 442 g and in each 40 l experiment tank the total biomass was 3.536 ± 0.019 kg, mean \pm SE, which is equivalent to a stocking density of 88 kg 1000 l⁻¹. The actual ammonia treatment concentrations were 0.2 (flow-through control), 0.3, 32.1, 60.3, 78.2 and 126.0 mg l⁻¹ NH₄-N.

6.3.12 Statistical Analyses

Probit analysis was used to determine the 96 h LC50 toxicity of ammonia in holding water, using SASS. A nested ANOVA, with tank nested in ammonia treatment, was used to test for tank effects and the effect of holding water ammonia concentration on haemolymph characteristics: ammonia, glucose, lactate, pH, refractive index and delta ion (osmolality). One-way ANOVA was used to analyse the effect of holding water ammonia concentration on ammonia excretion by lobsters. Two-way ANOVA was used to analyse the effects of ammonia and handling stress on oxygen consumption, and haemolymph characteristics in Experiment 2. Tukey-Kramer multiple comparison of means ($P = 0.05$) was used to separate means when treatment effects were significant. Means from the static renewal ammonia treatments were also compared with the flow-through control treatment using Dunnett's method. All ANOVAs and means comparisons were conducted with JMP 5.1 (© SAS Institute Inc.). Where necessary, data were transformed to satisfy Cochran's test for homogeneity of variance and the Shapiro-Wilk test for normal distribution of residuals (replicate value – tank or treatment mean). Where transformed data did not meet the assumptions of ANOVA, a Welch ANOVA for unequal variances was conducted.

As unionised ammonia is known to be the toxic component of total ammonia nitrogen, the toxicity data were analysed to look at this component. The amount of unionised ammonia present in the ammonia treatments was determined from a reference table of percentage unionised ammonia at different water temperatures and salinities (Trussell, 1972). The unionised ammonia value was then used to determine the equivalent total ammonia nitrogen concentration, or commonly measured component, at normal seawater pH of 8.2 and the same temperature as the experiments. Recommended "safe" levels of ammonia for long – term holding of both species of rock lobsters were calculated as 10% (Sprague, 1971), of the 96 h LC50 for animals held as a group.

6.4 Results

6.4.1 Experiment 1 – ‘unstressed’ *Jasus edwardsii*

The 96 h LC50 for *J. edwardsii* was 83 mg l⁻¹ NH₄-N (Fig. 1). Lobsters exposed to ammonia became increasingly aggressive with increasing levels of ammonia, and physical injuries were observed on several dead lobsters at the higher ammonia concentrations.

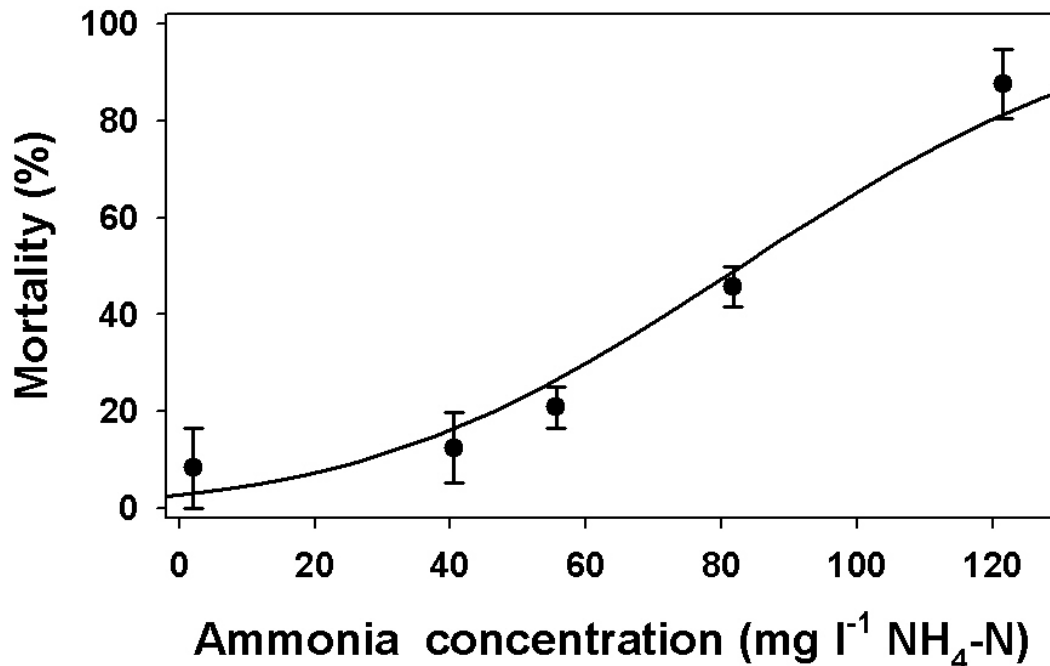


Figure 1. The 96 h mortality curve of unstressed southern rock lobsters, *Jasus edwardsii* exposed to varying levels of ammonia. A probit curve was used to determine the 96 h LC50 value of 83 mg l⁻¹ NH₄-N. Values are mean ± SE, n = 3 replicates, initially 8 lobsters per replicate. (Experiment 1)

Both ammonia concentration in lobster haemolymph and ammonia excretion rates increased incrementally with increasing ammonia concentration of the holding water (Fig. 2) (Haemolymph ammonia Nested ANOVA, N 5 ammonia levels, 3 replicate tanks, 3 lobsters, F = 45.24, df 14, 30, P < 0.0001; Ammonia excretion ANOVA N 5, 3, F = 73.50, df 4, 10, P < 0.0001). There was a significant tank effect on lobster haemolymph ammonia, due to the high variation among tanks at the 40.5 mg l⁻¹ NH₄-N treatment (Appendix 6). In the static renewal treatments with ammonia added, lobster haemolymph ammonia and ammonia excretion rates were higher than in the flow-through control. Haemolymph ammonia measured in 3 surviving lobsters in the highest ammonia treatment (121.6 mg l⁻¹ NH₄-N) was 101.0 ± 5.4 mg l⁻¹ (not shown on graph), 1.6 times higher than at 81.8 mg l⁻¹ NH₄-N. There were insufficient numbers of surviving lobsters to assess ammonia excretion at the highest ammonia treatment level.

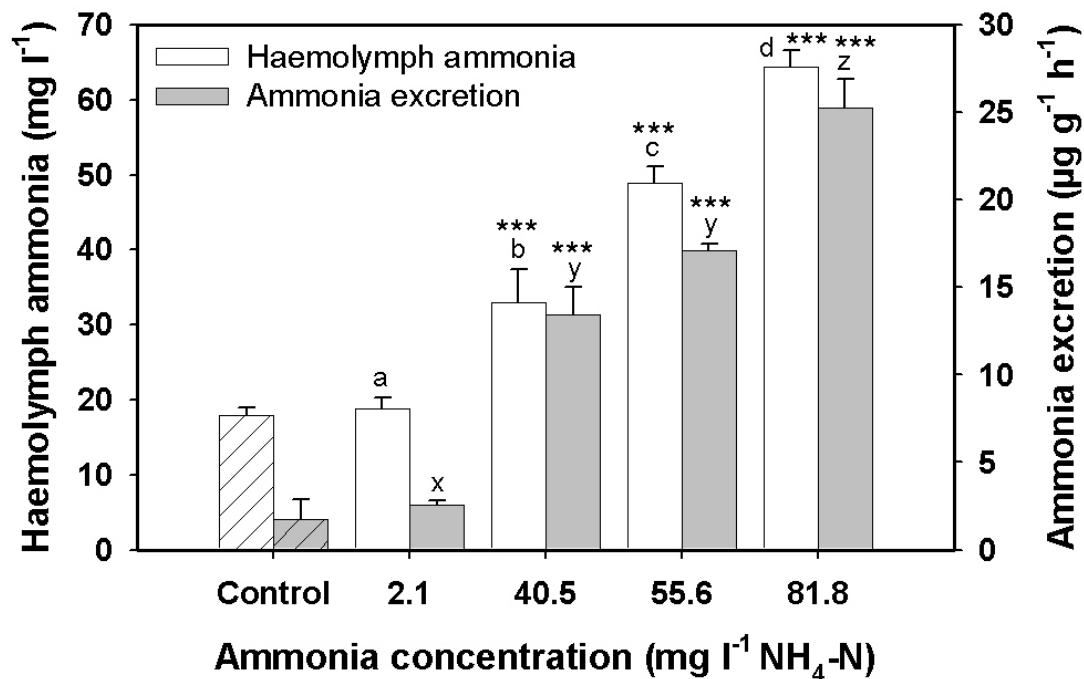


Figure 2. Haemolymph ammonia concentration (white bars) and ammonia excretion rates (grey bars) for surviving *Jasus edwardsii* following 96 h exposure to different external ammonia concentrations. Values are mean \pm SE, for haemolymph ammonia $n = 9$ (3 lobsters per replicate tank), and for ammonia excretion $n = 3$ replicates. Striped bars indicate flow-through control treatment; open bars are static renewal ammonia treatments. Different letters indicate significant differences among static renewal treatments for haemolymph ammonia (a, b, c, d) and ammonia excretion (x, y, z). Asterisks indicate values significantly different to the control (***) ($P < 0.001$). (Experiment 1)

Both ammonia and stress had a significant effect on oxygen consumption of *J. edwardsii*, however the interaction between ammonia and stress was also significant (Two-way ANOVA, $N = 5, 2, 3, F = 41.24, df = 9, 20, P < 0.0001$). Standard oxygen consumption increased incrementally with increasing ammonia concentration (ANOVA, $N = 5, 3, F = 12.35, df = 4, 10, P = 0.0007$), however compared with the control lobsters, consumption was only significantly higher in the $81.8 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ treatment (Fig. 3). Oxygen consumption measured following a stress event was not significantly different between treatments (ANOVA, $N = 5, 3, F = 3.36, df = 4, 10, P = 0.0545$).

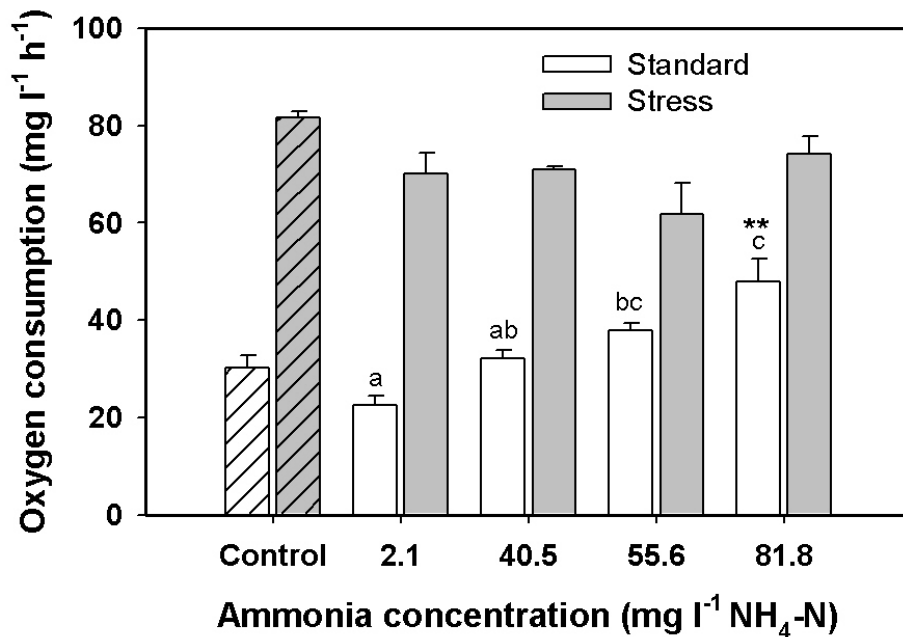


Figure 3. Oxygen consumption of *Jasus edwardsii*, following 96 h exposure to different external ammonia concentrations, under standard (2 h test, white bars) and following stress (25 min, grey bars) conditions. Striped bars indicate flow-through control treatment. Values are mean \pm SE, $n = 3$ replicates. Different letters indicate significant differences among static renewal treatments (a, b, c). Asterisks indicate values significantly different to control (** $P < 0.01$). (Experiment 1)

Haemolymph glucose concentration was similar in lobsters held in different concentrations of ammonia. In contrast, lactate tended to increase with ammonia concentration, and reached a maximum of 1.0 mmol l^{-1} ($n = 3$) in lobsters surviving in the highest treatment, $121.6 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ (Table 1), however variation between tanks within ammonia treatments was significant (Nested ANOVA, $N 5, 3, 3$, $F = 2.47$, $df 14, 30$, $P = 0.0185$). Haemolymph pH decreased significantly with ammonia concentration (Nested ANOVA, $N 5, 3, 3$, $F = 6.34$, $df 14, 30$, $P < 0.0001$) and there were no significant differences in haemolymph refractive index or delta ion between treatments.

Table 1. Haemolymph characteristics of *Jasus edwardsii* following 96 h exposure to different external ammonia concentrations. Values are given as mean \pm SE for $n = 9$, except for $121.6 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ where $n = 3$. Different letters indicate significant differences among static renewal treatments (a, b, c). Asterisks indicate values significantly different to control (* $P < 0.05$, *** $P < 0.001$).

Haemolymph Characteristic	Treatment Concentrations (mg l ⁻¹ NH ₄ -N)					
	0 (control)	2.1	40.5	55.6	81.8	121.6
Glucose (mmol l ⁻¹)	0.29 \pm 0.07	0.24 \pm 0.05	0.20 \pm 0.03	0.30 \pm 0.10	0.28 \pm 0.07	0.22 \pm 0.15
Lactate (mmol l ⁻¹)	0.12 \pm 0.04	0.04 \pm 0.01	0.08 \pm 0.02	0.07 \pm 0.01	0.14 \pm 0.06	1.01 \pm 0.48
pH	7.78 \pm 0.02	7.82 \pm 0.01 (a)	7.69 \pm 0.01 (bc ***)	7.71 \pm 0.01 (b *)	7.65 \pm 0.02 (c ***)	7.76 \pm 0.06
Refractive Index (N _D)	1354 \pm 1	1352 \pm 0	1352 \pm 1	1352 \pm 1	1350 \pm 1	1352 \pm 3
Delta Ion (Osmolality)	--	-18.7 \pm 7.6	-24.4 \pm 5.2	-21.7 \pm 2.4	-11.7 \pm 2.7	-43.5 \pm 3.5

6.4.2 Experiment 2 – ‘stressed’ *Jasus edwardsii*

The handling and transport ‘stresses’ imposed on *J. edwardsii* had no effect on the 96 h LC50, 83 mg l⁻¹ NH₄-N (Fig. 4) compared with the LC50 value derived from unstressed lobsters (83 mg l⁻¹ NH₄-N Fig. 1).

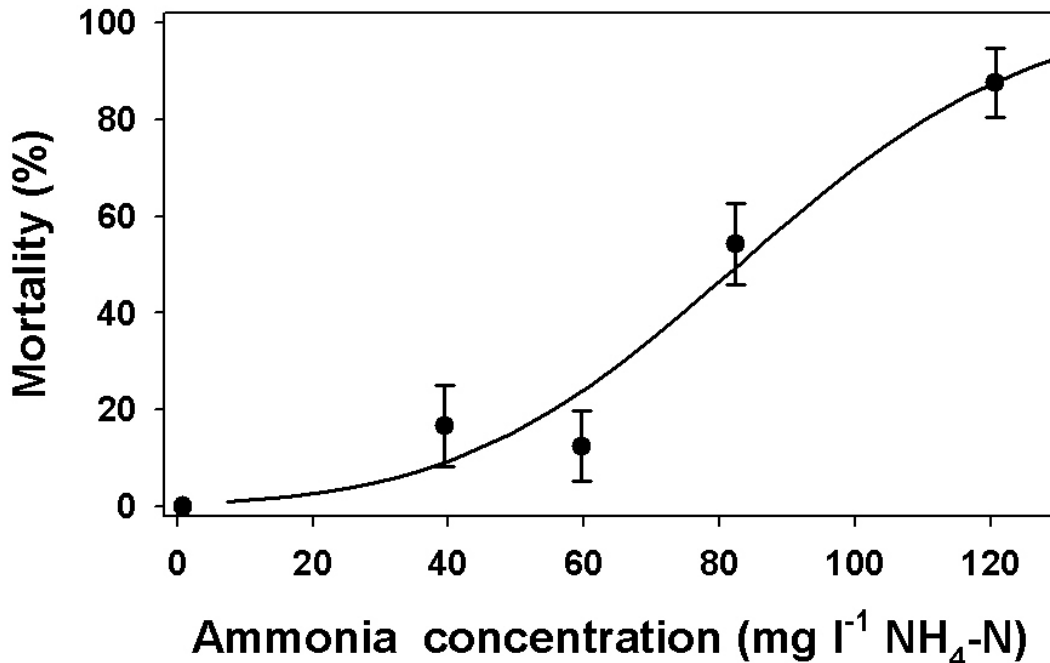


Figure 4. The 96 h mortality curve of “stressed” adult southern rock lobsters, *Jasus edwardsii*, exposed to varying levels of ammonia. A probit curve was used to determine the 96 h LC50 value of 83 mg l⁻¹ NH₄-N. Values are mean ± SE, n = 3 replicates, initially 8 lobsters per replicate. (Experiment 2)

Ammonia concentration of the holding water and acute handling stress both significantly increased ammonia concentration in *J. edwardsii* haemolymph (Fig. 5) (Two-way Nested ANOVA N 5, 3, 2, F = 52.34, df 19, 38, P < 0.0001). Haemolymph ammonia reached a maximum concentration of 105.7 ± 3.8 mg l⁻¹ (n = 3) in lobsters held for 96 h at the highest ammonia level, 120.8 mg l⁻¹ NH₄-N, however, due to high mortality reducing replication at this level this data was not included in statistical analyses. Haemolymph ammonia was similar in stressed lobsters (Fig. 5) compared with unstressed lobsters (Exp 1, Fig. 2) held for 96 h at comparable ammonia concentrations.

