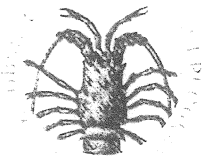

Physiological studies of stress and morbidity during post-harvest handling of western rock lobsters (*Panulirus cygnus*)

I. Physiological stress indicators.

B.D. Paterson, G.W. Davidson and P.T. Spanoghe

Queensland Government
Department of Primary Industries

CENTRE FOR FOOD TECHNOLOGY

FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Project No. 96/345

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Professor Bruce Phillips, Manager of the FRDC Rock Lobster Post Harvest Handling Sub-program , chaired the project steering committee. The companion project, led by the Aquatic Sciences Research Unit, also based at Curtin, was FRDC 96/344 "II. Standard Autopsy Techniques and Immune System Competency." That project, led by Associate Professor Louis Evans and involving Drs Japo Jussila, Elena Tsvetnencko and Jeff Jago, considered the health status and immune system parameters of lobsters and in many cases participated in joint experiments.

96/345 Physiological studies of stress and morbidity during post harvest handling of western rock lobsters (*Panulirus cygnus*), I. Physiological stress indicators

1 NON-TECHNICAL SUMMARY

1.1 PRINCIPAL INVESTIGATOR

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1.2 OBJECTIVES

1. Identify key physiological stress parameters that either describe stress levels and/or predict likely further mortality in lobsters after harvest and apply these parameters in studies aimed at improving post-harvest handling practises. This overall objective will be achieved by...
2. Obtaining baseline measurements of physiological parameters in resting undisturbed lobsters, with reference to interactions between season and locality and the effects of moult stage and other biological variables (Sub-objective 1)
3. Identifying physiological parameters, through field studies aimed at studying the effect of harvest and post-harvest handling on lobsters, which can be used to evaluate deviations from baseline values (stress responses) in captive lobsters. (Sub-objective 2)
4. Identifying physiological parameters, through controlled laboratory experiments using identified stressors (from 3. above) which can be used to evaluate deviations from baseline values (stress responses) in captive lobsters. (Sub-objective 3)
5. Develop simple methods of measuring one or more of the stress parameters identified in sub-objectives 2 and 3 for use in lobster processing factories in the evaluation of stress levels in selected lobster shipments (Sub-objective 4)
6. Apply the results and understanding of harvesting and post-harvest handling gained from field work in sub-objective 2, and the stress parameters identified in sub-objectives 2 and 3 in a study or studies of lobster post-harvest handling practices aimed at developing improved post-harvest procedures, (Sub-objective 5)
7. Use the findings of earlier sub-objectives to make recommendations for improvements in handling practices described in the recently published code of practice. (Sub-objective 6)
8. Use the findings to develop detailed knowledge and understanding of the physiological processes involved in stress responses in lobsters which can be used by processing companies and fishers to devise improved methods of post-harvest handling and transport. (Sub-objective 7)

1.3 SUMMARY

Lobsters removed from factory tanks because they have weakened during live storage have probably been stressed too much. Factory-based trials in this project have shown, in conjunction with FRDC 344, that when changes were seen in certain physiological/immunological parameters in the blood of lobsters during a 6h storage treatment then they were more likely to die within the next week. The key physiological indicators found here, lactate and magnesium, reinforce the point that storage in air at ambient temperature is highly detrimental to lobsters- even when seawater sprays are provided. These indicators were not simple enough for routine factory use, but they were still useful in monitoring the responses of lobsters to alternative storage/transport methods to ensure that the deleterious effects of out-of-water storage are minimised.

These findings vindicate the initial premise of this research, that stress during post-harvest handling was responsible for losses of rock lobsters during storage in factory tanks. The objective of this study was to find indicators of that stress so that they could be either used by some factories to grade live product for export or used in studies of alternative handling practices so that fewer lobsters were stressed.

The study showed that during the post-harvest handling of rock lobsters, several physiological parameters deviated from baseline levels (established by sampling lobsters on the sea floor using SCUBA and by sampling captive acclimated lobsters). These changes parallelled the respiratory problems seen when rock lobsters were kept out of water in laboratory experiments. Using these findings as a basis, a series of factory-based experiments, some using alternative ambient temperature storage methods, were used to establish which of these physiological changes could be linked to later mortality in stressed lobsters. Immediately after an imposed stress, the lobsters that eventually died over the following week were significantly different from future survivors with respect to some blood parameters (particularly lactate and magnesium concentration). Using discriminant analysis of several blood test results, it was possible to correctly classify the fate of 80-90% of lobsters. However with each replicate of the study, a different set of blood tests were required, though there was some consistency from time to time, particularly regarding lactate's importance to the analysis.

Key indicators for commercially significant stress clearly existed, but none of these were simple enough to apply in a factory context. When comparing the performance of lobsters in alternative storage methods, the most accurate method was clearly to count the actual mortality, but the results of human grader collaborating in this study could also be used to rank treatments from best to worst. The advantage of stress indicators was that they showed why lobsters were dying and suggested the changes required to improve outcomes.

If reduced deviations in these key indicators were used as the proviso for choosing storage/transport environments, then this criterion would continue to emphasise the need for submerged storage/transport of lobsters. The storage environment trials also showed that the prognosis was excellent for lobsters stored in recirculated seawater for 6 hours.

Changes to the code of practice would seem warranted. In recommending submerged transport as the best method for short term movement of lobsters, this work suggests that as long as aeration is maintained that water quality deterioration is not a major issue, and provision of biological filtration of the water does not seem to be a priority.

Submerged transport will not always be possible or practical. This research shows that contrary to what you might first expect, that spraying seawater onto lobsters in air at ambient temperature serves no apparent benefit in terms of lobster condition over and above that provided by simple humid air. Further work may be required, if there is a call for it, to establish exactly why sprays fail to benefit lobsters in air.

These observations regarding lobster transport and storage are confined to ambient temperature and hence are strictly applicable to bulk transport of lobsters (eg. on carrier boats). Cooling the lobsters down (eg. truck transport) introduces another variable, that of reduced metabolic rate, which may alter the lobster's responses.

1.4 KEYWORDS

Western rock lobster, post-harvest handling, live storage and transport, mortality.

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3 BACKGROUND

Spiny lobsters, known locally as rock lobsters, crayfish or "crays" are Australia's highest value export fishery, accounting for almost a third of the 1997/98 total export value (ABARE 1998). Exports of whole rock lobster (including live product) are targeted largely at established markets in Asia such as Japan, Hong Kong, and Taiwan, and in recent years, sales to China have burgeoned.

These fisheries are mainly based on two species, the western rock lobster *Panulirus cygnus*, and the southern rock lobster *Jasus edwardsii*, (the latter species also dominates the fishery in New Zealand). The western rock lobster is caught along the south-western coast of Western Australia, where the annual catch is on average around 10-11 thousand tonnes. In South Australia and Tasmania, the combined annual catch of the southern rock lobster is just under half that of the western species (eg. in 1997/98 just over 4 thousand tonnes) (ABARE 1998). There is also a minor dive fishery for tropical rock lobsters (eg. *P. ornatus*) in northern Australian waters.

All of these fisheries export significant quantities of live lobsters, but production of whole cooked and tailed lobsters persists. The marketing of live lobsters perhaps causes the most excitement, with lobsters being air-freighted to buyers thousands of kilometres away. However, it is noteworthy that only approximately a third of the product harvested in the Western Australian fishery is destined for live export. This is not due to a difficulty in live export technology, but rather reflects the profitability of various product forms. The situation may however differ between processors, some of whom may specialise in live marketing and want to export as much of their product in live form as possible.

The air freight of live rock lobsters is only a relatively recent innovation in terms of the development of these fisheries. Fortunately, rock lobsters have proved relatively easy to send long distances to markets in Asia, with only minor or indeed negligible losses. However, this excellent record is bolstered to some extent by rigorous selection of "healthy" or strong lobsters prior to packaging them for transport. Stress during post harvest handling has been blamed, anecdotally, for the condition of lobsters that are deemed unsuitable for live export.