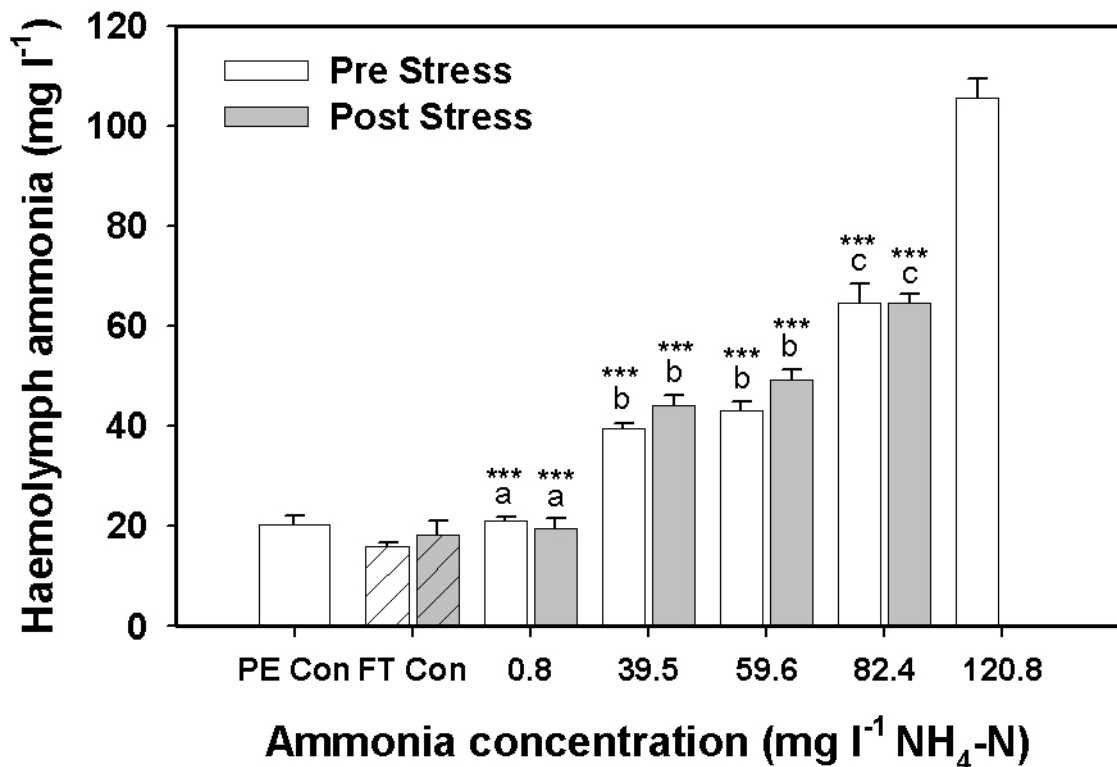


Figure 5. Haemolymph ammonia concentration of surviving *Jasus edwardsii* following 96 h exposure to different external ammonia concentrations and handling, before (open bars) and after (grey bars) an acute stress event. Values are mean \pm SE, $n = 6$ lobsters, except for $120.8 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ where $n = 3$. PE Con = control samples prior to 96 h toxicity trial; FT Con (diagonal striped bars) = lobsters held in flow-through water during 96 h toxicity trial. Different letters indicate significant differences among static renewal treatments (a, b, c). Asterisks indicate values significantly different to FT Con (***) $P < 0.001$. (Experiment 2)

Standard oxygen consumption increased incrementally with increasing ammonia concentration (Fig. 6) (ANOVA, $N 5, 3, F = 11.00, df 4, 10, P = 0.0011$), although there was no significant effect on oxygen consumption following an acute stress event (ANOVA, $N 5, 3, F = 1.53, df 4, 10, P = 0.2663$).

Haemolymph pH and refractive index were lower in lobsters exposed to higher ammonia concentrations for 96 h (Table 2) (Haemolymph pH Nested ANOVA, $N 5, 3, 2, F = 4.52, df 14, 29, P = 0.0032$; haemolymph refractive index Nested ANOVA $N 5, 3, 2, F = 2.77, df 14, 29, P = 0.0297$). Following exposure to acute stress, haemolymph pH decreased and was lowest in lobsters from the 49.5 and 59.6 mg.l^{-1} ammonia treatments (Nested ANOVA $N 5, 3, 2, F = 3.35, df 14, 28, P = 0.0154$). There were no treatment effects observed on haemolymph delta ion (osmolality).

Table 2. Haemolymph characteristics of *Jasus edwardsii* following 96 h exposure to different external ammonia concentrations and handling, before (Pre-) and after (Post-) an acute stress event. Values are mean \pm SE, n = 6, except for 120.8 mg.l⁻¹ NH₄-N where n = 3. Different letters indicate significant differences among static renewal treatments within stress level (a, b). Asterisks indicate values significantly different to control (** P<0.01, *** P<0.001).

Haemolymph Characteristic	Treatment Concentrations (mg l ⁻¹ NH ₄ -N)					
	0 (control)	0.8	39.5	59.6	82.4	120.8
pH Pre-stress	7.74 \pm 0.04	a 7.81 \pm 0.00	b 7.73 \pm 0.02	b 7.72 \pm 0.02	ab 7.77 \pm 0.02	7.74 \pm 0.02
pH Post-stress	7.75 \pm 0.02	a 7.72 \pm 0.02	b ** 7.62 \pm 0.02	b *** 7.61 \pm 0.04	a 7.75 \pm 0.02	--
Refractive Index (N _D) Pre-stress	1350 \pm 1	a 1353 \pm 1	ab 1350 \pm 1	ab 1350 \pm 1	b 1347 \pm 1	1351 \pm 2
Refractive Index (N _D) Post-stress	1350 \pm 1	1350 \pm 2	1350 \pm 2	1351 \pm 1	1348 \pm 1	--
Delta Ion (Osmolality) Pre-stress	3.8 \pm 4.9	4.0 \pm 4.5	4.5 \pm 6.1	5.8 \pm 2.5	4.5 \pm 4.3	-0.2 \pm 1.2
Delta Ion (Osmolality) Post-stress	2.6 \pm 5.3	10.5 \pm 6.7	4.2 \pm 3.2	5.0 \pm 4.5	0.7 \pm 6.5	--

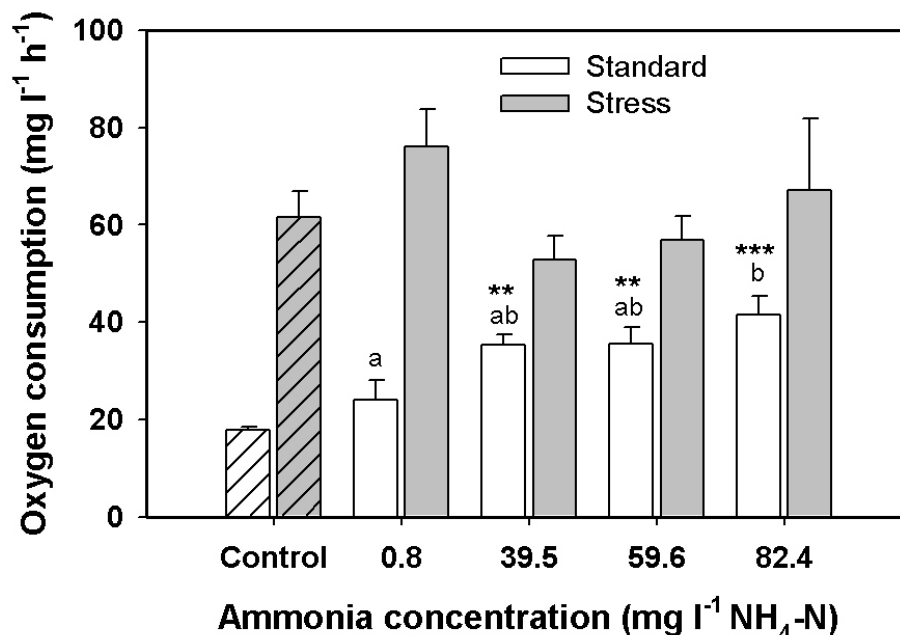


Fig. 6. Oxygen consumption of stressed *Jasus edwardsii*, following 96 h exposure to different external ammonia concentrations, under standard (2 h test) and following stress conditions (30 min). Striped bars indicate flow-through control treatment. Values are mean \pm SE, n = 3 replicates. Values are mean \pm SE for n = 3 in each case. Different letters indicate significant differences among static renewal treatments (a, b, c). Asterisks indicate values significantly different to control (** P<0.01, *** P<0.001). (Experiment 2)

Haemolymph lactate was significantly elevated in post-stress lobsters compared with pre-stress animals (Fig. 7) (Two-way Nested ANOVA N 5, 2, 3, $F = 3.70$, $df 19, 38$, $P = 0.0003$). However, the 96 h ammonia treatment did not have a significant effect on haemolymph lactate. Haemolymph glucose concentration decreased significantly with external ammonia concentration (Two-way Nested ANOVA N 5, 3, 2, $F = 3.25$, $df 19, 38$, $P = 0.0010$), and there were no significant differences between pre- and post-stress lobsters (Fig. 8).

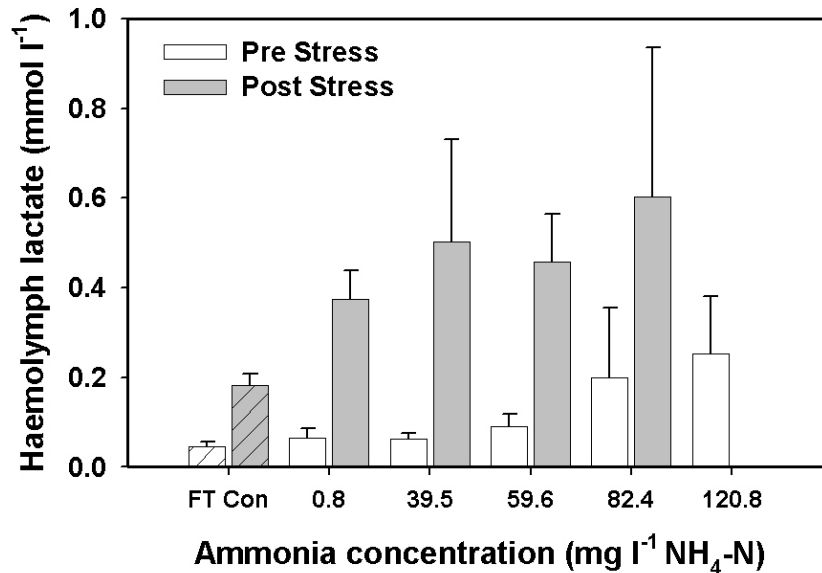


Figure 7. Haemolymph lactate concentration of surviving *Jasus edwardsii* following 96 h exposure to different external ammonia concentrations and handling, before (open bars) and after (grey bars) an acute stress event. Values are mean \pm SE, $n = 6$ lobsters. FT Con (diagonal striped bars) = lobsters held in flow-through water during 96 h toxicity trial. (Experiment 2)

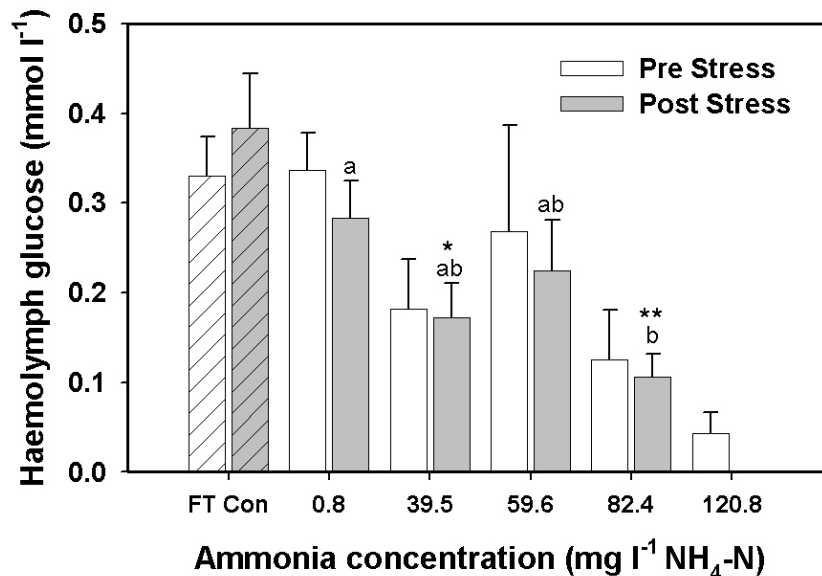


Figure 8. Haemolymph glucose concentration of surviving *Jasus edwardsii* following 96 h exposure to different external ammonia concentrations and handling, before (open bars) and after (grey bars) an acute stress event. Values are mean \pm SE, $n = 6$ lobsters. FT Con (diagonal striped bars) = lobsters held in flow-through water during 96 h toxicity trial. Different letters indicate significant differences among static renewal treatments (a, b). Asterisks indicate values significantly different to control (* $P < 0.05$, ** $P < 0.01$). (Experiment 2)

6.4.3 Experiment 3 – ‘unstressed’ *Panulirus cygnus*

As for *J. edwardsii*, the *P. cygnus* exposed to ammonia became very aggressive, and the level of aggression increased with the level of ammonia. Again, physical injuries were apparent on some dead lobsters suggesting that the behavioural changes induced by ammonia may have resulted in some of the mortalities observed, rather than the ammonia directly. The 96 h LC50 for unstressed *P. cygnus* held as a group was 39 mg l⁻¹ NH₄-N (Fig. 9).

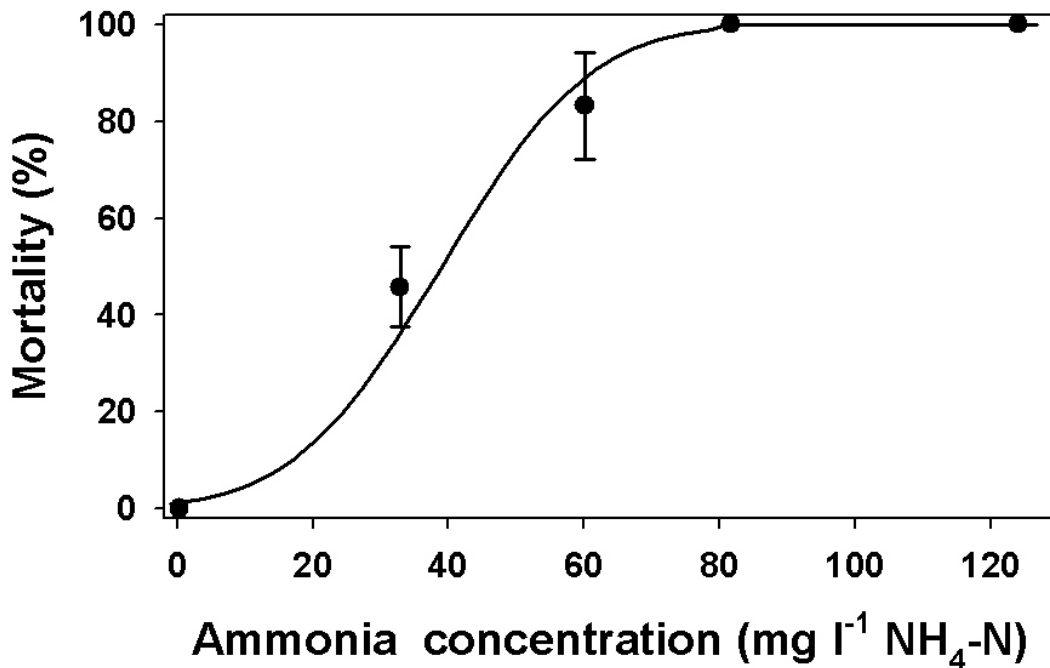


Figure 9. The 96 h mortality curve of “unstressed” adult western rock lobsters, *Panulirus cygnus*, exposed to varying levels of ammonia. A probit curve was used to determine the 96 h LC50 value of 39 mg l⁻¹ NH₄-N. Values are mean ± SE, n = 3 replicates, initially 8 lobsters per replicate. (Experiment 3)

There were too few lobsters surviving in the higher ammonia treatments (4 lobsters at 60.1 mg l⁻¹ NH₄-N, and none at 81.7 and 124.0 mg l⁻¹ NH₄-N) to allow assessment of oxygen consumption at high ammonia concentrations. Standard oxygen consumption of *P. cygnus* increased with exposure to increasing levels of ammonia between 0 and 32.9 mg l⁻¹ NH₄-N (Fig. 10) (ANOVA, N 3, 3, F = 13.34, df 2, 6, P = 0.0062). The oxygen consumption of lobsters exposed to stress was 1.5 – 3 times higher than under standard conditions (Two-way ANOVA N 3, 2, 3, F = 22.29, df 19, 38, P < 0.0001), and there were no differences between ammonia treatment levels when lobsters were exposed to stress (Welch ANOVA Unequal variances, N 3, 3, F = 0.2011, Prob > F = 0.8276).

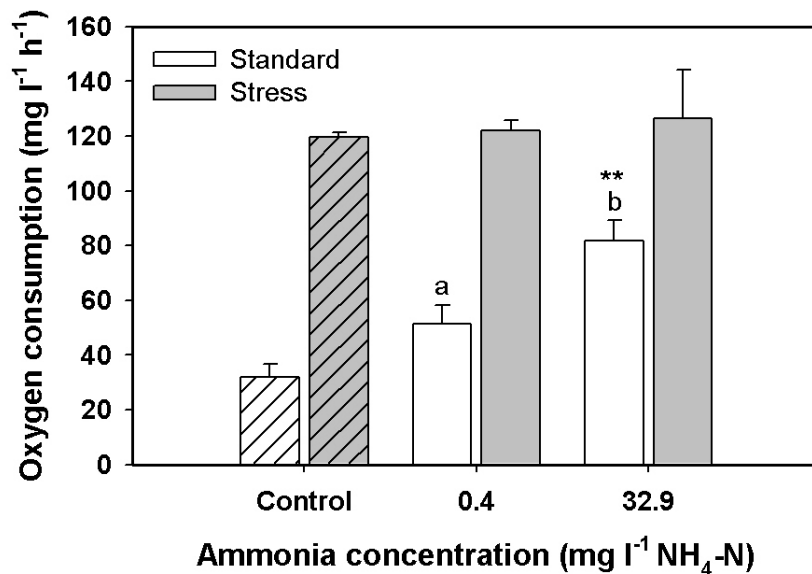


Figure 10. Oxygen consumption of stressed *Panulirus cygnus* following 96 h exposure to different external ammonia concentrations, under standard (35 min) and following stress (20 min) conditions. Striped bars indicate flow-through control treatment. Values are mean \pm SE for $n = 3$ in each case. Different letters indicate significant differences among static renewal treatments (a, b). Asterisks indicate values significantly different to control (** $P < 0.01$). (Experiment 3)

6.4.4 Experiment 4 – ‘stressed’ *Panulirus cygnus*

The 96 h LC₅₀ for stressed *P. cygnus* held as a group was 38 mg l⁻¹ NH₄-N (Fig. 11), equivalent to the value obtained for unstressed lobsters, 39 mg l⁻¹ NH₄-N (Exp 3, Fig. 9).

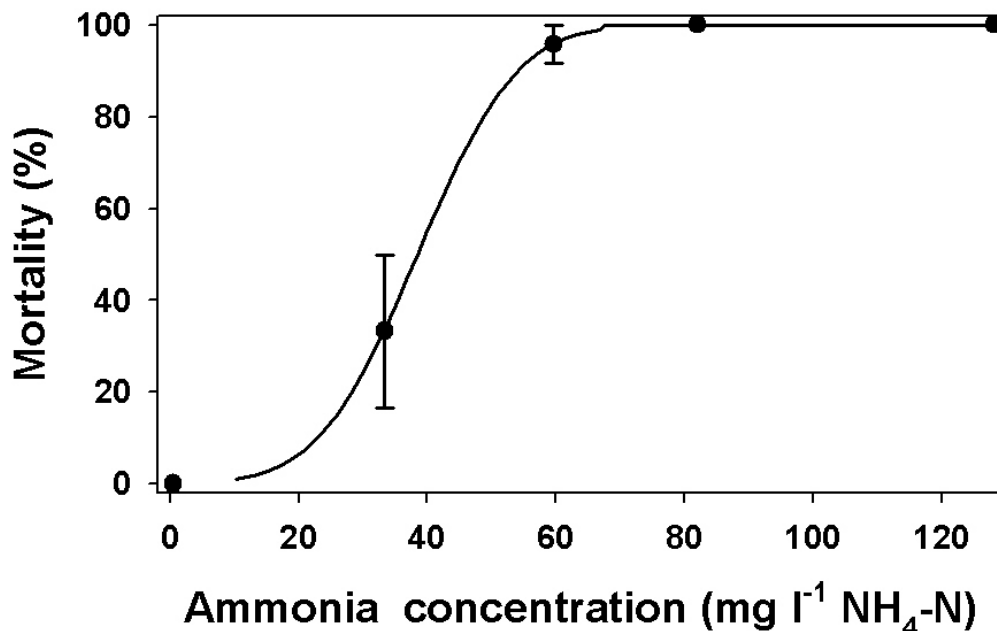


Figure 11. The 96 h mortality curve of stressed adult western rock lobsters, *Panulirus cygnus*, exposed to varying levels of ammonia. A probit curve was used to determine the 96 h LC₅₀ value of 38 mg l⁻¹ NH₄-N. Values are mean \pm SE, $n = 3$ replicates, initially 8 lobsters per replicate. (Experiment 4)

6.4.5 Experiment 5 – individually held *Panulirus cygnus*

The 96 h LC50 of individually held *P. cygnus* was 61 mg l⁻¹ NH₄-N (Fig. 12), over 50% higher than for lobsters held as a group, 39 mg l⁻¹ NH₄-N (see Fig. 9).

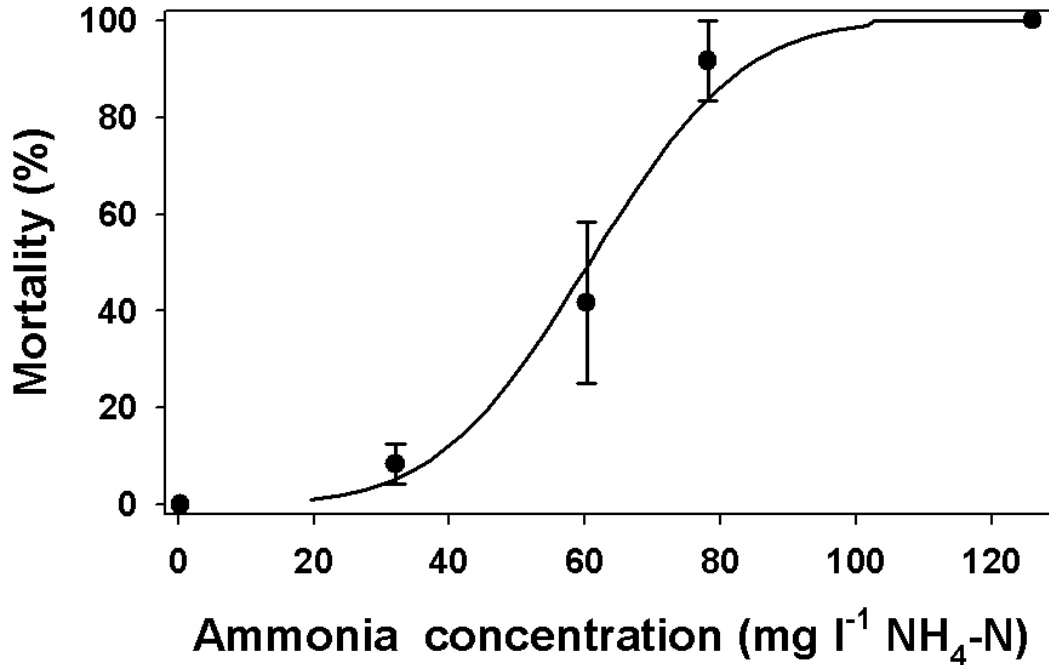


Figure 12. The 96 h mortality curve of individually held, unstressed adult western rock lobsters, *Panulirus cygnus*, exposed to varying levels of total ammonia. A probit curve was used to determine the 96 h LC50 value of 61 mg l⁻¹ NH₄-N. Values are mean ± SE, n = 3 replicates, initially 8 lobsters per replicate. (Experiment 5)

P. cygnus haemolymph ammonia and ammonia excretion rates increased with increasing levels of external ammonia (Fig. 13) (Haemolymph ammonia Nested ANOVA, N 4, 3, 3, F = 44.53, df 11, 23, P < 0.0001; Ammonia excretion ANOVA N 4, 3, F = 53.12, P < 0.0001). The highest concentration of haemolymph ammonia was recorded from the 2 surviving animals in the 78.2 mg l⁻¹ NH₄-N treatment (not included in analysis). There were no lobsters surviving in the highest ammonia treatment, 126.0 mg l⁻¹ NH₄-N.

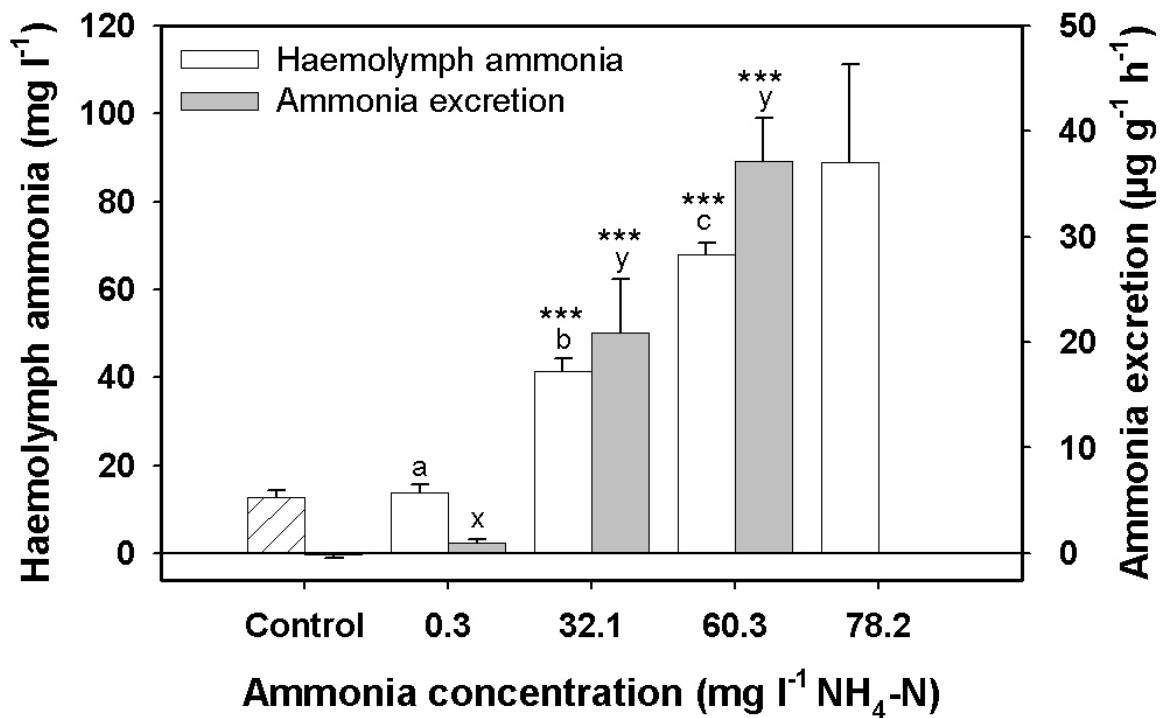


Figure 13. Haemolymph ammonia concentration (white bars) and ammonia excretion rates (grey bars) for surviving *Panulirus cygnus* following 96 h exposure to different external ammonia concentrations. Values are mean \pm SE, for haemolymph ammonia $n = 9$ lobsters except for 78.2 mg l⁻¹ NH₄-N where $n = 2$, and for ammonia excretion $n = 3$ replicates. Striped bars indicate flow-through control treatment, open bars are static renewal ammonia treatments. Note Y-axes do not start at zero. Different letters indicate significant differences among static renewal treatments for haemolymph ammonia (a, b, c) and ammonia excretion (x, y). Asterisks indicate values significantly different to flow-through control (***) ($P < 0.001$). (Experiment 5)

Standard oxygen consumption in *P. cygnus* held individually in different concentrations of ammonia increased with ammonia concentration (Fig. 14) (ANOVA N 4, 3, $F = 15.49$, $df 3, 8$, $P = 0.0011$). The oxygen consumption of lobsters exposed to stress was significantly higher than under standard conditions (Two-way ANOVA N 4, 2, 3, $F = 17.94$, $df 7, 16$, $P < 0.0001$). However, there were no differences in oxygen consumption between ammonia treatment levels when lobsters were exposed to stress (ANOVA N 4, 3, $F = 2.06$, $P = 0.1846$).

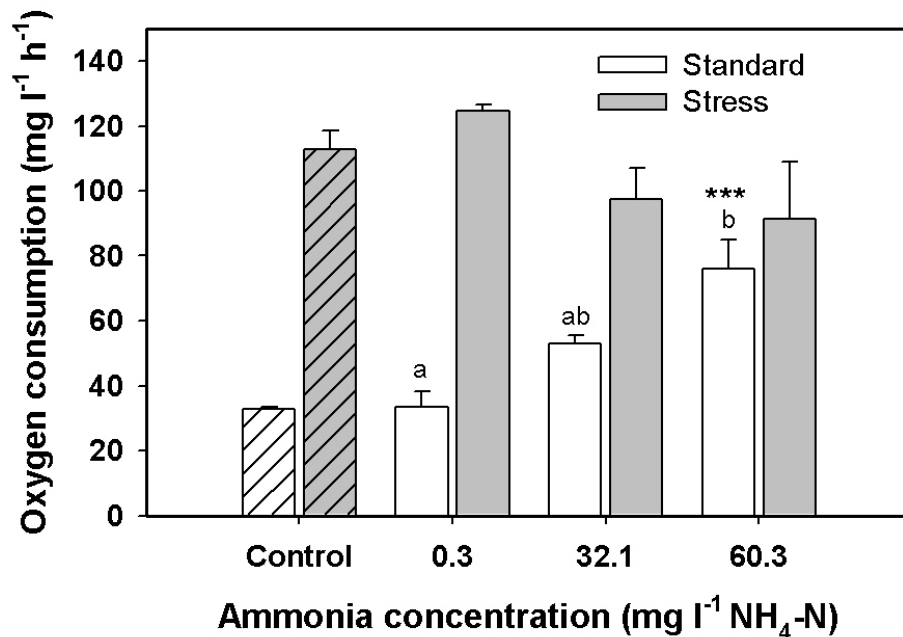


Figure 14. Oxygen consumption of individually held *Panulirus cygnus* following 96 h exposure to different external ammonia concentrations, under standard (25 min) and following stress (20 min) conditions. Striped bars indicate flow-through control treatment. Values are mean \pm SE for $n = 3$ in each case. Different letters indicate significant differences among static renewal treatments (a, b). Asterisks indicate values significantly different to control (***) ($P < 0.001$). (Experiment 5)

Haemolymph lactate was significantly lower in the 0.3 and 32.1 mg l⁻¹ NH₄-N treatments than in lobsters from the flow-through control (Fig. 15) (Nested ANOVA N 4, 3, 3, $F = 3.35$, df 11, 22, $P = 0.0076$). Within the static renewal ammonia treatments, haemolymph lactate tended to increase with external ammonia concentration and the single surviving lobster analysed from the 78.2 mg l⁻¹ NH₄-N treatment had a lactate concentration of 7.22 mmol l⁻¹. External ammonia concentration had no significant effect on haemolymph glucose concentration (Fig. 16) (Nested ANOVA N 4, 3, 3, $F = 1.17$, df 11, 22, $P = 0.3630$) although it declined with ammonia concentration to 0.106 mmol l⁻¹ in one lobster from the 78.2 mg l⁻¹ NH₄-N treatment.

Haemolymph pH decreased significantly with external ammonia concentration (Table 3) (Nested ANOVA N 4, 3, 3, $F = 2.28$, df 11, 22, $P = 0.0484$), and there was no significant effect of ammonia on haemolymph refractive index (Nested ANOVA N 4, 3, 3, $F = 1.47$, df 11, 21, $P = 0.2157$).

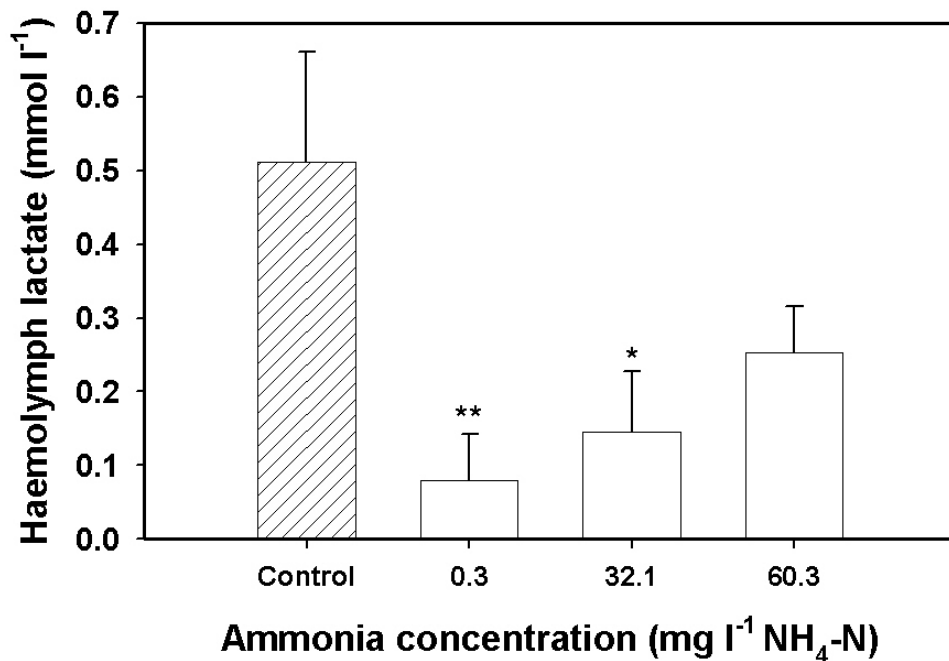


Figure. 15. Haemolymph lactate concentration of surviving *Panulirus cygnus* following 96 h exposure to different external ammonia concentrations. Striped bar indicates flow-through control treatment. Values are mean \pm SE, n = 8 or 9 lobsters. Asterisks indicate values significantly different to control (* P<0.05, ** P<0.01). (Experiment 5)

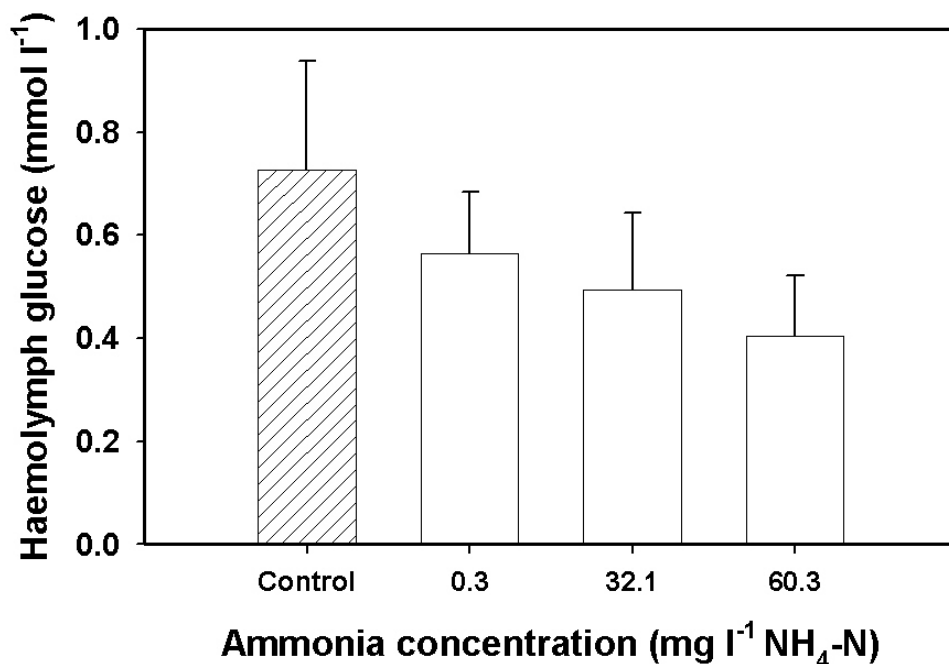


Figure. 16. Haemolymph glucose concentration of surviving *Panulirus cygnus* following 96 h exposure to different external ammonia concentrations. Striped bar indicates flow-through control treatment. Values are mean \pm SE, n = 8 or 9 lobsters. (Experiment 5)

Table 3. Haemolymph characteristics of surviving *Panulirus cygnus* following 96 h exposure to different external ammonia concentrations. Values are given as mean \pm SE for n = 8 or 9 lobsters, except for 78.2 mg l⁻¹ NH₄-N where n = 1. Different letters indicate significant differences among static renewal treatments (a, b). Asterisks indicate values significantly different to flow-through control lobsters (** P<0.01).