Post-harvest processing in each of these fisheries involves some degree of handling, transport and storage of live rock lobsters. Western rock lobsters are caught in baited pots and begin to tail flap vigorously as they leave the water and during sizing and handling on the boat. It is recommended that the rock lobsters are exposed to air on the boats for as short a period as possible to reduce stress (Brown and Caputi 1983), though very little is known about the physiological effects of keeping *P. cygnus* in air on boats. Lobsters generally cannot respire properly in air and this problem is compounded by high temperatures, disturbance and handling (Taylor and Whiteley 1989; Whiteley and Taylor 1990).

The western rock lobster catch is stored on board commercial boats in seawater holding tanks. These tanks must be well supplied with well-oxygenated sea-water because disturbed lobsters have elevated respiration rates (Crear and Forteath 1997). Following storage on the boats, some lobsters are unloaded from the boat directly at the factory. However, others arrive at the factory after a road trip, (stored cool in air or under a cool seawater spray). Others still are stored in floating pens and taken by fast carrier boats, again under seawater sprays or in tanks, to the factory.

At the factory, the lobsters are weighed in and sorted for size, injury and vigour; a process that involves further disturbance and handling in air. Some lobsters will be processed immediately. Others will be placed in storage tanks where they are kept for several days to several weeks before they are graded and packed for live export. The storage tanks provide an environment that allows lobsters to recover from the physiological stress accompanying harvesting and transportation. This recovery should occur relatively rapidly (Spanoghe and Bourne 1997; Taylor and Whiteley 1989; Whiteley and Taylor 1992), unless the lobsters have suffered permanent injury and will later weaken and die in captivity.

Overall, the post-harvest handling of western rock lobsters involves periods of storage or transport in air alternating with periods of submerged recovery. This apparent recovery needs to be carefully studied, particularly as some lobsters are already graded weak on arrival at the factories while others

may weaken and die subsequently. Anecdotal evidence suggests that the proportion of western rock lobsters arriving at factories in a condition that is fit for live export is somewhere around 85-90%. Lobsters can be rejected for a number of reasons, including injury, excessive leg loss (autotomy) or because they appear weak or exhausted.

In order to find out why lobsters weaken after harvest, and whether stress plays a role, we need to be able to quantify stress in rock lobsters. Little is known about stress in western rock lobsters but there is literature of stress in other crustaceans (Paterson and Spanoghe 1997a), and commercial species in particular (DeFur *et al.* 1988; Paterson *et al.* 1997c; Paterson and Spanoghe 1997a; Uglow *et al.* 1986; Vermeer 1987; Whiteley and Taylor 1990). A lobster can be said to be experiencing stress if its internal physiology has been pushed beyond normal bounds by a factor or stressor acting upon it (Barton and Iwama 1991). Of course, it is not enough to know that a lobster is simply stressed, there is clearly an imperative to identify stresses severe enough to lead to future mortality in lobsters.

4 NEED

Stress is often cited anecdotally as an issue in lobster handling. Yet, it is difficult to assess these calls for action because we understand little about the impact of handling stress in rock lobsters. Does stress matter? The parameters usually chosen in studies of crustacean post-harvest handling may be physiologically significant, but it is not certain that these "indicators" describe a stress severe enough to be of commercial significance to lobster handlers and processors.

Following a review of post-harvest issues in the Australian rock lobster industry the FRDC saw a need for a broad research effort with two general thrusts. Firstly, to investigate the basic physiology of stress in the western rock lobster in order to identify parameters that highlighted commercially important stress. These key stress indicators could then be used in focused studies of alternative handling methods. Secondly, to establish whether any of these key stress indicators were simple enough to be used to grade product, for example, by premises devoted to maximising export of live lobsters.

This study had to be as broad, and as multi-disciplinary in scope as possible, so as not to presuppose the outcome. For this reason, the study was set up as two sub-projects, this one, focussing on physiological impacts on lobsters and a collaborating sub-project (FRDC 96/344) studying lobster health status.

Because of the need to ensure an evolving process of collaboration and industry feedback, these projects, along with a number of other more specific post-harvest projects, were brought together as the FRDC Rock Lobster Post-harvest Sub-program.

5 OBJECTIVES

5.1 Original objectives

1. Identify key physiological stress parameters that either describe stress levels and/or predict likely further mortality in lobsters after harvest and apply these parameters in studies aimed at improving post-harvest handling practises. This overall objective will be achieved by...

2. Obtaining baseline measurements of physiological parameters in resting undisturbed lobsters, with reference to interactions between season and locality and the effects of moult stage and other biological variables (Sub-objective 1)

3. Identifying physiological parameters, through field studies aimed at studying the effect of harvest and post-harvest handling on lobsters, which can be used to evaluate deviations from baseline values (stress responses) in captive lobsters. (Sub-objective 2)

4. Identifying physiological parameters, through controlled laboratory experiments using identified stressors (from 3. above) which can be used to evaluate deviations from baseline values (stress responses) in captive lobsters. (Sub-objective 3)

5. Develop simple methods of measuring one or more of the stress parameters identified in sub-objectives 2 and 3 for use in lobster processing factories in the evaluation of stress levels in selected lobster shipments (Sub-objective 4).

6. Apply the results and understanding of harvesting and post-harvest handling gained from field work in sub-objective 2, and the stress parameters identified in sub-objectives 2 and 3 in a study or studies of lobster post-harvest handling practices aimed at developing improved post-harvest procedures, (Sub-objective 5)

7. Use the findings of earlier sub-objectives to make recommendations for improvements in handling practices described in the recently published code of practice. (Sub-objective 6)

8. Use the findings to develop detailed knowledge and understanding of the physiological processes involved in stress responses in lobsters which can be used by processing companies and fishers to devise improved methods of post-harvest handling and transport. (Sub-objective 7)

5.2 Changes

Changes.
As it became clear that a simple stress indicator had not need identified, the milestone relating to objective 5 was changed to..:
- develop, validate and document a set of practical indicators of stress and future mortality in rock lobsters

6 METHODS

6.1 Baseline physiology of free-ranging lobsters (Sub-objective 1)

6.1.1 Collection of samples

Several dive sampling trips were undertaken during 1997 - 1999. All trips were carried out in waters around the Abrolhos Islands and Geraldton, Western Australia. The location and timing of each trip was often chosen to provide baseline values for concurrent experiments using captive animals (see sub-objectives 2, 3 and 5).

6.1.1.1 Underwater

Lobsters were sampled by SCUBA divers working in pairs. One diver used a wire loop snare to catch lobsters. Following capture, one diver held the lobster with its abdomen flexed to expose the arthrodial membrane between the ventral margin of the carapace and the tergum of the first abdominal segment. The other diver used a disposable 2.5 mL syringe fitted with a 23 gauge hypodermic needle to withdraw a hemolymph sample (2.0 - 2.5 mL) from the pericardial sinus via the exposed membrane. This sample was collected within 1 minute of the animal first being disturbed. Immediately after sampling, the animal and hemolymph sample were brought to a boat on the surface.

6.1.1.2 At the surface

On the boat, the needle was removed from the syringe and the bulk of the hemolymph sample was dispensed equally to a pair of numbered 1.5 mL microcentrifuge vials. The tip of one pleopod was removed and placed in a microcentrifuge tube containing 10% formalin in 3% saline solution for later moult stage determination (see Section 6.8). Sex and carapace length was recorded. Sampled lobsters were stored on-board in flow-through live tanks. Once diving at a site was complete, all animals were returned to the ocean alive.

6.1.2 Initial measurements and sample storage

A few drops of whole hemolymph were dispensed immediately from the syringe and the protein concentration measured as refractive index, using a Shibuya S-1 hand-held refractometer, (see Section 6.9.2). A 250 µL sub-sample was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1 mol perchloric acid (PCA), to precipitate protein. The vials of whole and PCA-treated hemolymph were then promptly capped and frozen in liquid N_2 for later analysis (see Section 6.10).

6.1.3 Hemolymph chemistry assays

Lactate and glucose concentrations of these neutralised perchlorate extracts (NPE) were determined spectrophotometrically using diagnostic kits. The concentrations of calcium, magnesium and potassium were determined in serum prepared from the whole hemolymph samples. For details of sample handling and assays conducted refer to Section 6.10.

6.2 Acclimation and physiological responses of lobsters to captivity (sub-objective 1)

Two experiments were conducted to address this sub-objective and specifically, the aspects investigated were:

i) whether lobsters could be maintained in captivity for relatively long periods under conditions which allowed their physiological state to return to that observed in undisturbed lobsters in the wild and,

ii) to establish whether repetitive sampling of individual lobsters was a practical method of tracking physiological changes in the animals.

6.2.1 Experiment 1: Single and repetitive sampling of western rock lobsters held in a recirculating seawater system for 42 days

6.2.1.1 Collection and storage of lobsters

A consignment of 54 kg of red "A" grade lobsters, obtained from a local commercial supplier, was held in a 3000 L recirculating sea water system (18º C, 35ppt) at the Geraldton College of TAFE for a period of 7 weeks. During the holding period the animals were fed twice weekly with fresh mussels and water quality parameters were monitored daily.

The process of acclimation to captivity was examined by taking hemolymph samples from groups of 15 lobsters at different times during the experiment (single group), on arrival and after one and two days recovery in the tank and thereafter, at weekly intervals. These lobsters were only sampled once. A further fifteen lobsters (serial group) were sampled on arrival and then sampled again each time that the other groups of lobsters were sampled.

The sampling procedure was conducted as follows: baskets were lifted gently from the tank and placed above another basket in such a way that all animals will remain immersed. A sheet of dark plastic was placed above the basket, with a view to restrict visual disturbance. Lobsters were caught quickly by hand and using a 5 mL hypodermic syringe fitted with a 22 gauge needle, a hemolymph sample was withdrawn from the pericardial sinus by flexing the abdomen and inserting the needle through the arthrodial membrane at the junction of the ventral margin of the carapace and the tergum of the first abdominal segment. All hemolymph samples were collected within 30 seconds of the animal first being caught.

The animals were subjected to a visual assessment with reference to their vigour (VI) (Spanoghe and Bourne 1999) and to a general examination, with data such as size, gender, missing appendages and the occurrence of shell damages being recorded. A pleopod tip was taken from each lobster for moult stage determination (see Section 6.8).

Hemolymph samples were used for determination of hemolymph pH, protein (by refractive index), lactate, glucose and a number of ionic constituents. Total hemolymph cell count was also determined.

6.2.1.2 Initial measurements and sample storage

The needle was removed from the syringe and the bulk of the hemolymph sample was added to a pair of numbered 2 mL microcentrifuge vials. The protein concentration of whole hemolymph was measured immediately from the syringe as refractive index, using a UR-2 hand-held refractometer, (see Section 6.9.2). The pH of the hemolymph sample in one vial was measured immediately at ambient temperature using a pH meter (TPS WP 81) connected to a temperature compensated electrode (tapered glass bulb).

Using an automatic pipette, a 250 µL subsample of whole hemolymph was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1 mol L^{-1} perchloric acid (PCA), to precipitate protein. The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and then frozen in liquid N_2 for later analysis.

6.2.1.3 Hemolymph chemistry assays

Lactate and glucose levels were determined later from the deproteinated samples using spectrophotometric kits. Serum was produced from the whole hemolymph and used to establish levels of calcium, magnesium and potassium. For details of sample handling and assays conducted refer to section 6.10.

6.2.2 Experiment 2: Acclimation and physiological responses of the western rock lobster to captivity over a 67 days period

6.2.2.1 Collection and storage of lobsters

Approximately 220 kg of western rock lobsters (WRL, *Panulirus cygnus*, carapace length 77 ± 0.22 mm, N= 181) were obtained from the Western Australian Department of Fisheries (Fisheries WA), in November 1997. These animals were caught during a population management survey conducted by Fisheries WA in the commercial WRL fishing zone situated around the reefs fringing the Houtman Abroholos Islands, offshore from Geraldton, W.A. (28° 45' S, 144° 35' E). Immediately after being harvested, and for all the journey back to shore, the lobsters were stored on board the research vessel, in tanks flushed with seawater pumped from the ocean. Immediately after reaching the harbour at Fremantle, the animals were transported out of water to the nearby South Metropolitan College of TAFE, where they were placed in a black rectangular fibreglass tank (3.0 x 1.2 x 0.9 m; 2.66 m^3, Tank West, Canning Vale W.A.). This tank had been filled with fresh seawater filtered by recirculation and gravity through a biological filter system. The filtration system was composed of; i) a five hundred litre cylindrical container (Hawke Bros Pty. Ltd.) filled with OviFlow Biological Filter medium, ii) a foam fractionator (Aquatic Technologies, Perth W.A.) supplied with ozone (Ozotech Inc.) and a heat exchanger maintaining the water temperature close to 19 ± 0.2ºC. Plastic crates (Nally Lug Box 610*419*312, Viscount Plastic, Canning Vale W.A.) were placed on their sides in the tank to provide shelter for the animals. The tank was covered by a black plastic tent to reduce ambient light levels and visual disturbance. During the holding period, the animals were fed twice weekly with fresh mussels (*Mytilus* sp.). Water temperature, ammonia, nitrate, nitrate, dissolved oxygen, salinity, and pH were monitored daily during the holding period.

6.2.2.2 Sampling of lobsters

Samples of 14 animals were selected at random for study prior to introduction to the tank (day 0) and after 2, 4, 8, 15, 22, 29, 39, 43, 50, 57 and 67 days, respectively. The sampling procedure, initial measurements and sample storage, and hemolymph chemistry assays were conducted as described above (see Section 6.2.1)

6.3 Responses during postharvest handling (Sub-objective 2)

6.3.1 Collection of samples

The objective was for the catches of three boats to be studied on three occasions (January 1997, April 1997, and June 1997).

Hemolymph samples were taken from groups of 15 ungraded red 'A' lobsters from each boat at the following times:

1) Immediately following pot retrieval (*Pot*).
2) Upon arrival at the processing factory jetty and after 1 - 6 h storage in onboard holding tanks (*Jetty*).
3) After "sham" grading (45 min. air exposure with disturbance) at the factory (*Belt*).
4) After 24 h storage in live holding tanks in the factory (*24 h*).
5) After 48 h storage in live holding tanks in the factory (*48 h*). While handling methods may vary between facilities, the above description is generally true for most processors.

The catch of two boats (A, B) from the pots, following storage in the deck tanks and following sham grading, were studied on three occasions during the season, approximately 2-3 months apart (January, April, and June 1997). The catch of a third boat, Boat C was studied during the April and June trips. The third boat on January trip (now Boat F) was no longer available during these later trips.

When available, extra lobsters from these boats were also taken, usually from the previous or following days catch, at random from the belt, and placed in baskets in the seawater tanks for one and two days recovery. The idea at this stage was to see what levels of parameters occurred in lobsters that had been in captivity for at least a day, to see if they would be at baseline levels, and not to bias the samples by grading out the weak lobsters.

In January, the one and two day samples were obtained from a preceding days catch for boat A. Arrangements for the other one and two day samples had already been made with skippers of two additional boats (here known as Boats D and E), but these had to withdraw from the trial, necessitating a change to boats B and F for the pot/jetty/ belt part of the study.

During the more typical, April field trip, boat F was no longer available so additional boat (here called C) was drawn into the study. Lobsters were kept aside from all three boats (A,B and C) to be able to keep a sample from the particular day's catch and judge the actual recovery of that catch, following the belt treatment, after one day in the tank. Another day's catch from these boats had to be used for the two day sample.

In June, both the one and two day recovery samples came from different fishing days for the boats A,B and C.

A follow up trip was arranged to for December 1998 (the so-called 'whites' run), though only boats A and B were available to participate.

All hemolymph samples (2.5 mL) for this experiment were withdrawn from the base of one of the 5th walking legs of each animal using a syringe and hypodermic needle, instead of from the pericardial sinus.

The animals were subjected to a visual assessment with reference to their vigour (VI) (Spanoghe and Bourne 1997) and to a general examination, with data such as size, gender, missing appendages and the occurrence of shell damages being recorded. A pleopod tip was taken from each lobster for moult stage determination (see Section 6.8).

6.3.2 Initial measurements and sample storage

The needle was removed from the syringe and the bulk of the hemolymph sample was added to a pair of numbered 1.5 mL microcentrifuge vials. The protein concentration of whole hemolymph was measured immediately from the syringe as refractive index, using a either a UR-2 or Shibuya S-1 hand held refractometer, (see Section 6.9.2). As the very first field trip (January 1997) preceded the adoption of this method, no protein data was obtained from that trip. The pH of the hemolymph sample in one vial was measured immediately at ambient temperature using a pH meter (TPS WP 81) connected to a temperature compensated electrode (TPS IJ42).