Haemolymph Characteristic	Treatment Concentrations (mg l ⁻¹ NH ₄ -N)				
	0 (control)	0.3	32.1	60.3	78.2
pH	7.84 \pm 0.02	7.84 \pm 0.02	7.77 \pm 0.03	7.70 \pm 0.03	7.82
Refractive Index (N _D)	1351 \pm 1	1353 \pm 2	1354 \pm 2	1355 \pm 2	1355

6.4.6 96 h LC50 values corrected for changes in pH

The 96 h LC50 values needed to be re-analysed to account for changes in pH in the experimental tanks. It was difficult to maintain the pH at normal seawater levels (8.1-8.2) in the static renewal ammonia treatments. The pH generally fell to 7.7-7.9 and this lowered the percentage of the ammonia present in the unionised form. There are a number of ways in which the equivalent unionised ammonia concentrations can be calculated depending upon the temperature and pH chosen to represent typical conditions in the experiment (either mean across the whole experiment or mean for the ammonia treatment closest to the LC50). Using these options, the toxic levels of unionised ammonia were 0.76 to 1.17 mg l⁻¹ NH₃-N for *J. edwardsii* and 0.84 to 1.87 mg l⁻¹ NH₃-N for *P. cygnus* (see Table 4 for details). Re-calculation of the data to adjust for pH and temperature under “normal” holding conditions resulted in a range of 96 h LC50 values for *J. edwardsii* of 21 to 37 mg l⁻¹ NH₄-N, for *P. cygnus* held as a group of 14 to 22 mg l⁻¹ NH₄-N, and for *P. cygnus* held individually of 25 to 34 mg l⁻¹ NH₄-N.

Table 4. Calculation of unionised nitrogen equivalent to 96 h LC50 TAN from toxicity experiments, for rock lobsters *Jasus edwardsii* and *Panulirus cygnus*, and total ammonia nitrogen concentration to induce 50% mortality at ‘normal’ holding temperature and pH.

		Measured					“Normal” conditions			
		96 h LC50 TAN (mg l ⁻¹)	Temp (°C)	pH	% unionised NH ₃ *	N in unionised form NH ₃ LC50 (mg l ⁻¹)	Temp (°C)	pH	% unionised NH ₃	Equivalent TAN #
Species & Experiment										
<i>J. edwardsii</i>										
Experiment 1 (unstressed)	A	83	12.53	7.80	1.41	1.17	12	8.2	3.32	35
	B	83	12.53	7.66	1.03	0.85	12	8.2	3.32	26
	C	83	12.53	7.66	1.03	0.85	13	8.2	3.59	24
Experiment 2 (stressed)	A	83	12.49	7.82	1.48	1.23	12	8.2	3.32	37
	B	83	12.49	7.61	0.91	0.76	12	8.2	3.32	23
	C	83	12.49	7.61	0.91	0.76	13	8.2	3.59	21
Previously reported values [^]		83	12	7.8	1.4	1.16	12	8.2	3.3	35
<i>P. cygnus</i>										
Experiment 3 (unstressed)	A	39	19.71	7.91	3.07	1.20	19	8.2	5.50	22
	B	39	19.71	7.82	2.51	0.98	19	8.2	5.50	18
	C	39	19.71	7.82	2.51	0.98	20	8.2	5.94	16
Experiment 4 (stressed)	A	38	20.34	7.90	3.12	1.19	19	8.2	5.50	22
	B	38	20.34	7.74	2.20	0.84	19	8.2	5.50	15
	C	38	20.34	7.74	2.20	0.84	20	8.2	5.94	14
Experiment 5 (individual)	A	61	20.31	7.89	3.06	1.87	19	8.2	5.50	34
	B	61	20.31	7.78	2.40	1.46	19	8.2	5.50	27
	C	61	20.31	7.78	2.40	1.46	20	8.2	5.94	25
Previously reported values [^]		61	19	7.8	2.26	1.38	19	8.2	5.50	25

* – values or estimated values from table in R.P. Trussell (1972), J. Fish. Res. Bd Can. 29(10): 1505-1507.

– LC50 TAN (normal conditions) = (unionised N LC50, mg l⁻¹) / (% unionised under normal conditions)

[^] – Values from initial calculations reported in project milestone reports and at conferences and workshops 2000-2003

A – mean temp and pH for experiment

B – mean temp for experiment and mean pH for treatment closest to 96 h LC50, normal temperature 12 for SRL or 19 for WRL.

C – mean temp for experiment and mean pH for treatment closest to 96 h LC50, normal temperature 13 for SRL or 20 for WRL.

6.5 Discussion

The current study establishes for the first time the 96 hour median lethal concentration (96 h LC50) for acute toxicity to ammonia for the two most valuable commercial species of rock lobster in Australia. Ammonia is very toxic to aquatic animals and controlling the concentration of unionised ammonia in holding tanks is a primary objective of recirculating systems (Losordo et al., 1998; Crear et al., 2003). Knowing the toxic level of ammonia for rock lobsters provides a benchmark for commercial operators of holding systems.

Southern rock lobsters, *J. edwardsii*, were found to be more tolerant to TAN than western rock lobsters, *P. cygnus*. The 96 h LC50 for unstressed lobsters held as a group was 83 mg l⁻¹ NH₄-N for *J. edwardsii*, and 39 mg l⁻¹ NH₄-N for *P. cygnus*. However, 96 h LC50 for the highly toxic unionised ammonia for unstressed lobsters held as a group are similar for both species: 0.85 to 1.17 mg l⁻¹ NH₃-N for *J. edwardsii*; and 0.98 to 1.20 mg l⁻¹ NH₃-N for *P. cygnus* (Table 4). The results indicate that rock lobsters are able to handle similar levels of ammonia to other crustaceans. For example, 96 h LC50 for penaeid prawns typically ranges from 0.5 to 1.7 mg l⁻¹ NH₃-N. Comparative studies on lobsters are scarce with incipient LC50 of 1.4 mg l⁻¹ NH₃-N for *H. americanus* larvae (Delistraty et al., 1977). Although Young-Lai et al. (1991) found a much higher level for *H. americanus* adults of 3.25 mg l⁻¹ NH₃ corresponding to a 96 h LC50 of 219 mg l⁻¹ NH₄-N. The reason for the differences between *J. edwardsii* and *P. cygnus* relate primarily to the different water quality variables, particularly pH and temperature. An important consideration in determining the toxicity of ammonia to animals is the concentration of hydrogen ions in the water. As the pH increases above 7.2 there is a reduction in hydrogen ions and consequently a greater proportion of the highly toxic unionised ammonia (NH₃) in solution (Forteath et al., 1993). The pH of seawater is usually around 8.2 but due to the accumulation of CO₂, through respiration and other biological processes, and its reaction with water to form carbonic acid, the pH of lobster holding systems are typically lower, from 7.8 to 8.2 (Crear et al., 2003). Similarly in this study, the pH in the static renewal experiments was reduced ranging from 7.7 to 7.9. Depending on how you calculate pH and temperature, the 96 h LC50 values adjusted for pH, when held as a group were; 21 to 37 mg l⁻¹ NH₄-N for *J. edwardsii* and 14 to 22 mg l⁻¹ NH₄-N for *P. cygnus* (Table 4).

The temperatures used in the current study, the densities and the size of lobsters are all designed to reflect those in commercial holding tanks. These are important factors as (1) there is a trend for increased ammonia tolerance with decreasing temperature and (2) we demonstrate behavioural effects on ammonia tolerance between individual and group held lobsters. The species differences in the current study highlight the need to determine LC50 values for each species and to take into account the different holding conditions. The different temperatures and sizes of lobsters used, 13°C and 576 to 703 g for *J. edwardsii* and 19°C and 442 to 446 g for *P. cygnus* have a direct bearing on the increased 96 h LC50 TAN for *J. edwardsii* over *P. cygnus*. Crear and Forteath (2002) found that the ammonia excretion of *J. edwardsii* and *P. cygnus* increases significantly and exponentially with temperature. They calculated Q_{10} values for *J. edwardsii* and *P. cygnus*, at the temperatures used in the current study, to be within the range considered normal for biological processes (Schmidt-Nielsen, 1990).

Ammonia levels within commercial holding and transport facilities commonly exceed the toxic levels determined in our study (B. Crear unpublished data). Even at the low treatment concentration of 40 mg l⁻¹ NH₄-N there was some mortality of lobsters in the experimental

tanks. That fact, coupled with the increased aggression of lobsters exposed to ammonia, means that ammonia concentration should be maintained as low as possible to avoid detrimental impacts on the lobsters. The increased aggression (to each other and to researchers) observed in both species not only resulted in mortality, but also significant limb loss/damage, which can have significant impact on the value of the lobsters. Major stressors in commercial handling of lobsters are emersion and accompanying factors such as disturbance and temperature (Paterson and Spanoghe, 1997). Ammonia is excreted in lobsters through the gills by diffusion and $\text{Na}^+/\text{NH}_4^+$ exchange across the epithelium and rapidly accumulates in animals removed from the water (Kormanik and Cameron, 1981; Regnault, 1987; Crear and Forteach, 2002). However, there were only minor differences in the 96 h mortality curves for stressed and unstressed lobsters for *J. edwardsii* (Figs 1 and 4) and *P. cygnus* (Figs 9 and 11). From an industry perspective the results are positive as they show that the physiological consequences of ammonia accumulation due to handling are not detrimental. Our study supports the findings of Crear and Forteach (2002), which showed that TAN in *J. edwardsii* and *P. cygnus* accumulated in haemolymph during handling but was released very quickly after re-immersion. Haemolymph ammonia concentration was double the ammonia excretion rate for both species over the range of ammonia concentrations tested (Figs 2 and 13). Haemolymph ammonia levels were found to be considerably higher than previously obtained data for prawns *Nephrops norvegicus* (Schmitt and Uglow, 1997). The haemolymph ammonia content of *J. edwardsii* and *P. cygnus* in this study was around 2 to 4 times higher than that of *N. norvegicus* at equivalent high external ammonia concentrations (34 – 68 mg l⁻¹), but around 10 times higher in lobsters than prawns held under control (low ammonia) conditions. Both lobster species had significantly greater levels of ammonia in haemolymph and excreted more ammonia when exposed to higher ammonia concentrations (>40 mg l⁻¹). The effects of longer air exposure and the long-term chronic effects of exposure to sub-lethal levels of ammonia need further investigation.

When lobsters were exposed to ammonia they became very aggressive and had higher standard oxygen consumption rates than animals held in low ammonia. This could lead to further deterioration of the water quality in the holding tanks. Low dissolved oxygen can lower the acute lethal concentrations of ammonia in penaeid prawns (see review by Allan, 1992). Oxygen consumption increased with ammonia concentration for both stressed and unstressed groups for both species (Figs 3,6,10 and 14). Stressed *J. edwardsii* exposed to ammonia tended to have lower maximum oxygen consumption than unstressed *J. edwardsii* in post experiment stress tests (Figs 3 and 6) and similar trends were apparent in individually held *P. cygnus*. There were clear differences between species in the consumption of oxygen with *P. cygnus* consuming approximately double the oxygen of *J. edwardsii* under different temperatures but otherwise similar treatment conditions. In general, there were few changes in haemolymph metabolites measured with increasing ammonia concentrations (Tables 1-3). Glucose concentration in crustacean haemolymph generally rises in response to stress from handling and emersion (Dall, 1974). However, there was no significant increase in haemolymph glucose for *J. edwardsii* following 96 h exposure to a range of ammonia concentrations (Table 1) and there was a tendency for glucose levels to fall with increasing exposure to high ammonia (Figs 8 and 16). Similarly, the haemolymph lactate in lobsters exposed to ammonia was lower than in the control lobsters. Further results from haemolymph studies of lobsters exposed to ammonia, particularly the acid-base responses to ammonia toxicity are presented and discussed in the next chapter.

Our study indicates that *J. edwardsii* and *P. cygnus* can handle higher levels of ammonia during short-term (<24 h) exposure (data not provided). However, during longer term

holding (>24 h) some mortality occurs at low levels (30–40 mg l⁻¹ TAN). Ideally, acute ammonia toxicity tests should continue for extended periods to enable the concentration at which acute toxicity stops increasing with time i.e., the incipient lethal concentration or the lethal threshold concentration to be determined (Sprague, 1990; Allan, 1992). However, longer-term experiments are confounded by mortality (reducing density), cannibalism and associated starvation. If the mortalities during toxicity experiments plateau, for example at 72 h then the 96 h LC50 may indicate that a threshold has been reached (Allan, 1992). However, this was not the case in the current study where mortality continued to increase in most experiments for treatments either side of the LC50 up to 96 h. It is desirable to maintain as low a level of ammonia as possible in lobster holding or transport tanks but at times it is difficult for commercial operators to avoid short-term high concentrations (Crear et al., 2003). At these times, knowing the tolerance of lobsters to ammonia as measured by 96 h LC50 is critical and provides options when transporting or holding them. For example, when fishing boats encounter poor seawater quality (e.g., entering low salinity in Macquarie Harbour, Tasmania) they can safely close their tanks to incoming water knowing that it is unlikely that the ammonia will rise to a dangerous level over a few hours, provided other conditions e.g., oxygen levels are maintained. Similarly, the presence of elevated levels of ammonia for short periods after a tank is stocked should not necessarily be a cause for concern. However, a continuously elevated level of ammonia is an indication that changes to the design or management of the system should be considered (Crear et al., 2003). Longer-term holding, especially when feeding the lobsters is desirable, requires the maintenance of much lower levels of ammonia. “Safe” long-term exposure to ammonia for crustaceans has commonly been based on multiplication of LC50 values by an ‘application factor’ of 0.1 or 10% (Delistraty 1977; Chen et al 1990; Allan 1992). This is an arbitrary approach, which is not always in agreement with the effective concentration that reduces growth (EC values) but does provide a useful benchmark in the absence of further data (Sprague, 1971; Allan, 1992). In seawater with a pH of 8.2, the calculated safe levels for exposure were 2.1 to 3.7 mg⁻¹ NH₄-N for *J. edwardsii* and 1.4 to 2.2 mg l⁻¹ NH₄-N for *P. cygnus*. More accurate assessment of safe long-term exposure levels requires further study where the effects of feeding and growth are determined.

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7 CHAPTER 2. SUB-LETHAL AMMONIA CONCENTRATIONS: PHYSIOLOGICAL CONSEQUENCES - HAEMOLYMPH ACID-BASE RESPONSES TO AMMONIA TOXICITY

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7.1 Introduction

The effect of external ammonia on the physiology of crustaceans has received recent attention. The main reasons behind this appear to be as a result of the requirement for crustacea to be maintained until the most optimal time for release on to the live market, or else shipped live to markets. In the latter case, there have been investigations into the acid-base disturbances that arise from exposing subtidal crustaceans, such as Palinurid lobsters, to aerial exposure (emersion) (Taylor and Waldron 1997; Morris and Oliver 1999ab).

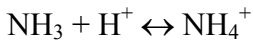
Economically it is reasonable to maximise the catch of lobsters during the open season, then to hold them for release on the market at times when a premium price can be commanded. The need to optimise holding conditions and maximise the amount of stock being held often means that lobsters are held at high stocking densities and thus water quality degradation is a potential risk. Degradation of water quality would likely result in increases in ammonium (NH_4^+), and nitrite (NO_2^-) concentrations as well as potentially leading to hypoxia if oxygen demand is not met. Particular risk occurs when large numbers of lobsters are stocked in to a holding facility over a short period of time. The shock loading of the holding system would likely lead to acute increases in ammonium followed by increases in nitrite.

The effects of environmental ammonia, and to a lesser extent nitrite, have been examined in decapod crustacea, primarily penaeid prawns (Chen et al. 1990; Chen et al. 1993; Chen and Cheng 1993; Lin et al. 1993; Chen et al. 1994a,b; Chen and Lin 1995; Cheng and Chen 1998; Cheng and Chen 2001).

In most aquatic organisms (including crustacea) ammonia is the endpoint of protein breakdown through transamination or deamination. Ammonia (NH_3) is approximately 1000 times more soluble than CO_2 , with an aqueous diffusion coefficient similar to that of CO_2 resulting in the diffusivity of NH_3 being about 1000 times greater than that for CO_2 (Wood 1993; Walsh 1999). Therefore, $\text{NH}_3/\text{NH}_4^+$ is the favoured form in which nitrogenous wastes are excreted in many aquatic organisms. In an aqueous environment, NH_3 acts as a weak acid and thus it is possible to ascribe Henderson-Hasselbach equation characteristics to the dissociation of NH_3 and the ionised NH_4^+ according to:

$$\text{NH}_4^+ = \frac{\text{TAN}}{1 + \text{antilog}(\text{pH} - \text{pK})} = \text{TAN} - \text{NH}_3$$

where TAN is the total ammonium nitrogen concentration, pH is the pH of the solution and pK is the dissociation constant for:



The pH, temperature and ionic strength of the solution will determine the extent of NH_3 or NH_4^+ predominance. In most aquatic organisms, NH_4^+ dominates and due to its relatively low permeability through lipid membranes is most likely to diffuse through aqueous pores or in association with similar sized ions through ion channels in epithelia. NH_3 on the other hand is more mobile, diffusing through lipid and aqueous pores readily (Wood 1993).

The aims of this study were to examine the acid-base physiological responses of *Jasus edwardsii* to external concentrations of ammonium chloride and examine the recovery from exposure to ammonium chloride and nitrite and hypoxia that mimic the types of toxic insult likely to be encountered in a lobster holding facility.

7.2 Methods

Table 1. Treatments used for each of the experiments to examine haemolymph acid-base responses to ammonia toxicity in *Jasus edwardsii*.

Experiment	Treatment	Measured after time (hours)
1	0,10,30,60,135 mg L ⁻¹ TAN	1
2	60 mg L ⁻¹ TAN	0, 1, 3, 6, 24
3	60 mg L ⁻¹ TAN for 24 h	0, 1, 3, 6, 24
4	60 mg L ⁻¹ TAN + 10 nitrite for 24 h	0, 1, 3, 6, 24
5	60 mg L ⁻¹ TAN for 24 h then 3 h low oxygen ^a	0, 1, 3, 6, 24

^a water oxygen saturation level maintained below previously calculated critical oxygen saturation level for active lobsters of 59.4% (Crear and Forteach, 1998)

7.2.1 Ammonia exposure conditions

Adult intermoult rock lobsters from two populations of two different sizes (mean mass \pm se of 583.8 ± 17.4 g and 199.6 ± 11.0 g, respectively) were used in the experiments. Lobsters were maintained in holding tanks (4m³) supplied with flowing seawater at an ambient water temperature of 12°C.

For ammonia exposure experiments, lobsters were removed from their holding tanks and maintained within a 350 L tank holding a maximum of 6 lobsters per tank. Each of these tanks was replicated 6 times and each tank was supplied with flowing seawater at 12°C. Lobsters were exposed to environmental ammonia by stopping the water flow and adding NH_4Cl solutions to achieve concentrations of 0 to 135 mg L⁻¹ TAN. Based on previous studies (Section 6) the value of 60 mg L⁻¹ TAN was chosen for static exposures of lobsters; this concentration is just below the 96h LC50 of 83 mg L⁻¹ TAN for unstressed *J. edwardsii*. Aeration was maintained in each tank at 90-100% air saturation of oxygen. Ammonia exposures lasted 24 h, then lobsters were sampled or the flow of ammonia free ambient seawater restored.