Using an automatic pipette, a 250 µL sub-sample of whole hemolymph was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1M perchloric acid (PCA), to precipitate protein. The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and then frozen in liquid N_2 for later analysis.

6.3.3 Hemolymph chemistry assays

Lactate and glucose concentrations were assayed in neutralised perchlorate extracts. Serum was prepared from whole hemolymph samples transferred to microcentrifuge tubes and stored at -20ºC before analysis. Serum samples were analysed for chloride, sodium, calcium, potassium and magnesium. For details of sample handling and assays conducted refer to the section of the methods entitled 'Preparation and analysis of hemolymph samples.'

6.3.4 Statistics

All samples were compared by ANOVA. If ANOVA yielded a significant result, differences between sample means were identified using Dunnett's test ($\propto = 0.05$). All results are presented as means 1 ± s.e.m.

6.4 Responses to controlled emersion (Sub-objective 3)

6.4.1 Collection and storage of lobsters

Four hundred animals (both sexes, mean carapace length (CL) = 78.0 ± 0.1 mm) were used. Animals were purchased from local commercial suppliers in groups of 100 and were transported in air to the laboratory. Animals were placed in a 6000 L recirculating seawater system (20ºC, 12h light/12 h dark) for 3 weeks to acclimate. During acclimation the animals were fed twice weekly with chopped fish and fresh mussels (*Mytilus edulis*). After the initial acclimation period animals were taken from the recirculating system and a small hole was drilled through the dorsal cuticle overlying the pericardial sinus of each animal. After drilling the holes animals were transferred to 3 identical recirculating seawater systems each comprised of 8 glass aquaria housed in a controlled temperature room (20ºC, 12h light/12 h dark). Each aquarium contained 4 oyster mesh baskets. Individual lobsters were placed in each of the baskets where they were left to acclimate for a further 7 days. Feeding was discontinued 3 days before the end of the acclimation period. On the 6th day of the acclimation period animals in 2 randomly selected tanks were sampled to provide pre-treatment baseline values.

6.4.2 Experimental protocol

On the 7th day of the acclimation period animals from a further 2 randomly selected tanks were sampled to demonstrate that a stable baseline had been reached. Immediately following the second pre-treatment sampling, 10 of the remaining 20 tanks were drained of water, emersing the lobsters. One hour after draining the tanks, one tank of emersed animals (treatment) and one tank of submerged animals (control) were sampled as above. This process was repeated at 3, 12 and 24 h after emersion. After 24 h of exposure to air the remaining 6 tanks of emersed lobsters were refilled with aerated seawater. Pairs of tanks of treatment and control lobsters were sampled at 1, 3, 8, 12, 24 and 48 h after reimmersion. By this method one replicate of the experiment yielded n = 4 for each sample time. The experiment was repeated 3 times to give a final sample size of 8 - 12 for each sample time. All animals used were judged to be in Stage C of the moult cycle (Lyle and McDonald 1983).

6.4.3 Hemolymph sampling

To sample an animal, approx. 1 mL of post-branchial hemolymph was obtained for acid-base and respiratory gas analysis by carefully inserting a 23 ga hypodermic needle into the pericardial sinus via the hole in the dorsal cuticle. This was done while the animals were still underwater and could usually be achieved without disturbing the animals. Each animal was then removed for the basket and a sample of pre-branchial hemolymph (~ 1 mL) was obtained for gas analysis from the sinus at the base of the gills via the arthrodial membrane between the base of the 3rd and 4th legs. The position and approximate dimensions of this sinus were determined by corrosion casting with Batson's #17 methacrylate casting medium (unpublished data).

A second hemolymph sample (~2 mL) was then taken from the pre-branchial site for later analysis for various metabolites and electrolytes (see 6.4.5). A sample of abdominal flexor muscle was taken by flexing the abdomen and inserting a Mdtech Ultra-cut automated core biopsy instrument (14 ga x 10 cm) into one of the large abdominal flexor muscles via the arthrodial membrane between the terga of the 2nd and 3rd abdominal segments. This tissue sample was immediately frozen in liquid nitrogen for later extraction and analysis of lactate and adenylate nucleotide levels (ATP,ADP and AMP).

Immediately after sampling, the animals were subjected to a visual assessment with reference to their vigour (VI) (Spanoghe and Bourne 1997) and to a general examination, with data such as size, gender, missing appendages and the occurrence of shell damages being recorded. A pleopod tip was taken from each lobster for moult stage determination (see Section 6.8).

6.4.4 Hemolymph gas measurements

The pH of pre- and post-branchial hemolymph was determined on each sample using a Radiometer and thermostatted (20ºC) G299A glass capillary pH electrode connected to a TPS 1952 programmable ion analyser. The hemolymph was aspirated directly into the electrode from the syringe after first

removing the needle. Syringes of hemolymph delivered to the hemolymph gas equipment that could not be assayed immediately because measuring a previous sample was in progress were kept on ice to retard clotting.

Immediately after filling the pH capillary electrode with a fraction of a given sample, the partial pressures of oxygen and total carbon dioxide content in the remaining sample was determined simultaneously. For measuring oxygen partial pressure, part of the hemolymph was injected into a Strathkelvin MC 100 thermostatted (20ºC) cell housing a 1302 Po_2 oxygen electrode connected to a Strathkelvin 781B meter. Total carbon dioxide values of hemolymph samples were determined (Cameron 1971) using a Radiometer E5037 Pco_2 electrode housed in a custom built thermostatted (40ºC) glass cell and connected to an Orion Research EA940 expandable ion analyser. HCO_3^- concentrations and Pco_2 of samples were calculated using rearrangements of the Hendersen-Hasselbach equation. Values of the constants determined for *Carcinus maenas* at the appropriate salinity and temperature were used (Truchot 1976).

6.4.5 Other initial measurements and sample storage

The needle was removed from the syringe and the bulk of the hemolymph sample was added to a pair of numbered 1.5 mL microcentrifuge vials. The protein concentration of whole hemolymph was measured immediately from the syringe as refractive index, using a Shibuya S-1 hand held refractometer, (see Section 6.9.2).

Using an automatic pipette, a 250 µL subsample of whole hemolymph was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1M perchloric acid (PCA), to precipitate protein. Using another automatic pipette, a solution of 50 µL of whole hemolymph from each lobster was diluted in 950 µL of distilled water in a third numbered 1.5 mL vial for later estimation of hemocyanin concentration (see Section 6.9.3).

The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and then frozen in liquid N_2 for later analysis.

6.4.6 Hemolymph chemistry assays

Lactate and glucose levels were determined later from the deproteinated samples using spectrophotometric kits. Serum was produced from the whole hemolymph and used to establish levels of calcium, magnesium and potassium. For details of sample handling and assays conducted refer to section 6.10.

6.5 Validation of indicators (pilot study) (subobjective 4)

The objective of the experiment was to relate hemolymph chemistry of individual lobsters to their fate when stored in the factory. The nature of the stress applied, and exactly what factors lead to stress in western rock lobsters could not be adequately studied in this trial. A handling regime was chosen that was likely to stress lobsters but without leading to unusually high levels of mortality. Rather than reflecting practices in a given factory, it was decided instead to adopt a treatment that was at the far end of the existing spectrum of commercial handling, to maximise the chances of seeing changes in a range of possible stress indicators

6.5.1 Experimental protocol

Lobsters arriving directly from 6 lobster boats at a rock lobster factory at Geraldton, Western Australia, left in baskets in the pack-out shed for 1 h and then placed in a spray truck module, typical of many used for post-harvest road transportation of lobsters, for four hours. The air temperature in the pack-out shed was initially 39°C, cooling toward the afternoon and evening, and the thermostat of the spray truck module was set to 15°C.

The simulator used was an insulated truck-mountable module with sealed rear opening doors that was cooled by forced-draft chiller refrigeration. Seawater was sprayed via a manifold of garden irrigation micro-sprays through the interior of the module and drained into a sump underneath the module. The sump contained a submersible pump to recirculate the small amount of water used by the module back through the sprays.

An experienced grader, employed by the factory, graded the lobsters upon removal from the simulator, and then hemolymph samples were taken from equal numbers of accepted and rejected lobsters. Lobsters rejected because of physical injury at this stage were not sampled. Following sampling, the lobsters were tagged, to allow later identification of individuals, using coloured, individually-numbered, livestock ear-tag anchored firmly to the distal segment of the antennal peduncle using cable ties (100 x 2.5 mm). The trailing end of the cable tie was cut away using small pliers.

The lobsters were then placed in one of the factory's storage tanks and allowed to recover in free-flowing seawater and their fate followed for up to 8 days. The tank was monitored in the course of the factories routine operation and any lobsters that died ('Dead in tank') or weakened in the tank ('Weak in tank') were removed and their tag numbers recorded. After 8 days, the remaining lobsters were graded for simulated live export. Lobsters were either rejected as weak ('Weak at packing') or because of leg-loss ('Leg-loss at packing) or categorised as acceptable for live export ('Packed-out').

The lobsters were packed as if for export but were kept for 38 hours and then unpacked, and the outcome of simulated transport followed for 48 hours.

The experiment was repeated on the following day using lobsters freshly caught from four of the same boats sampled on the previous day. As the lobsters were very lethargic during handling on the first day, the regime was made less extreme on the second day by using the air-conditioning (25°C) in the packing shed, and increasing the thermostat of the spray truck module to 17°C.

Immediately after sampling, the animals were subjected to a visual assessment with reference to their vigour (VI) (Spanoghe and Bourne 1997) and to a general examination, with data such as size, gender, missing appendages and the occurrence of shell damages being recorded. A pleopod tip was taken from each lobster for moult stage determination (see Section 6.8).

6.5.2 Initial measurements and sample storage

The needle was removed from the syringe and the bulk of the hemolymph sample was added to a pair of numbered 1.5 mL microcentrifuge vials. The protein concentration of whole hemolymph was

measured immediately from the syringe as refractive index, using a Shibuya S-1 hand held refractometer, (see Section 6.9.2).
Using an automatic pipette, a 250 µL subsample of whole hemolymph was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1M perchloric acid (PCA), to precipitate protein. The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and then frozen in liquid N_2 for later analysis.

6.5.3 Hemolymph chemistry assays

Lactate and glucose levels were determined later from the deproteinated samples using spectrophotometric kits. Serum was produced from the whole hemolymph and used to establish levels of calcium, magnesium and potassium. For details of sample handling and assays conducted refer to section 6.10.

6.5.4 Statistics

Initially, ANOVAs were performed to seek significant differences between categories of lobsters. A step-wise discriminant function analysis was then conducted (Statistika, Version 5.5, StatSoft, Inc. Tulsa, Oklahoma, USA). Individual lobsters were then re-classified into groups on the basis of posterior probabilities calculated from the squared Mahalanobis distance from group centroids (Klecka 1980).

6.6 Validation of indicators including a test of alternative environments- November 1998 (sub-objectives 4 and 5)

6.6.1 Collection and storage of animals

Pink A sized jetty lobsters (approx. 445 g wet weight) that had been held in the factory for 24 h were used in this study. The 24 h period of acclimation in factory live tanks had the added benefit of standardising the condition of the lobsters prior to them being used in the experiment. The animals were fed with chopped fish (pilchards) the night before being used in the experiments, to simulate feeding on baits in pots that would normally occur in freshly caught lobsters.

6.6.2 Experimental protocol

6.6.2.1 The storage environments

The experimental treatments were set up in five insulated cubicles constructed for the purpose at a cooperating lobster factory (Geraldton Fisherman's Cooperative). The basic approach was to stress lobsters for a given period, sample hemolymph from each before tagging them and returning them to the factory so that the tag numbers of lobsters which were later found weak or dead by tank monitors could be tallied against hemolymph test results.

Rather than simply providing for a single treatment as used in the pilot trial (above) the cubicles were arranged to provide the following five storage treatments.

Treatment 1: Submerged-flowing controls
Treatment 2: Submerged-recirculated
Treatment 3: Moist air (100% RH)
Treatment 4: Spray-flowing
Treatment 5: Spray-recirculated (same lobster/water ratio as 2)

For the submerged-flow through cubicle (Treatment 1) the two baskets of lobsters (about 10.5 kg each) were placed in a black plastic tank, similar to a common style of tank used on lobster fishing boats. To raise the stocking density, unoccupied volume in the tank was displaced using a water-filled plastic container. Seawater continually flowed into the tank (10 L/min) through a manifold at the bottom of the tank and overflowed to waste. Aeration was provided via an air-stone to assist oxygenation.

The submerged-recirculated cubicle (Treatment 2) was identical to treatment 1 except that instead of the seawater flowing continuously into the tank containing two baskets of lobsters from the ocean inflow, a closed circuit was established after filling the tank. The aim of this treatment is to see, by comparing with treatment 1, whether recirculating the water and allowing water quality to deteriorate would influence condition and survival. Flow rate was matched to that of treatment 1. To recirculate the water, overflow draining from the cubicle was collected in a small sump and returned to the lobster tank in the cabinet using a submersible bilge pump. The lobster tank was aerated to ensure adequate oxygenation. The ratio of lobster:water was maintained at 21 kg/ 79 L.

In the moist air cubicle (Treatment 3), the two baskets of lobsters were kept in the cabinet and the atmosphere was kept humid by bubbling air through a bucket of water inside the cubicle. The aim of this treatment was to see, by comparing with treatment 1, what condition and survival would ensue if the lobsters were kept in air but not sprayed.

For the spray-flow through cubicle (Treatment 4), two baskets of lobsters were placed in the cubicle, as in treatment 3, but flowing seawater was sprayed over the lobsters using an irrigation spray head. The aim of this treatment was to see, by comparing with treatment 1, whether the manner of delivering flowing seawater to the lobsters influenced their condition and survival. Early checks were

made to ensure that the spray coverage was effectively covering the baskets. The water flow rate was matched to that of treatment 1.
The spray-recirculated cubicle (Treatment 5) was similar to treatment 4 in that two baskets were placed in the cubicle. The same ratio of lobster weight to water volume was used as in treatment 2. However, unlike this other recirculated treatment, the sump had to accommodate the bulk of the water since the lobsters were not stored in a tank. The water flow rate was matched to that of treatment 1. The aim of this treatment was to see, by comparing with treatment 4, whether recirculating the sprayed seawater influenced the lobsters' condition and survival.

6.6.2.2 Application of stress

On the morning of the experiment, approximately 21 kg of lobsters (about 46 individuals) were weighed out and were placed in each treatment cubicle (above) in pairs of plastic mesh baskets for six hours. This required about 105 kg of lobsters, or approximately 230 individuals. This quantity included enough for 20 lobsters to be sampled from each of the treatments, and for an additional 20 lobsters in each treatment to act as controls for the hemolymph sampling process. About half a dozen spare lobsters were included to compensate for occasions were lobsters died in the treatment or were too damaged to sample.
Because of the time taken to prepare each set of lobsters and to sample and handle the lobsters following treatment, the beginning and end of each treatment was staggered by hourly intervals.
All treatments were maintained at the ambient seawater temperature (22º C) at the time the experiment was conducted. The temperature in each treatment was monitored using digital thermometers. Maintaining temperature was simple in treatments 1 and 4, which received the seawater inflow from the live lobster factory. The temperature of the humid air treatment was controlled by altering the temperature of the water used to humidify the incoming air. Temperature tended to increase in the recirculated seawater treatments (no. 2 and 5) due to heating by the pumps. This was countered by periodically adding sealed bottles of frozen water to the sump of the pump. These were replaced as they thawed.
Water quality (DO, pH) was checked at hourly intervals during the storage period, using hand-held meters and electrodes. At each sample time a water sample was also taken and refrigerated for later determination of ammonia using the Berthelot method, (Varley 1967)