Haemolymph samples were taken from one lobster per tank (n = 6) without the use of any anaesthesia agent to avoid inducing acute acid-base disturbances due to anaesthesia. A post-

branchial haemolymph sample was taken from the pericardial sinus using a 21 gauge needle and a chilled 3 mL syringe. The entire sampling procedure took less than 2 minutes. The lobsters were then placed in a separate tank of clean flowing seawater to recover.

Haemolymph samples were taken from the two populations of lobsters used in this study prior to any experimental use and acid-base, ammonia and urea parameters measured as described below. These samples will be referred to as the untreated controls.

7.2.2 *Haemolymph acid-base, ammonia and urea measurements*

All measurements of haemolymph acid-base variables were determined from post-branchial haemolymph samples. Measurements of haemolymph ammonia and urea were made on both pre- and post-branchial haemolymph samples. Haemolymph pH was determined on a 70 μ L haemolymph sample using a Hanna HI1330 pH electrode connected to a Cameron Instrument Company (Port Aransas, Texas) BGM 200 blood gas analyser and calibrated to ± 0.005 pH units using Radiometer S1500 and S1510 precision buffers. Haemolymph total carbon dioxide content was determined from a 100 μ L sample of haemolymph using a Capni-con 5 (Cameron Instrument Company) total carbon dioxide analyser calibrated against a 20 mM NaHCO_3 standard. For determination of haemolymph total ammonia concentration, 1 mL of haemolymph was diluted in 9 mL of chilled de-ionised water (10x) and frozen at -20°C for further analysis. The remaining haemolymph was frozen at -20°C for determination of haemolymph urea concentration.

Haemolymph carbon dioxide tension (PCO_2) and haemolymph bicarbonate concentration (HCO_3^-) were calculated from pH and total haemolymph carbon dioxide content according to a rearrangement of the Henderson-Hasselbach equation:

$$\text{PCO}_2 = \frac{\text{CCO}_2}{\alpha\text{CO}_2 [1 + \text{antilog}(\text{pH} - \text{pK}')]}$$

$$\text{HCO}_3^- = \text{CCO}_2 - (\alpha\text{CO}_2 * \text{PCO}_2)$$

where CCO_2 is the total haemolymph carbon dioxide content, pH is the haemolymph pH, pK' is the dissociation constant for carbonic acid in the haemolymph of a lobster (6.013 from Taylor and Waldron 1997) and αCO_2 is the solubility of CO_2 in haemolymph at 12°C (0.0555 from Cameron 1986).

Haemolymph NH_4^+ concentrations were determined from diluted haemolymph samples using the salicylate hypochlorite method (Verdouw et al 1978). This method was adapted for use on a 96 well microtitre plate using a Tecan A-502 Rainbow thermo microplate reader for measuring absorbance at 680 nm against a standard curve determined using concentrations of NH_4Cl . Haemolymph urea concentration was determined from frozen undiluted haemolymph using the method of Price and Harrison (1987).

In Experiment 3, post-branchial haemolymph oxygen tension (PO_2) was measured on a sample of haemolymph injected into a Radiometer BMS Mk 2 blood gas machine using a Cameron Instrument Company E101 oxygen electrode connected to a Cameron Instrument Company BGM200 blood gas analyser. The electrode was calibrated against a 2% NaSO_3 solution (zero) and air saturated water (O_2 tension 158 mmHg). Due to the rapid clotting of the haemolymph within the blood gas analyser and on the electrode membrane, the electrode

was cleaned and re-calibrated for every sample. The time limitations of sampling meant that this measurement was only undertaken during Experiment 3.

7.2.3 Experiment 1 – Haemolymph acid-base disturbances after 1 h exposure to ammonia

Lobsters were allocated to each tank (6 lobsters in each of 5 tanks) and the water flow through the tanks stopped. NH_4Cl was then added to each tank to an appropriate concentration of 0 (control), 10, 30, 60 and 135 mg L^{-1} TAN. Lobsters were maintained with constant aeration for 1 h, then haemolymph sampled as above. In this experiment to determine the acute impact of NH_4Cl exposure on haemolymph acid-base parameters it was believed that all the lobsters in a given treatment would experience the same level of exposure. The unit of interest in this study was the individual lobster and tank effects were not considered.

7.2.4 Experiment 2 – Haemolymph acid-base disturbance of lobsters to sublethal ammonia over 24 h

Lobsters were allocated to each of 6 tanks (6 lobsters per tank). The water flow was stopped but aeration maintained and NH_4Cl was added to each tank to achieve a level of 60 mg L^{-1} TAN. A lobster from each tank was removed at 0, 1, 3, 6 and 24 h exposure and haemolymph samples removed as described above.

7.2.5 Experiment 3 – Recovery of haemolymph acid-base disturbances after 24 h exposure to sublethal ammonia

Lobsters were allocated to each of 6 tanks (6 lobsters per tank). The water flow was stopped but aeration maintained and NH_4Cl was added to each tank to achieve a level of 60 mg L^{-1} . Lobsters were maintained under these conditions for 24 h after which the flow of ammonia free ambient seawater was restored. Lobsters were sampled at 0, 1, 3, 6 and 24 h following the restoration of water flow.

7.2.6 Experiment 4 – Recovery of haemolymph acid-base disturbances after 24 h exposure to sublethal ammonia and nitrite

Lobsters were allocated to each of 6 tanks (6 lobsters per tank). The water flow was stopped but aeration maintained and NH_4Cl was added to each tank to achieve a level of 60 mg L^{-1} . In addition NaNO_3 was added to each tank to achieve a final concentration of 10 mg L^{-1} NO_3^- . Lobsters were maintained under these conditions for 24 h after which the flow of ammonium-free ambient seawater was restored. Lobsters were then sampled at 0, 1, 3, 6 and 24 h following the restoration of water flow.

7.2.7 Experiment 5 – Recovery of haemolymph acid-base disturbances after 24 h exposure to ammonia and subsequent 3 h hypoxia

Lobsters were allocated to each of 6 tanks (6 lobsters per tank). The water flow was stopped but aeration maintained and NH_4Cl was added to each tank to achieve a level of 60 mg L^{-1} . Lobsters were maintained under these conditions for 24 h after which the water was replaced with fresh aerated seawater then bubbled with nitrogen gas to reduce the oxygen saturation to an average of 45.5% (\pm se 0.8%) for 3 h. After 3 h of hypoxia, the water flow of ambient aerated seawater was restored. Lobsters were then sampled at 0, 1, 3, 6 and 24 h following the restoration of water flow.

7.2.8 *Data analysis*

Calculated PCO_2 , haemolymph HCO_3^- concentrations and haemolymph pH values were used to construct pH-bicarbonate diagrams for the subsequent experiments. These diagrams were used to interpret changes in acid-base status of the haemolymph under the various experimental conditions.

One way analysis of variance was used to examine differences in time for a given treatment. A Bonferroni planned contrast *post-hoc* analysis was used to compare differences in means to unexposed controls. P-values of less than 0.05 were considered to be significant.

7.3 Results

7.3.1 Experiment 1 – Haemolymph acid-base disturbances after 1 h exposure to ammonia

There was a concentration dependent increase from 7.68 to 7.77 in haemolymph pH with external NH_4Cl concentrations up to 30 mg L^{-1} after 1 h of exposure (Fig. 1a). However, at concentrations of 60 and 135 mg L^{-1} , there was no reduction in haemolymph pH after 1 h of exposure. There were concentration dependent increases in total haemolymph CO_2 (C_{CO_2}), CO_2 tension (P_{CO_2}) and haemolymph bicarbonate (HCO_3^-) after 1 h exposure to NH_4Cl (Fig. 1b, c, and d).

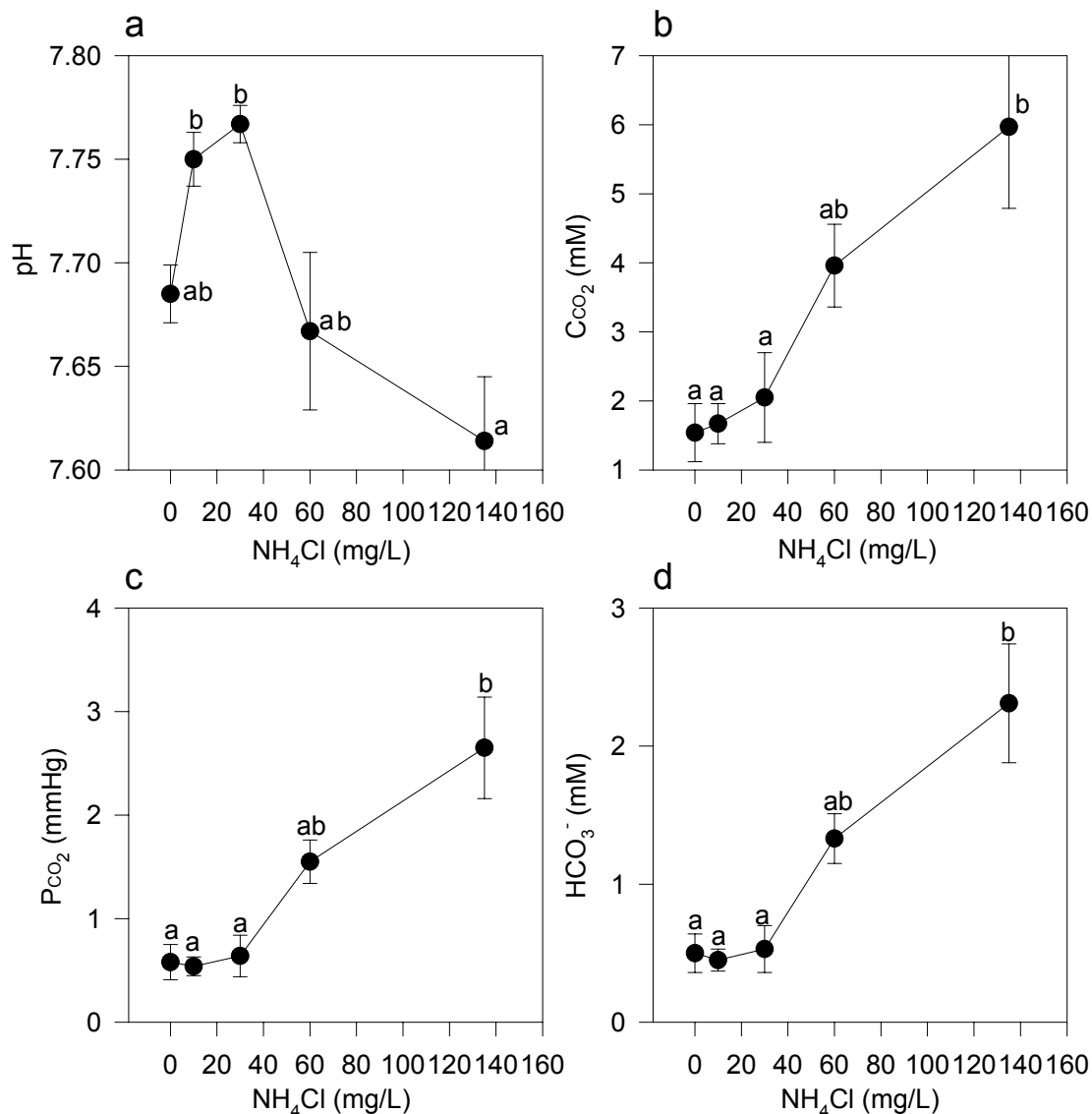


Figure 1. The effect of 1 h exposure to different concentrations of NH_4Cl on the post-branchial haemolymph pH (a), total CO_2 , C_{CO_2} (b), PCO_2 (c), and bicarbonate, HCO_3^- (d) concentrations of southern rock lobsters. Points sharing a common letter do not significantly differ ($P < 0.05$).

There was a concentration dependent increase in the concentration of NH_4^+ in the haemolymph after 1 h exposure to different concentrations of environmental NH_4Cl (Fig. 2a). However, there was no relationship between the concentration of haemolymph urea and NH_4Cl treatment (Fig. 2b).

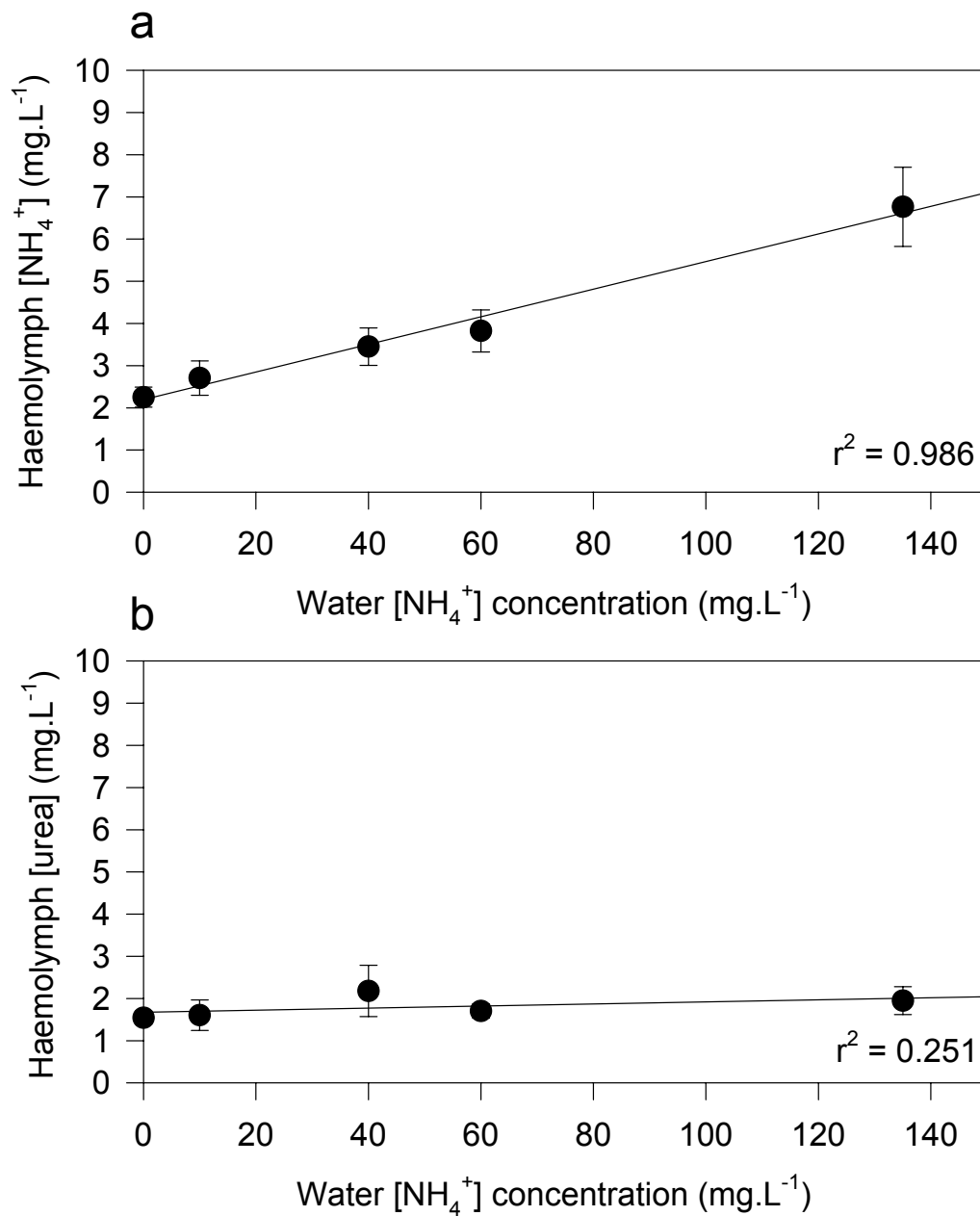


Figure 2. The relationship between water concentration NH_4Cl and post-brachial haemolymph ammonia, NH_4^+ (a), and urea (b) concentrations of southern rock lobsters exposed for 1 h.

7.3.2 Experiment 2 – Haemolymph acid-base disturbance of lobsters to sublethal concentrations of NH_4Cl over 24 h

Over the duration of the NH_4Cl exposure there was considerable variation in the responses of individual animals. This resulted in there being no statistical differences detected between means for all variables. However, it was apparent that during the initial 6 h of exposure there was an increase in haemolymph pH (Fig.3a). Similar increases in haemolymph CCO_2 , PCO_2 and HCO_3^- occurred after the first hour of exposure, followed by a reduction to basal level by 24 h exposure (Fig. 3b, c and d).