6.6.2.3 Grading, sampling and storage after the treatments

After 6 hours had elapsed for each treatment, the cubicle was opened and the animals inside were graded by an experienced factory grader into dead, damaged, weak and accepted categories. Any lobsters in the “dead” category that showed a heart beat upon further examination were transferred into the weak category for the purposes of hemolymph sampling. Half of the sample of weak/rejected lobsters had hemolymph samples removed from them (see Section 6.6.3).
Immediately after sampling, the ‘reject’ lobsters were subjected to a visual assessment with reference to their vigour (VI) (Spanoghe and Bourne 1997) and to a general examination, with data such as size, gender, missing appendages and the occurrence of shell damages being recorded. A pleopod tip was taken from each lobster for moult stage determination (see Section 6.8).
The sampled lobsters were then tagged, to allow later identification of individuals, using coloured, individually-numbered, livestock ear-tag anchored firmly to the distal segment of the antennal peduncle using cable ties (100 x 2.5 mm). The trailing end of the cable tie was cut away using small pliers.
The remainder of the lobsters required for hemolymph sampling was made up to a sample size of 20 using accepted lobsters. These lobsters were also assessed for sex, moult stage and vigour and then tagged, (see Section 6.8).
Note that the initial intention was to sample equal numbers (10) of rejected and accepted lobsters from each treatment. This approach was used in an earlier pilot trial to examine differences between the

two groups. As the experiment proceeded it became apparent that this would not always be possible. In some treatments the number of rejected or accepted animals was less than the required number. This practice was discontinued in the next trial (see Section 6.7) because it compounds the effects of the treatments themselves and complicates interpretation of the results.
The other half of the group of weak/rejected lobsters from each treatment plus sufficient unsampled accepted lobsters to bring the total to 20 from that treatment were kept aside. These animals were not tagged, and they are therefore controls for the effects of hemolymph sampling and tagging.

6.6.2.4 Monitoring during recovery

After sampling, the tagged and un-tagged control lobsters were returned to a tank in the factory. Tank monitors were kept away from the tank for several hours to prevent them rejecting lobsters that were temporarily fatigued by the sampling procedure.
The experiment using the five treatments was replicated on 3 consecutive days, such that the total number of lobsters sampled was 300. The lobsters emerging from each day of tests was assigned to a separate tank in the factory. The lobsters were left in these tanks for 7 days after the day of the last experiment. During this recovery period, tank monitors removed moribund and dead animals. All animals removed by tank monitors were weighed to allow tallying with the factory of the original quality of lobsters used. The tag number was recorded in order to identify the corresponding hemolymph samples.

6.6.2.5 Simulated load-out
All lobsters (tagged and untagged) were then packed as if for commercial export and kept aside for 36 hours. The number of untagged, and the identification of any tagged lobsters found dead upon unpacking was recorded. The surviving animals were unpacked and placed in a factory tank to recover for 24 h. Particulars of any animals dying during this period were also recorded. After the 24 h recovery period, the surviving lobsters were examined and a tally of tag numbers associated with survivors was made.

6.6.3 Hemolymph sampling and initial measurements

Two hemolymph samples were drawn from the pericardial cavity of each animal. A 2.5 mL sample was drawn for analysis of hemolymph constituents. The hypodermic was removed and the bulk of the hemolymph sample was added to a pair of numbered 1.5 mL microcentrifuge vials. The protein concentration of whole hemolymph was measured immediately from the syringe as refractive index, using a Shibuya S-1 hand held refractometer, (see Section 6.9.2).
Using an automatic pipette, a 250 µL subsample of whole hemolymph was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1 mol L^{-1} perchloric acid (PCA), to precipitate protein. The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and then frozen in liquid N_2 for later analysis.
The second sample (1 mL) was drawn using an ice cold syringe, and stored on ice prior to analysis of the enzymatic activity of the hemolymph.

6.6.4 Hemolymph chemistry assays

Lactate and glucose levels were determined later from the deproteinated samples using spectrophotometric kits. Serum was produced from the whole hemolymph and used to establish levels of calcium, magnesium and potassium. For details of sample handling and assays conducted refer to section 6.10.

6.7 Validation of indicators including a test of alternative environments- March 1999 (sub-objective 4 and 5)

6.7.1 Collection and storage of animals

Pink A sized jetty lobsters (approx. 445 g wet weight) that had been held in the factory for 24 hours were used in this study. As described for the November study, the lobsters were preconditioned to simulate feeding on a bait the night before the experiment.

6.7.2 Experimental protocol

6.7.2.1 The storage environments

The experimental treatments were set up as before in five insulated cubicles as described above (Section 6.6.2.1). The only difference was that new spray heads were used that gave a better coverage of the lobsters.

Treatment 1: Submerged-flowing controls
Treatment 2: Submerged-recirculated
Treatment 3: Moist air (100% RH)
Treatment 4: Spray-flowing
Treatment 5: Spray-recirculated (same lobster/water ratio as 2)

These treatments have been described above (See Section 6.6.2.1).

6.7.2.2 Application of stress

This replicate is identical to the preceding study, except that the ambient seawater temperature was now higher (26°C). Water quality was monitored as before. Temperature control arrangements to counter pump heating were similar. However this time, the ambient air temperature in the room was lower than the seawater temperature, meaning that we had to heat the humid air treatment. We attempted to do this using hot water in the bucket being used to maintain humidity.

6.7.2.3 Grading, sampling and storage after the treatments

As in the earlier trial, after 6 hours had elapsed for each treatment, the cubicle was opened and the animals inside were graded by an experienced factory grader into accepted, rejected and damaged lobsters. This time, however, rejected lobsters were not favoured during the sampling protocol. Half of all rejected lobsters had hemolymph samples removed from them (see section 6.7.2.6) and half of all accepted lobsters had hemolymph samples taken. The unsampled lobsters remained as controls for the sampling and tagging process. Following sampling, the sex, moult stage and vigour stage of each animal was determined (Section 6.8) and the lobsters tagged as before.

6.7.2.4 Monitoring during recovery

After sampling, the tagged and untagged control lobsters were returned to a tank in the factory and monitored as for the previous trial, identifying lobsters that became dead and moribund. Again, the experiment using the five treatments was replicated on 3 consecutive days, with each day getting its own tank.

The lobsters were left in these tanks for 7 days after the day of the last experiment and then loaded out as follows.

6.7.2.5 Simulated load-out

In a departure from the previous trial, all lobsters (tagged and untagged) were put into the commercial packing shed at the factory and packed. Lobsters rejected by the staff as unsuitable for export were kept aside and also packed in marked boxes.
Hereafter, a similar procedure was followed as used in section 6.6.2.5. The boxes were kept aside for 36 hours, mortality assessed first at unpacking and then after 24 hour recovery before recovering tags from the survivors.

6.7.2.6 Hemolymph sampling and initial measurements
Two hemolymph samples were drawn from the pericardial cavity of each animal. A 2.5 mL sample was drawn for analysis of hemolymph constituents. The hypodermic was removed and the bulk of the hemolymph sample was added to a pair of numbered 1.5 mL microcentrifuge vials. The protein concentration of whole hemolymph was measured immediately from the syringe as refractive index, using a Shibuya S-1 hand held refractometer, (see Section 6.9.2).
Using an automatic pipette, a 250 µL subsample of whole hemolymph was drawn from each vial of whole hemolymph and added to duplicate vials containing 500 µL of 1 mol L^{-1} perchloric acid (PCA), to precipitate protein. The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and then frozen in liquid N_2 for later analysis.
The second sample (1 mL) was drawn using an ice cold syringe, and stored on ice prior to analysis of the enzymatic activity of the hemolymph.

6.7.3 Hemolymph chemistry assays

Lactate and glucose levels were determined later from the deproteinated samples using spectrophotometric kits. Serum was produced from the whole hemolymph and used to establish levels of calcium, magnesium and potassium. For details of sample handling and assays conducted refer to section 6.10.

6.8 Biological information

Carapace length (mm) was measured using plastic calipers. Sex of lobsters was established by inverting lobsters and establishing the location and shape of reproductive organs. In order to identify moult stage, the tip of one abdominal pleopod was removed and placed in a vial containing 3% NaCl solution. This pleopod edge was staged using published descriptions (Lyle and McDonald 1983). The vigour of each animal was assessed, (Spanoghe and Bourne 1997). By this method animals are ranked on a scale from 0 - 5, with 5 being the most vigorous and 0 being a dead animal.