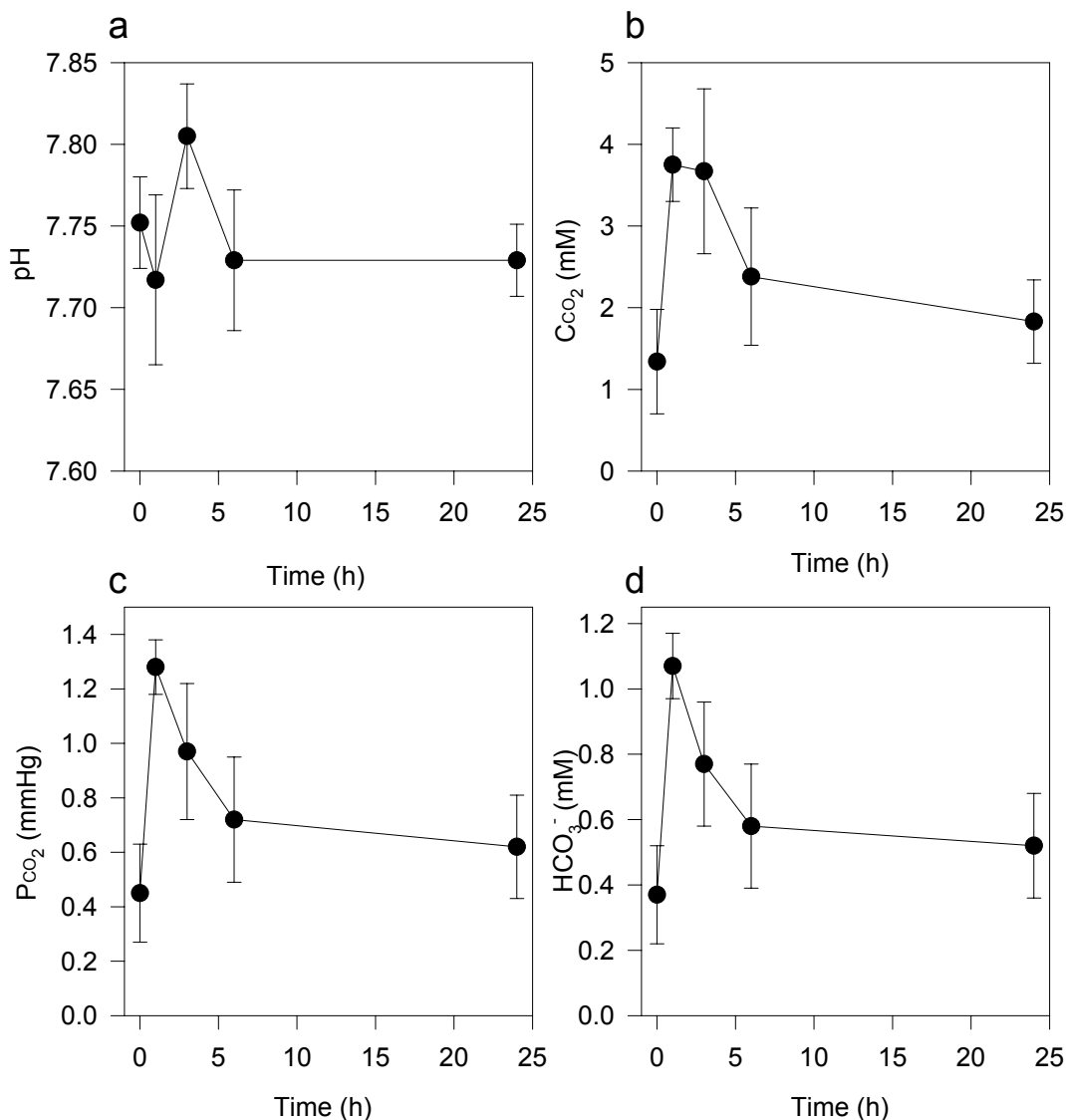


Figure 3. The effect of 24 h exposure to 60 mg l⁻¹ NH_4Cl on the post-brachial haemolymph pH (a), total CO_2 , CCO_2 (b), PCO_2 (c), and bicarbonate, HCO_3^- (d) concentrations of southern rock lobsters. Asterisks represent significance from 0 h exposure ($P < 0.05$).

There were significant increases in haemolymph NH_4^+ concentration during the 24 h of exposure compared with those concentrations of unexposed lobsters ($P < 0.05$). The biggest increase in haemolymph NH_4^+ concentration occurred after the first hour of exposure with gradual increases in concentration, stabilising at maximal levels by 24 h of exposure (Fig. 4a). There were gradual increases in haemolymph urea concentrations over the 24 h exposure duration of the experiment. However, these apparent increases were not statistically significant from baseline levels in unexposed lobsters (Fig. 4b).

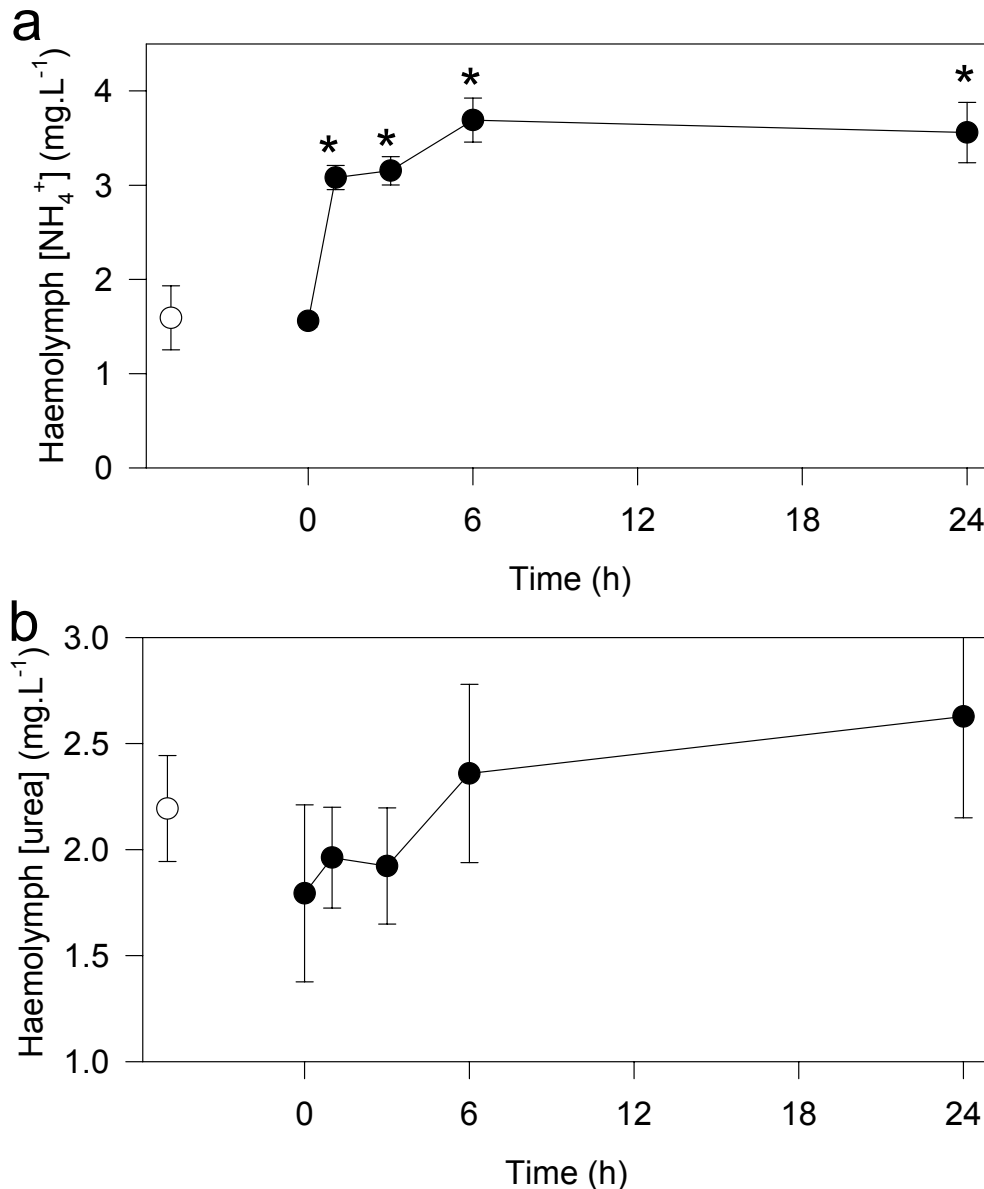


Figure 4. The effect of 24 h exposure to 60 mg.L⁻¹ NH₄Cl on the post-branchial haemolymph ammonia, NH₄⁺ (a) and urea (b) concentrations of southern rock lobsters. Open symbol represents concentrations in unexposed lobsters. Asterisks indicate significance from 0 h exposure ($P < 0.05$).

7.3.3 Experiment 3 – Recovery of haemolymph acid-base disturbances after 24 h exposure to sublethal ammonia

There were no significant changes in haemolymph pH or PO₂ following exposure to 60 mg L⁻¹ NH₄Cl (Fig. 5a, b). There were significant (P<0.05) reductions in haemolymph PCO₂ and CCO₂ levels following exposure compared to pre-exposure levels (Fig. 5c, d).

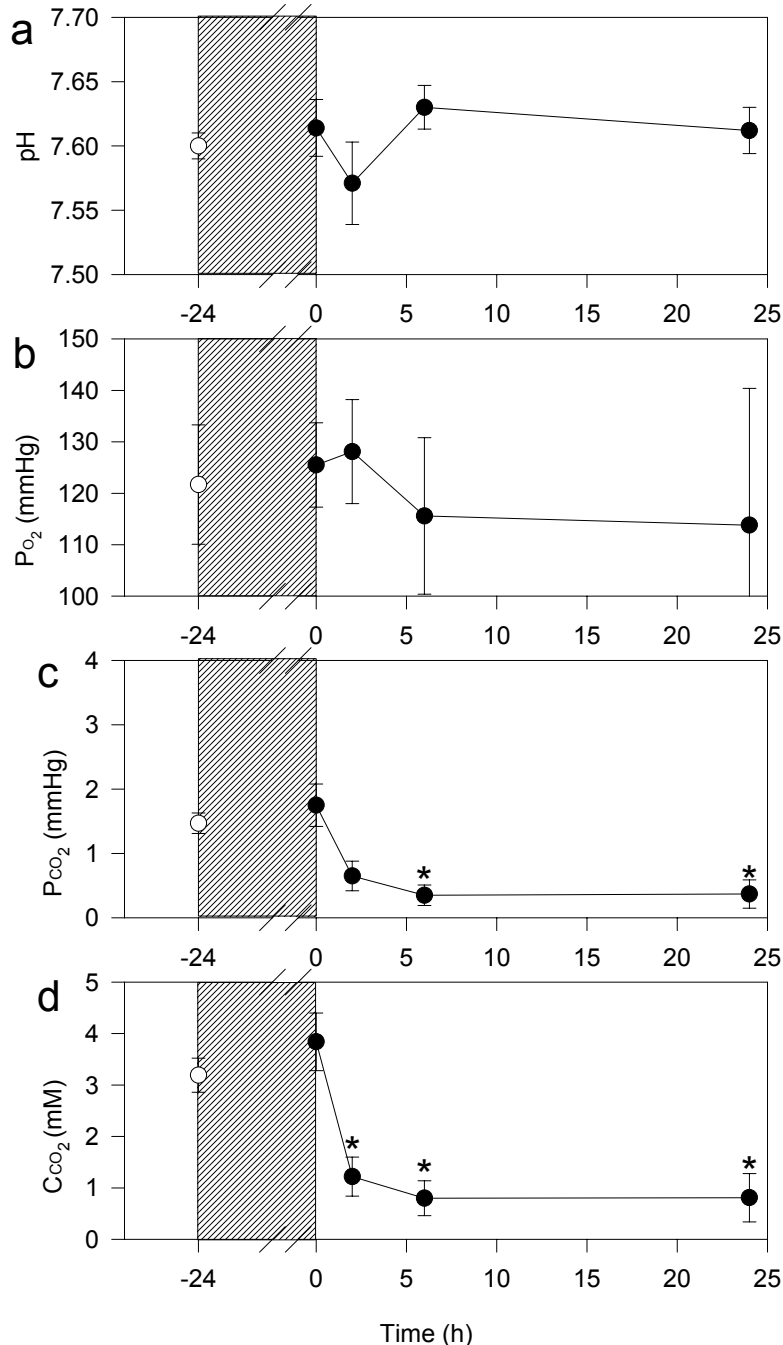


Figure 5. The recovery from 24 h exposure to 60 mg.L⁻¹ NH₄Cl (shaded bar) on the post-branchial haemolymph pH (a), PO₂ (b) PCO₂ (c) and total CO₂, CCO₂ (d) concentrations of southern rock lobsters. Open symbol represents levels measured in pre-exposed lobsters. Asterisks indicate significance from unexposed animals (P<0.05).

Haemolymph NH_4^+ concentrations were significantly ($P < 0.05$) elevated over baseline (pre-exposure) levels after 24 h exposure to NH_4Cl (Fig. 6a). These levels were similar to those observed in the previous experiment (Fig. 4a). Removal of environmental NH_4Cl resulted in a rapid decrease in haemolymph ammonia concentrations to below pre-exposure levels (Fig. 6a). Haemolymph urea concentrations were variable, however, it appeared that levels were elevated following 24 h exposure to NH_4Cl (consistent with the results from the previous experiment, Fig. 4b). Removal of the environmental NH_4Cl resulted in a rapid decrease in haemolymph urea concentrations to pre-exposure levels (Fig. 6b).

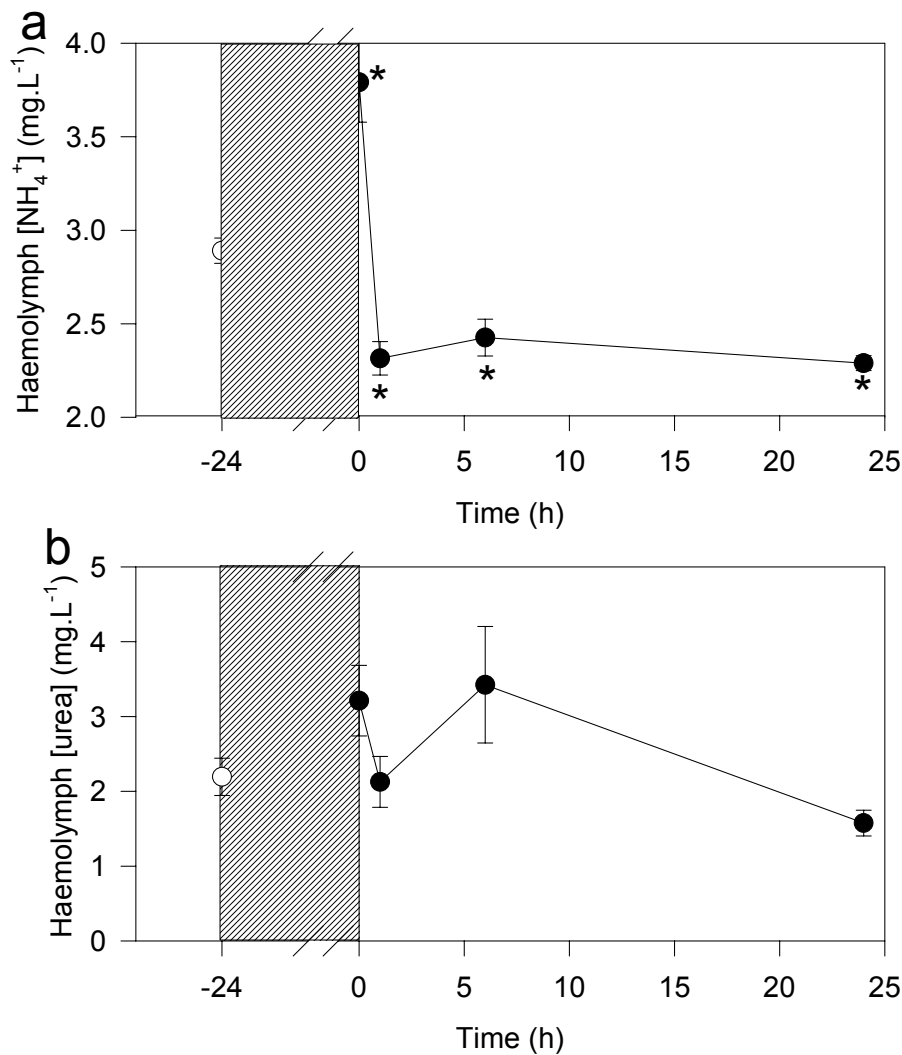


Figure 6. The recovery from 24 h exposure to $60 \text{ mg.L}^{-1} \text{NH}_4\text{Cl}$ (shaded bar) on the post-branchial haemolymph ammonia, NH_4^+ (a) and urea (b) concentrations of southern rock lobsters. Open symbol represents levels measured in pre-exposed lobsters. Asterisks indicate significance from unexposed animals ($P < 0.05$).

From the pH-bicarbonate plot it was evident that exposure of lobsters to $60 \text{ mg L}^{-1} \text{ NH}_4\text{Cl}$ resulted in an initial marked metabolic alkalosis (Fig. 7). Following removal of the NH_4Cl (between 0 and 2 h of recovery) there was a marked mixed metabolic acidosis and respiratory alkalosis resulting in a pronounced decrease in haemolymph HCO_3^- concentrations (Fig. 7). After 2 h of recovery, the apparent metabolic acidosis was overcome and a respiratory alkalosis persisted to 24 h (Fig. 7).

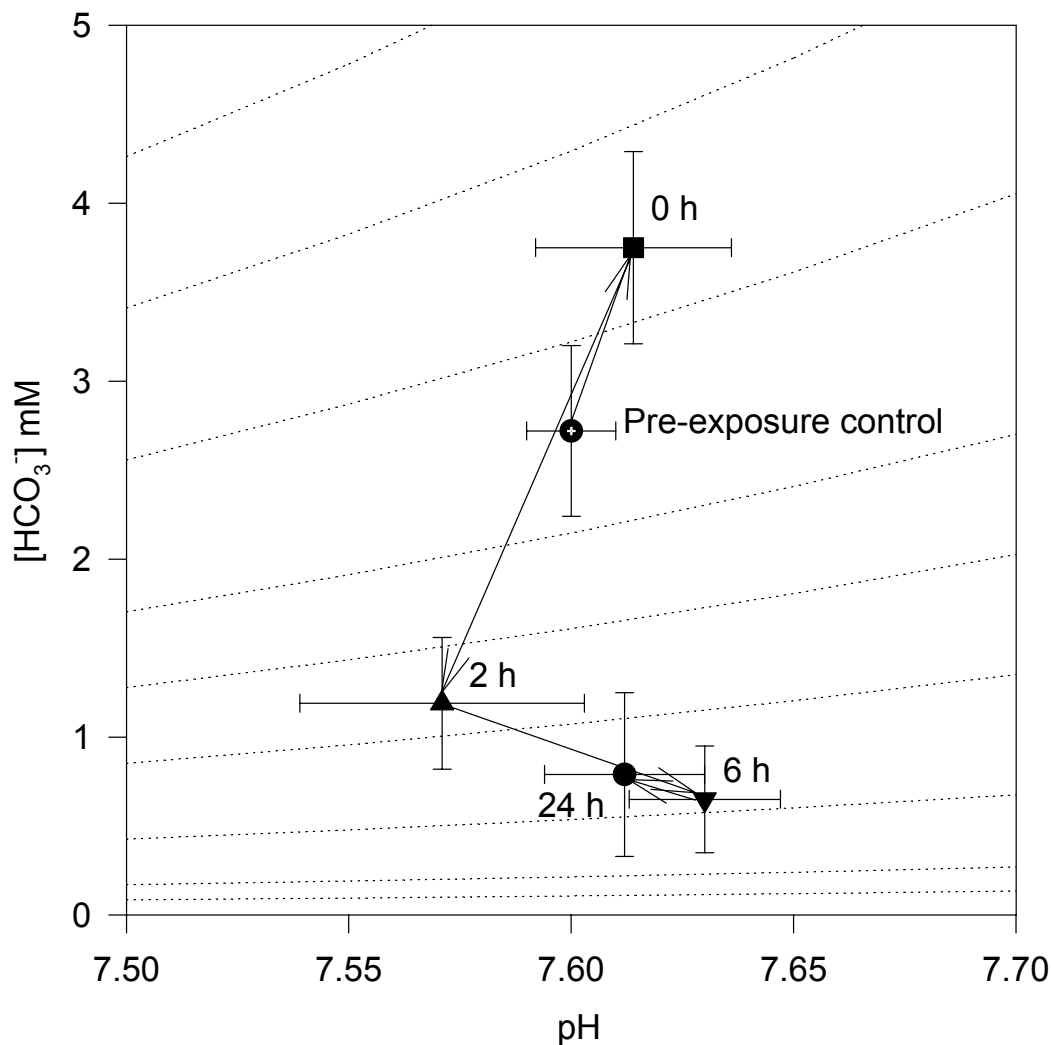


Figure 7. pH-bicarbonate diagram indicating the changes in acid-base status of southern rock lobster haemolymph during recovery from 24 h exposure to $60 \text{ mg.L}^{-1} \text{ NH}_4\text{Cl}$. Isopleths represent theoretical haemolymph PCO_2 values calculated from pH and total CO_2 data from unexposed lobsters.

7.3.4 Experiment 4 – Recovery of haemolymph acid-base disturbances after 24 h exposure to sublethal ammonia and nitrite

There was a slight but significant increase in haemolymph pH ($P < 0.05$) following exposure to $60 \text{ mg L}^{-1} \text{ NH}_4\text{Cl}$ and $10 \text{ mg L}^{-1} \text{ NaNO}_2$ (Fig. 8a). During the recovery period where environmental NH_4^+ and NO_2^- levels were negligible, haemolymph pH was not statistically significantly different to that in unexposed animals. Immediately following exposure to NH_4^+ and NO_2^- , haemolymph PCO_2 and CCO_2 levels were significantly reduced ($P < 0.05$) and did not recover during the 24 h period of the experiment (Fig. 8b, c).

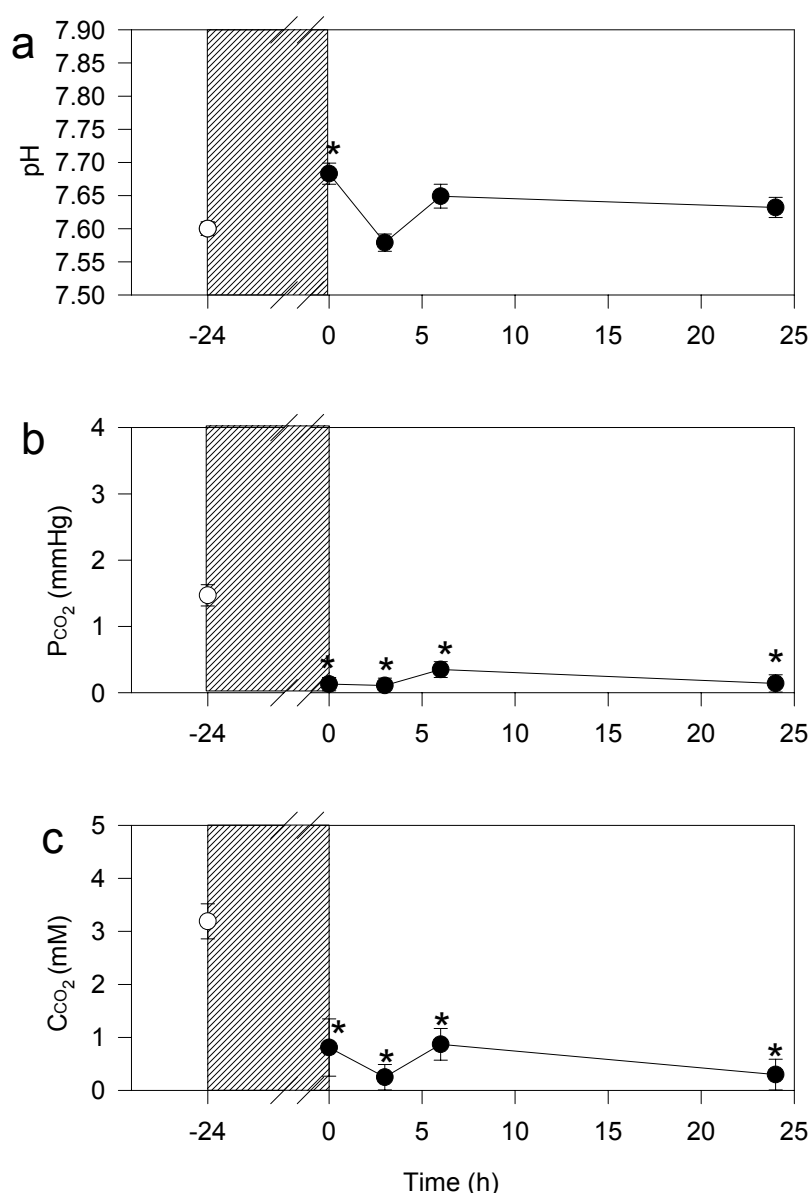


Figure 8. The recovery from 24 h simultaneous exposure to $60 \text{ mg.L}^{-1} \text{ NH}_4\text{Cl}$ and $10 \text{ mg.L}^{-1} \text{ NaNO}_3$ (shaded bar) on the post-branchial haemolymph pH (a), PCO_2 (b) and total CO_2 , CCO_2 (c) concentrations of southern rock lobsters. Open symbol represents levels measured in unexposed lobsters. Asterisks indicate significance from unexposed animals ($P < 0.05$).

Haemolymph NH_4^+ concentrations were reduced to negligible levels within the first hour of recovery following exposure to NH_4^+ and NO_2^- (Fig. 9a). Haemolymph urea concentrations were not significantly different from those measured in unexposed lobsters and there were no differences between pre and post branchial haemolymph samples (Fig. 9b).

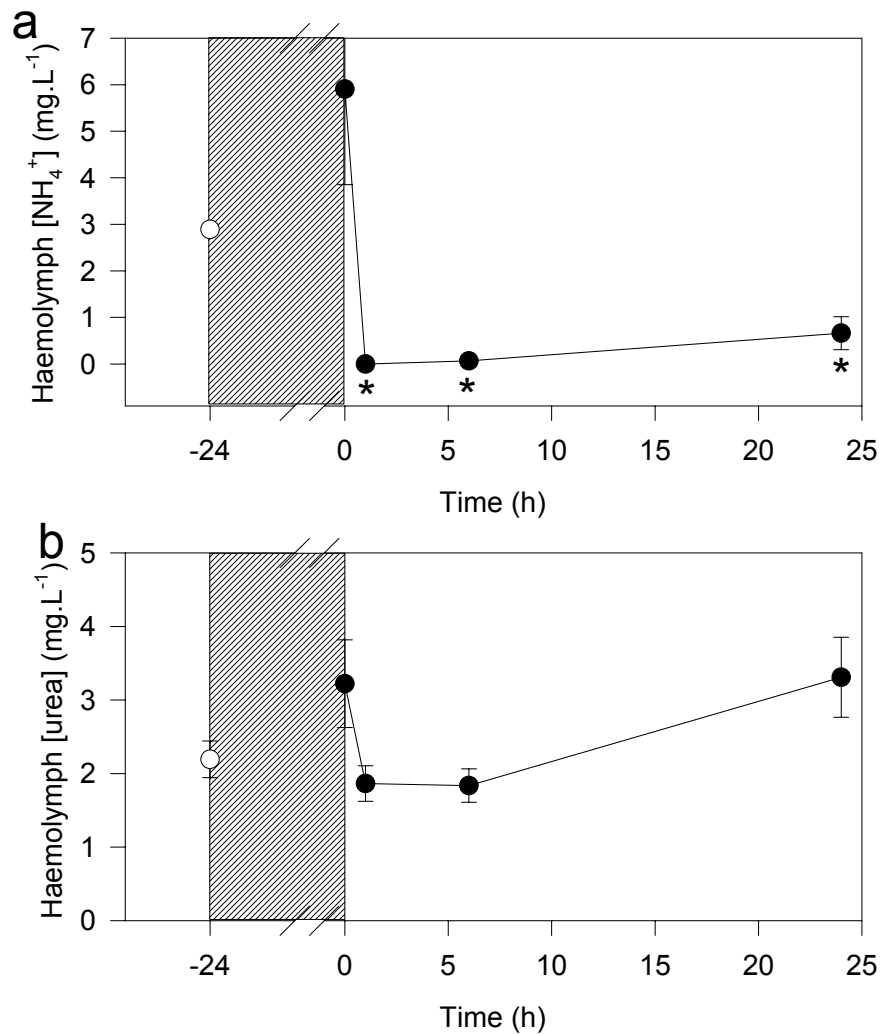


Figure 9. The recovery from 24 h simultaneous exposure to 60 mg.L⁻¹ NH₄Cl and 10 mg.L⁻¹ NaNO₃ (shaded bar) on the post-branchial haemolymph ammonia, NH₄⁺ (a) and urea (b) concentrations of southern rock lobsters. Open symbol represents levels measured in unexposed lobsters. Asterisks indicate significance from unexposed animals (P < 0.05).

Simultaneous exposure to NH_4Cl and NaNO_3 resulted in a pronounced respiratory alkalosis (Fig. 10). The removal of NH_4Cl and NaNO_3 (recovery) resulted in a marked metabolic acidosis (0-3 h recovery) followed by a small metabolic alkalosis (3-6 h recovery) (Fig. 10) and haemolymph HCO_3^- concentrations remained low to 24 h (Fig. 10).

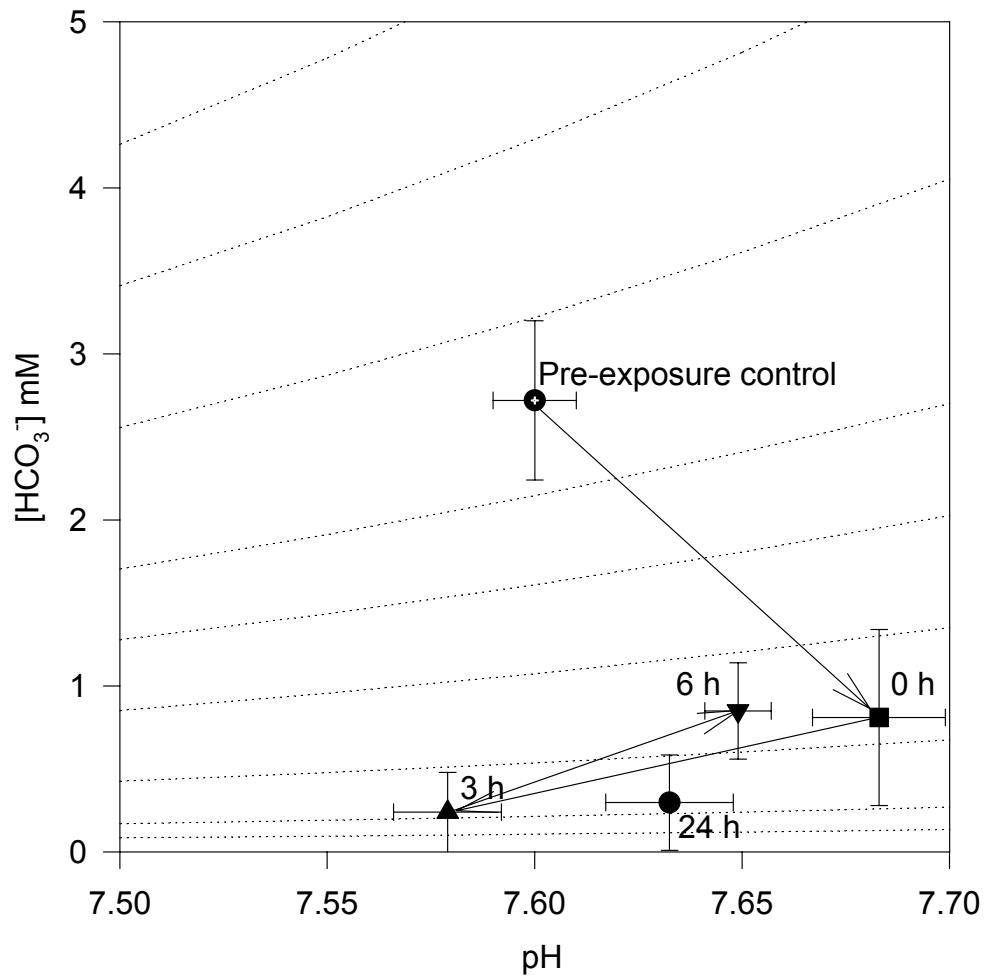


Figure 10. pH-bicarbonate diagram indicating the changes in acid-base status of southern rock lobster haemolymph during recovery from 24 h simultaneous exposure to $60 \text{ mg.L}^{-1} \text{ NH}_4\text{Cl}$ and $10 \text{ mg.L}^{-1} \text{ NaNO}_3$. Isopleths represent theoretical haemolymph PCO_2 values calculated from pH and total CO_2 data from unexposed lobsters.

7.3.5 Experiment 5 – Recovery of haemolymph acid-base disturbances after 24 h exposure to ammonia and subsequent 3 h hypoxia

Following exposure to environmental NH_4Cl then 3 h of hypoxia, haemolymph pH was slightly elevated over levels measured in unexposed animals (Fig. 11a). During the recovery phase when lobsters were maintained in ammonia-free normoxic water, haemolymph pH was reduced to levels similar to those seen in unexposed animals (Fig. 11a). Haemolymph PCO_2 levels were low throughout the experiment, similar to those measured in unexposed animals (Fig. 11b). However, CCO_2 levels were marginally increased (albeit not statistically significantly) after ammonia and hypoxia exposure the decreased to levels measured in unexposed lobsters. By 24 h of recovery, both PCO_2 and CCO_2 levels were below the limit of detection.

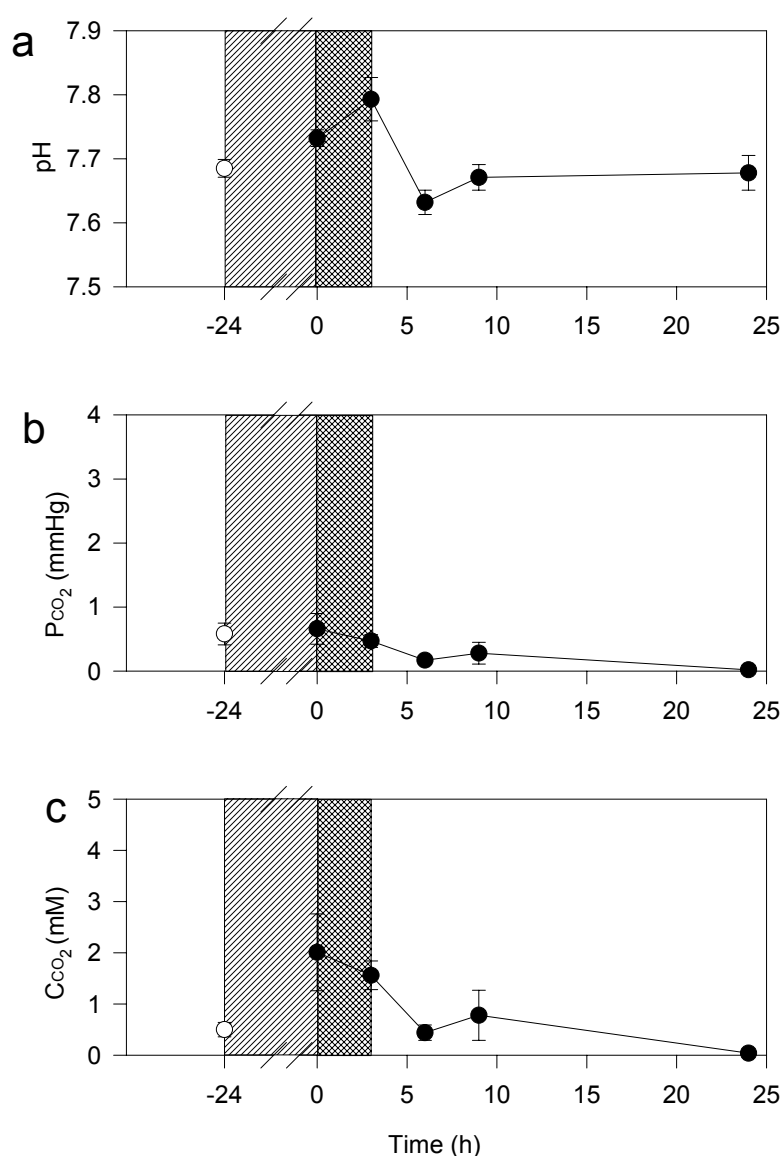


Figure 11. The recovery from 24 h exposure to $60 \text{ mg.L}^{-1} \text{ NH}_4\text{Cl}$ followed by 3h exposure to 45% air saturation hypoxia (shaded bars) on the post-branial haemolymph pH (a), PCO_2 (b) and total CO_2 , CCO_2 (c) concentrations of southern rock lobsters. Open symbol represents levels measured in unexposed lobsters. Asterisks indicate significance from unexposed animals ($P < 0.05$).

Following ammonia exposure and hypoxia haemolymph NH_4^+ concentrations were close to zero (Fig. 12a). Haemolymph urea concentrations remained similar to those measured in unexposed controls (Fig. 12b).