6.9 On the spot measurements of hemolymph

6.9.1 pH

The pH of fresh hemolymph was measured immediately after sampling by first dispensing the sample into a 1.5 mL microcentrifuge tube (supported in a microcentrifuge tube rack). The tapered glass bulb of a 'spear-tip' pH electrode (TPS IJ42) was then inserted into the vial. Care was taken to ensure that there was sufficient volume in the microcentrifuge to wet the potassium junction of the electrode. The results were displayed on a portable pH meter (TPS WP-81).

The results obtained using this technique are adequate for field measurements but the technique is not ideal. For example, carbon dioxide can diffuse out of open hemolymph samples during handling and dispensing. For this reason, hemolymph gas and pH measurements are normally conducted in the laboratory in an 'anaerobic' or 'gas-tight' method using an acid-base analyser such as the Radiometer BMS 3 Mk 2 (see section 6.4.4), that is, preventing contact between the sample being measured and the atmosphere. We took the opportunity during the program of hemolymph gas measurements in section 6.4 to compare the field technique (spear electrode) to the results obtained from duplicate samples using the Radiometer G299A microcapillary pH electrode. Contrary to the theoretical objections to the technique, the results obtained using the two methods agree surprisingly well (r^2=0.621, n=86), with no evidence of a systematic error in either direction caused by the crude sample handling methods of the field technique.

6.9.2 Total protein concentration (refractive index)

The refractive index of the hemolymph was converted to total protein concentration using a calibration obtained from the refractive index of hemolymph samples of known protein concentration (by Biuret method, using bovine serum albumen standards) (Paterson et al in press) (see Appendix 2).

6.9.3 Estimation of hemocyanin concentration

Hemocyanin concentration was estimated by measuring the absorbency at 340 nm of a 1/10th dilution of hemolymph in aerated distilled water (Hagerman 1983), using a Shimadzu UV 120-02 UV/Visible spectrophotometer.

We initially expected to convert these absorbances to concentration units (g L^{-1}) by applying an extinction coefficient determined for another crustacean species. However after considering the data (Nickerson and Van Holde 1971), it was apparent that the only criterion for selecting a particular coefficient was that it did not result in any values of hemocyanin: total protein greater than 1. This seemed somewhat artificial, hence the measure of hemocyanin is reported as absorbance units only.

Application of published extinction coefficients produced occasions when an 'impossible' outcome resulted, namely a hemolymph hemocyanin level that exceeded the total protein level.

6.10 Preparation and analysis of hemolymph samples

6.10.1 Preparation of serum and neutralised perchlorate extracts

6.10.1.1 Serum

Serum was prepared from whole hemolymph samples and allowing the samples to clot, then the clot was removed with a needle. After centrifugation (9000 rpm, 4ºC, 10 min.) the serum was transferred to microcentrifuge tubes for storage at -20ºC. Usually approximately 30% of the original sample volume was recovered as serum.

6.10.1.2 Neutralisation of perchlorate extracts

Acid extracted samples were centrifuged at 9000 rpm at 4ºC for 10 min. 500 µL of supernatant was removed and neutralised with 70 µL of 3 M KOH. Neutral perchlorate extracts (NPE) were stored at -25 ºC for subsequent analyses.

6.10.2 Analytical techniques

6.10.2.1 Lactate

Lactate was measured enzymatically using a Boehringer Mannheim kit (cat. no. 139 084) based on the LDH/GPT method (Noll 1974). This method is not subject to the problems with unstable end-points reported with the alternative LDH/hydrazine method (Engel and Jones 1978; Gutmann and Wahlefeld 1974). All blanks and standards were prepared as NPE.

6.10.2.2 Glucose

Glucose was measured enzymatically using an Instrument Laboratories Multistat III analyser and the Roche Unimate 5 glucose HK kit. All blanks and standards were prepared as NPE.

6.10.2.3 Serum electrolytes

Serum samples were analysed for calcium and magnesium using Trace Arsenazo III and Calmagite kit methods respectively, with an Olympus model AU500 autoanalyser while sodium and potassium was determined by atomic absorption spectrophotometry using a Varian AA-40 AAS. Cesium chloride was added to all samples to achieve a final concentration of 1000 mg L^{-1}. Chloride concentration was determined using a Corning model 925 chloride analyser.

6.10.3 Preparation and analysis of tissue samples

Samples of abdominal flexor muscle in PCA were defrosted at 4ºC before being homogenised on ice using an Ultra Turrax T25 homogeniser and centrifuged (10 000 rpm, 10 min. at 4ºC). Neutralised perchloric extracts of tissue homogenates were prepared on ice by adding a known volume of 3 molar KOH to 1000 µL of supernatant. Neutalised extracts were stored at -20ºC prior to analysis. Analysis of lactate concentration proceeded as for hemolymph extracts.

Muscle concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inosine monophosphate (IMP) in NPE of muscle were determined using reverse phase HPLC. The composition of the buffer used was as follows: 60 mM $NH_4H_2PO_4$, 5 mM Tetrabutylammoniumphosphate (TBAP), pH = 5.0. Two mobile phases were used during the elution: mobile phase A = 95% buffer, 5% methanol; mobile phase B = 65% buffer, 35% methanol. The following schedule was followed during the elution:

0 min 100% A
20 min. 100% B
27 min 100% A

Separation was performed using a C18 Column (Altech Cat. No. 28841, 250 x 4.6 mm Adsorbosphere) connected to a Waters 600 controller and Waters 486 tunable UV detector. Detector output was plotted using an HP 33967 series II integrator. The system was calibrated using standard solutions containing known concentrations of adenylates in buffer. Standards were prepared fresh daily.

Measured concentrations of the adenylates ATP, ADP and AMP were used to calculate the adenylate energy charge (AEC) of the muscle tissue (Atkinson 1977).

$$AEC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

6.11 Statistical analysis

All general statistical tests (eg. analysis of variance) were performed using a selection of software packages.

- JMP V. 3.2.2 (SAS Institute Inc. Cary, NC, USA).
- Statistix for Windows, Version 1.0 (Analytical Software, Tallahassee, Florida USA)

General discriminant analysis was conducted initially using Genstat 5, Release 4.1 (Lawes Agricultural Trust, Rothamsted Experimental Station). Statistica '99 version 5.5A (StatSoft, Inc. Tulsa, Oklahoma USA) was used for all stepwise discriminant function analyses, including reclassification of lobsters and prediction of group membership.

7 RESULTS AND DISCUSSION

7.1 Characterise the baseline physiology of free-ranging and captive lobsters (Sub-objective 1)

7.1.1 Summary

A lobster is stressed when its internal physiology deviates measurably from that of a resting, undisturbed or 'baseline' lobster. Before proceeding to describe stressed lobsters, the first step of this project was therefore to establish what lobsters are normally like.

Clearly, the first place to look for normal lobsters was in their natural environment, on the sea floor. We used SCUBA to obtain hemolymph samples from lobsters at a number of locations and at different times of year (for methods see section 6.1). This ground-breaking work showed that it was possible to obtain baseline measurements from submerged lobsters and gave some insights into the magnitude and causes of variation in these parameters as well as the effects of moulting. The disturbance associated with capture and sampling effected the levels of some parameters, particularly lactate.

After determining baseline values in the wild (Table 1, page 24), the next area to consider was whether lobsters that were brought into captivity recovered back to normal. Knowing this last point is an important pre-requisite for the experiments on captive lobsters planned later in the study (see Section 7.3). After a preliminary trial at Geraldton (see section 7.1.3), we obtained lobsters from Fisheries WA and installed them in a partially recirculating seawater system at Muresk Marine Laboratory in Fremantle, and showed that they did indeed recover in captivity to baseline levels for key parameters (section 7.1.4). The lobsters on arrival showed a hemolymph physiological profile expected of handling and disturbance (covered in some detail later, in section 7.2) but recovered quickly to baseline levels. And notwithstanding the fact that lobsters generally remained at baseline when held in the laboratory, there were still fluctuations in some parameters that are worthy of comment.

One case of note was a temporary rise in hemolymph glucose. Though not in itself statistically significant, this peak coincided with the peak in nitrite level during establishment of the biological filter. Glucose is a known stress indicator in crustaceans (Telford 1968), and therefore, the biological significance of this trend is cause for some concern. Certainly, it would be important to see if this effect recurs in other cases were lobsters encounter rising nitrite levels in holding systems.