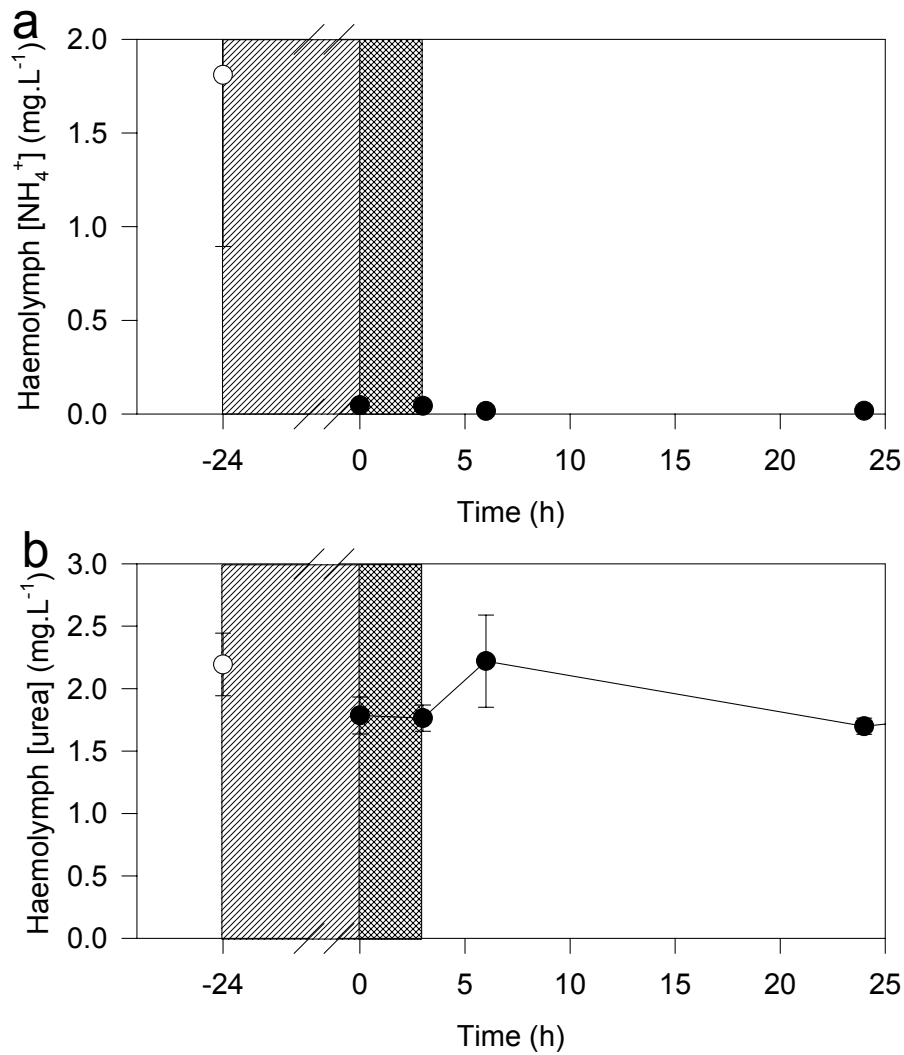


Figure 12. The recovery from 24 h exposure to 60 mg.L⁻¹ NH_4Cl followed by 3 h exposure to 45% air saturation hypoxia (shaded bars) on the post-branchial ammonia NH_4^+ (a), urea (b) concentrations of southern rock lobsters. Open symbol represents levels measured in unexposed lobsters. Asterisks indicate significance from unexposed animals ($P < 0.05$).

Following exposure to $60 \text{ mg L}^{-1} \text{ NH}_4\text{Cl}$ for 24 h there was a marked metabolic alkalosis (Fig. 13). Exposure to hypoxia induced a respiratory alkalosis (Fig. 13). The removal of environmental NH_4Cl and reinstatement of normoxia resulted in a pronounced metabolic acidosis by 3 h after which there was little acid-base disturbance to the end of the experiment at 24 h (Fig. 13).

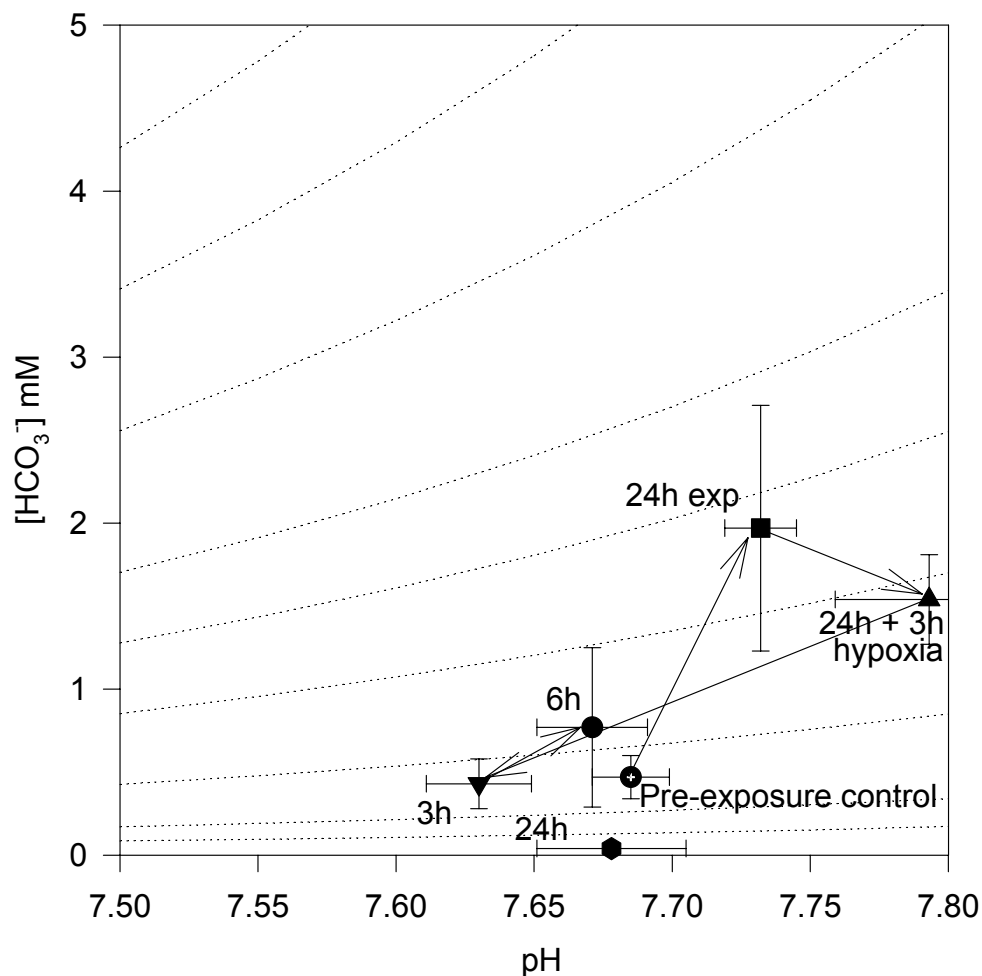


Figure 13. pH-bicarbonate diagram indicating the changes in acid-base status of southern rock lobster haemolymph during recovery from 24 h exposure to $60 \text{ mg.L}^{-1} \text{ NH}_4\text{Cl}$ and 3h exposure to 45% air saturation hypoxia. Isopleths represent theoretical haemolymph PCO_2 values calculated from pH and total CO_2 data from unexposed lobsters.

7.4 Discussion

Acute exposure to concentrations of NH_4Cl less than the 96 h LC50 (60 mg L^{-1}) elicited different acid base responses to exposures at or above the 96 h LC50. At these lower exposure concentrations there was an apparent alkalosis although no apparent changes in haemolymph bicarbonate concentration (HCO_3^-). This suggests that the alkalosis was not of respiratory origin arising as part of the bicarbonate buffering system of acid-base regulation. It is possible that this alkalosis may have reflected changes in the buffering capacity of the haemolymph, perhaps due to changes in haemocyanin buffering capacity. Chen et al. (1994) reported decreases in haemocyanin concentrations with increasing environmental ammonia concentrations in *Penaeus japonicus*. However, we have no evidence of changes in haemocyanin concentrations in our study.

Ammonia exposure and haemolymph ammonium concentration correlated closely; however, haemolymph urea could not be correlated with increasing concentration of environmental ammonium. Thus, in contrast to some other decapod crustaceans, there is no evidence from this study that the production of urea is a mechanism of detoxification of ammonium. Ureotelism, the accumulation and excretion of urea as a nitrogenous waste end product has been reported in *Penaeus japonicus* (Chen and Cheng 1993; Chen et al. 1993), *P. chiensis* (Chen and Lin 1993) and *P. monodon* (Chen et al. 1994) when exposed to elevated environmental ammonia.

Recovery from 24 h exposure to 60 mg L^{-1} NH_4Cl indicated that the haemolymph remained well buffered with no significant fluctuations in haemolymph pH. However, this was not as a consequence of bicarbonate (as indicated by the total haemolymph CO_2 content, which was significantly reduced). This suggests that bicarbonate buffering may not be the primary source of haemolymph buffering capacity. The high protein content of the haemolymph as a consequence of high concentration of haemocyanin free within it, suggests that non-bicarbonate buffering is more likely to account for the majority of the haemolymph buffering, at least following an acute sub-lethal environmental NH_4^+ exposure. There was a rapid excretion of NH_4^+ during the first hour of recovery, as has been observed for other crustacea (Crear and Forteach, 2002).

Recovery from NH_4Cl exposure suggests that a metabolic alkalosis developed in conjunction with a respiratory acidosis during exposure to environmental NH_4^+ . Upon removal of the environmental NH_4^+ , an immediate metabolic acidosis occurs until the haemolymph NH_4^+ load is reduced thereafter followed by a respiratory alkalosis most likely as a consequence of increased ventilation. This “alkalotic” state was maintained for over 24 h post-exposure with HCO_3^- levels not beginning to recover until at least 72 h post-exposure (unpublished data). This suggests that HCO_3^- is probably not the primary source of buffering in the haemolymph of the southern rock lobster under our conditions. It was also evident that HCO_3^- was not being used for the production of urea since haemolymph urea concentrations remained similar to those of the control lobsters. Work by a number of authors (as reviewed in Whiteley 1999) suggests that the majority of crustaceans take up HCO_3^- from the environment as a counter ion to Cl^- excretion. Thus the use of integumental HCO_3^- is limited only to situations when severe acidoses occur (e.g. emersion for extended periods). Our data therefore suggests that HCO_3^- is not being replaced following its use as a buffer either due to failure of branchial uptake or reduced reliance upon HCO_3^- buffering. The extreme respiratory and metabolic acidoses induced by emersion experiments using *Jasus edwardsii* (Morris and Oliver 1999ab; Taylor and Waldron 1997) and the Japanese rock lobster,

Panulirus japonicus (Huang and Chen 2001) clearly indicated increases in haemolymph HCO_3^- buffering. However, in our study the effect of NH_4^+ exposure was to reduce bicarbonate loads.

The concomitant exposure of lobsters to $60 \text{ mg L}^{-1} \text{ NH}_4\text{Cl}$ and $10 \text{ mg L}^{-1} \text{ NaNO}_3$ simulated the effects of shock loading a lobster holding facility where the biological filter of such a system may be unable to accommodate the increased NH_4^+ load. The increased haemolymph pH following exposure probably reflected increased ventilation of the gills since PCO_2 was low indicating the occurrence of a respiratory alkalosis. Nitrite is known to reduce the oxygen carrying capacity of oxygen transport pigments (e.g. haemoglobin and haemocyanin). This may lead to increases in ventilation to attempt to increase oxygen loading since, at least in teleosts, nitrite poisoning reduces oxygen carrying capacity, blood oxygen content being the primary stimulus for ventilation (Gilmour 1999). Indeed after 24 h exposure to nitrite *Penaeus japonicus* increased oxygen consumption and ammonia-N excretion (Cheng and Chen 1998). In reality, increases in ventilation are futile since the conformation of the oxidised transport pigment would result in methaemoglobin or methaemocyanin molecules that are unable to bind and transport oxygen. Recovery of lobsters resulted in a decrease in haemolymph pH and low CO_2 tensions and total CO_2 concentrations in the haemolymph. These changes coincided with a metabolic acidosis that occurred immediately at the onset of recovery followed by a metabolic alkalosis indicating changes in haemolymph pH and HCO_3^- concentrations to suggest compensation for the initial metabolic acidosis. The low concentration of haemolymph NH_4^+ in the first hour of recovery suggests that a concomitant exposure to both NH_4Cl and NaNO_2 may have either decreased the uptake of NH_4^+ or stimulated the excretion of NH_4^+ most likely through increased ventilation rate. In *Penaeus japonicus*, increased environmental NO_2^- decreased haemolymph NH_4^+ concentrations and stimulated NH_4^+ excretion as well as urea excretion (Cheng and Chen 2001).

Exposure of lobsters to acute hypoxia following 24 h exposure to $60 \text{ mg L}^{-1} \text{ NH}_4\text{Cl}$ resulted in an apparent increase in haemolymph pH and gradual decrease in PCO_2 and total CO_2 , leading to a respiratory alkalosis. Increased ventilation as a consequence of hypoxia results in the driving off of CO_2 from the gills due to high solubility of CO_2 in water and the increased flow of water across the gills facilitates the rapid removal of CO_2 from the blood by diffusion. In addition, crustacean gill epithelia have a basolaterally located carbonic anhydrase that facilitates the hydration of bicarbonate to molecular CO_2 (Henry, 1987) which can also be driven off by diffusion across the gill epithelium. The increased ventilation associated with hypoxia was likely to have also reduced the haemolymph NH_4^+ concentration rapidly. As with previous experiments, recovery from ammonium exposure results in a rapid attenuation of haemolymph concentrations. As the NH_4^+ ion is relatively impermeable to epithelial membranes (Wood 1993), diffusion must have occurred in the form of NH_3 . In light of the present evidence it would appear that *Jasus edwardsii* may be able to maintain branchial ammonia excretion by diffusion.

In conclusion, exposure to sublethal levels of environmental NH_4Cl results in the accumulation of NH_4^+ within lobsters. There is a rapid elimination of NH_4^+ from the haemolymph during recovery. It appears that elimination of NH_4^+ from the haemolymph is facilitated by hyperventilation. Acid-base disturbances within the lobster haemolymph are limited to changes in haemolymph pH with the rapid utilisation of haemolymph HCO_3^- . Once free HCO_3^- stores within the haemolymph have been exhausted, it would appear that they are not readily replaced (from fixed stores such as the carapace) but remain low to be

replaced slowly over several days (presumably by branchial HCO_3^- uptake [Whiteley 1999]). This indicates that HCO_3^- buffering of the lobster haemolymph is of minor consequence when metabolic and respiratory acidoses have been compensated for, and implies that pH buffering from non-bicarbonate sources (e.g. haemocyanin) may play an important role in acid-base balance in lobsters. Lobsters appear to be physiologically very well equipped to handle many of the water quality issues which may confront them in the live lobster industry.

7.5 Acknowledgments

We would like to acknowledge the assistance of Mr Grant Allen in sample collecting and Mr Mark Johns for assistance with haemolymph NH_4^+ and urea analysis.

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8 BENEFITS AND ADOPTION

The project has provided baseline information on toxicity levels for TAN in lobster holding systems, which will be of benefit to industry and scientists working with rock lobsters. The production of two high quality booklets on oxygen and recirculation systems incorporates the scientific data from the experimentation. The information is provided in a readily digestible form suitable for fishermen, transporters and holding facility operators. During the project Dr Crear and his associates also regularly visited commercial operators in Tasmania and Western Australia to conduct workshops and discuss the results of the study. Results were also communicated through popular articles and conference presentations (see Appendix 5). Adoption of better handling and holding conditions for rock lobsters has led to improved economic value through increased survival and condition of product to the market. The study has stimulated further interest into and research on the physiology of ammonia toxicity in rock lobsters and highlights the value of applied research to industry. The information from the project has been captured in the new Rock Lobster Industry Code of Practice.

9 FURTHER DEVELOPMENT

This and previous FRDC funded projects have determined the optimum water quality parameters for maintaining live rock lobsters. This project has further highlighted that rock lobsters appear to be physiologically very well equipped to handle many of the water quality issues that may confront them in the live lobster industry. However, lobster mortality issues still occur within the lobster industry mainly because of a poor understanding of the basics of water management and/or the physiological requirements of lobsters. Thus, the major requirement in terms of further development is to ensure that the information generated by the current project continues to be made readily available to and is taken up by industry.

10 PLANNED OUTCOMES

The project provided improved efficiency and increased economic returns to the rock lobster industry, via enhanced management of commercial rock lobster holding and transport systems. Specifically it achieved this outcome by:

1. Determining the appropriate maximum short and long-term safe levels of ammonia in holding and transport systems.
2. Better understanding the mechanisms and effects of ammonia toxicity in rock lobsters.
3. Production of two industry guides suitable for fishermen, transporters and holding facility operators.

11 CONCLUSION

The project establishes for the first time the acute ammonia toxicity for adult *J. edwardsii* and *P. cygnus* under conditions similar to those experienced in holding tanks. The 96 h LC50 values determined for both species under “stressed” and “unstressed” conditions provide invaluable information for commercial operators holding lobsters in high density recirculation facilities. Data on ammonia excretion rates, haemolymph pH, glucose and lactate provide useful information on the stress tolerance of the animals. Examining the acid-base physiological responses of *J. edwardsii* to external concentrations of ammonium chloride and examining the recovery from exposure to ammonium chloride and nitrite and hypoxia, to mimic the types of toxic insult likely to be encountered in a lobster holding facility, also provided useful baseline information. We now know that exposure to sub-lethal levels of environmental NH_4^+ results in the accumulation of NH_4^+ within lobsters. There is a rapid elimination of NH_4^+ from the haemolymph during recovery. Elimination of NH_4^+ from the haemolymph is facilitated by hyperventilation. Acid-base disturbances within the lobster haemolymph are limited to changes in haemolymph pH with the rapid utilisation of haemolymph HCO_3^- . Lobsters appear to be physiologically very well equipped to handle many of the water quality issues that confront them in the live lobster industry.

12 REFERENCES

References for research chapters are provided at the end of chapters 6 and 7. All other references in the report are provided here.

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13 APPENDIX 1: INTELLECTUAL PROPERTY

No intellectual property was generated through this project that requires protecting.

14 APPENDIX 2: STAFF LIST

Staff engaged on the project and funded by FRDC were:

Dr Bradley Crear (Research Fellow) 2000 to 2002	TAFI, MRL, Hobart	PhD	25%
Mr Grant Allen (Technician); 2000 to 2001	TAFI, MRL, Hobart	BSc	100%
Ms Joan Van Drunen (Technician) 2001 to 2002	TAFI, MRL, Hobart	BSc	100%

In kind contributions from TAFI were:

Dr Mark Powell (Lecturer); 2000 to 2003	TAFI, SoA, Launceston	PhD	20%
Dr Stephen Battaglene (Senior Research Fellow) 2002 to 2003	TAFI, MRL, Hobart	PhD	15%
Dr Jennifer Cobcroft (Junior Research Fellow) 2003	TAFI, MRL, Hobart	PhD	10%
Mr Edward Smith (Technician) 2002 to 2003	TAFI, MRL, Hobart	BSc(Hons)	25%

15 APPENDIX 3: OXYGEN – INDUSTRY GUIDE 1

The following Guide for the rock lobster industry was published in 2002 to complete objective 1 of this project. The guide, as formatted for publication, is included on the CD accompanying this final report.

Crear, B.J., and Allen, G. 2002. Optimising water quality - oxygen. Guide for the rock lobster industry. No. 2. Tasmanian Aquaculture and Fisheries Institute, University of Tasmania. 36p.

16 APPENDIX 4: AMMONIA – INDUSTRY GUIDE 2

The following Guide for the rock lobster industry was published in 2003 to complete objective 4 of this project. The guide, as formatted for publication, is included on the CD accompanying this final report.

Crear, B.J., Cobcroft, J.M., and Battaglione, S.C. 2003. Recirculating systems for holding rock lobsters. Guide for the rock lobster industry. No. 2. Tasmanian Aquaculture and Fisheries Institute, University of Tasmania. 29p.

**17 APPENDIX 5: A LIST OF PUBLICATIONS
ARISING FROM THIS PROJECT**

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