The other case of observed variation was a rise in average hemolymph calcium, magnesium and protein concentration in intermoult lobsters (stage C) during the first 39 days of the trial, followed by a fall in these parameters. Curiously, these very parameters change in concert during the crustacean moult cycle- suggesting that moulting is somehow leaving a signature in the hemolymph chemistry of intermoult lobsters in the tank. Little information on the changes in hemolymph chemistry during the moult cycle of *P. cygnus* have been obtained during this study, because of the general rarity of non-intermoult lobsters.

The change in calcium found here may simply be the cumulative effect of moulting during the sampling program. Firstly, the concentrations of these hemolymph constituents may rise toward the later stages of intermoult, accounting for the observed rise in the average. Lobsters that moult then leave the population of intermoult lobsters with high levels of these parameters and eventually re-appear in the 'intermoult' category with low levels of these parameters, accounting for the later fall in average concentration. This suggests that averages for a category like 'intermoult' lobsters may reflect changes in the rate at which lobsters enter and leave the category (the relative proportions of early and late intermoult) as much as actual changes in hemolymph chemistry of individuals.

This information provided us with benchmark for a number of biochemical hemolymph parameters in *P. cygnus*, the basis from which to address the next sub-objective; to establish if particular parameters were deviating as a result of various aspects of post-harvest handling (Section 7.2). Yet, in itself it has provided a number of interesting insights in long-term acclimation of rock lobsters, and the kinds of changes that might be seen in their hemolymph chemistry when attempting to interpret changes in

'condition.' More information is needed on the effects of moulting on hemolymph chemistry. Because of the difficulty of finding pre- and post-moult lobsters in the field, this work is best organised in the laboratory.

The rest of this section examines the baseline findings both for the seafloor sampling of lobsters and acclimation to captivity. The results of each of the acclimation studies are presented, the initial 42 day study at Geraldton and the later 67 day trial at Fremantle.

7.1.2 Baseline physiology of free-range lobsters

7.1.2.1 Success of the methodology

To our knowledge this is the first time that underwater sampling of spiny lobsters has been attempted, and this kind of sampling has only been attempted for a few large aquatic crustaceans (Houlihan and Mathers 1985).

Until this study, the best estimates of the physiological profiles of lobsters in the wild were obtained from animals immediately following capture in pots. The difficulty with using pot-caught animals to estimate undisturbed levels of parameters is that the effect of confinement in pot for periods of up to 12 h is unknown. In particular, animals confined in pots are susceptible to attack by predators, such as snapper (*Pagrus auratus*) or octopus (*Octopus tetricus*). Having said this, the profiles of free-ranging intermoult lobsters sampled by divers were virtually identical to those of intermoult animals sampled on commercial fishing boats immediately following pot retrieval (see Section 7.2).

The additional benefit of using divers to sample lobsters is that the catchability of lobsters using baited pots varies over the moult cycle (Chittleborough 1970). This changing catchability is presumably due to cessation of feeding during certain stages of the moult cycle (Thomas 1966). Thus divers can sample a wider range of moult stages than is possible when sampling animals caught in pots. Nevertheless, getting more than a couple of premoults has still been difficult.

The underwater sampling procedure was demonstrated to be simple and effective. In all, 48 male and 38 female lobsters were sampled (mean carapace length = 74 ± 1 mm). Examination of magnesium concentrations suggests that samples were free from contamination by sea water. The magnesium concentration of sea water, at 50-60 mmol L^{-1}, (Dall 1974a), is approximately 5 times higher than that of the hemolymph, thus sea water contamination would result in unusually high and variable hemolymph magnesium concentrations.

The results for each parameter are examined and factors influencing the results are discussed.

7.1.2.2 Baseline values in intermoult lobsters

7.1.2.2.1 Protein in hemolymph

Hemolymph total protein concentration in intermoult lobsters was extremely variable, ranging from 52 -148 g L^{-1}. This will become a familiar refrain throughout this report. The values of total protein reported here are generally similar to those measured previously in captive *P. cygnus* (Dall 1974b), however, the total protein concentration reported in that study for intermoult animals tended to be higher than in free-ranging animals in the present study. This difference is most likely due to the abundance of food available to captive animals, which were fed *ad libitum* (Dall 1974b).

7.1.2.2.2 Lactate in hemolymph

While hemolymph lactate was generally low in these lobsters (< 1.0 mmol L^{-1}, Table 1), the values we report here are somewhat higher than those measured later in resting lobsters in captivity, which were typically 0.3-0.5 mmol L^{-1} (see for example section 7.3). Lactate, a product of anaerobic metabolism, is readily released into the hemolymph of crustaceans as a result of exercise or oxygen deprivation (Albert and Ellington 1985; Phillips *et al.* 1977). The difference observed here might be attributable to both artificially low levels of spontaneous activity in captive semi-confined lobsters, or possibly to disturbance resulting from the divers taking several lobsters occupying the same crevice in the reef.

Here there is a clear case for interpreting 'baseline' results after examining both captive and wild lobsters.

7.1.2.2.3 Glucose in hemolymph

Hemolymph glucose concentrations in free-ranging intermoult lobsters were also low (0.1 - 0.5 mmol L^{-1}, Table 1). These values are consistent with those observed later in undisturbed *P. cygnus* in captivity (see sections 7.1.4.4, 7.3.5) and with those reported for unstressed decapods in general, (Telford 1974).

7.1.2.2.4 Serum electrolytes (calcium, potassium and magnesium)

Serum calcium and potassium concentrations from free-ranging intermoult lobsters (13.6 ± 0.2 and 10.3 ± 0.2 mmol L^{-1}, respectively, Table 1) were similar to values previously reported for whole hemolymph from captive intermoult western rock lobsters (~13.1 mmol L^{-1} and ~11.1 mmol L^{-1}, respectively) (Dall 1974a)). However, serum magnesium concentrations from free ranging intermoult lobsters (11.0 ± 0.3 mmol L^{-1}) were considerably lower than those reported previously for whole hemolymph of captive lobsters (~14.8 mmol L^{-1}) (Dall 1974a). The reason for this discrepancy is unknown, but it follows on from another discrepancy noted in total protein level, but this could simply be coincidental (see page 22). Clearly sea water contamination of the dive samples is not responsible as this would tend to elevate serum magnesium values.

7.1.2.3 Seasonal, geographical differences

Significant differences were found between dive trips in intermoult levels of lactate, magnesium, and potassium ($P<0.05$, Table 1). The reasons for these differences are unclear, but seasonal and geographical variation are two of the more obvious factors that may be influential. However, the small sample sizes preclude meaningful discussion of these results. It is sufficient to say that variation in levels of parameters was observed and should be recognised in future studies.
Glucose, calcium in intermoult lobsters, did not differ significantly by sampling trip or geographical region ($P>0.05$, Table 1).

7.1.2.4 Moult stage

Overall, total protein showed a trend to increase from early post-moult (stage AB) to late pre-moult (stage D_{3-4}) (Table 2). In free-ranging lobsters, concentrations of total protein were lowest immediately following a moult (Stage AB), increased gradually during the intermoult period and reached a maximum in late pre-moult animals (D_{3-4}). The increase in hemolymph protein during the intermoult period presumably indicates that the tissue mass of the animals is increasing as the animals grow to fill the shell (Dall 1974b).
However, calcium in late pre-moult (stage D_{3-4}) lobsters was significantly higher ($p < 0.05$) than in early post-moult (stage AB) and intermoult (stage C) animals. Similarly, hemolymph magnesium concentrations also showed a significant increase from post-moult to pre-moult stages ($p < 0.05$). These patterns are similar to those seen in other decapods.
There was no apparent effect of moult stage on potassium, lactate or glucose concentration ($p > 0.05$, Table 2). The latter finding contrasts with a reported increase in glucose levels in pre-moult individuals of freshwater crayfish, *Orconectes propinquus* and *Procambarus robustus*, (Telford 1974). However, caution is urged in the interpretation of the results of the present study due to the very small numbers of non-intermoult lobsters sampled.

Table 1. Values of hemolymph metabolites and selected serum electrolytes (average ± SE) from free-ranging intermoult (Stage C) western rock lobsters *Panulirus cygnus*. Superscripts indicate values that are significantly different ($p < 0.05$, ANOVA, Tukey-Kramer HSD). Sample size in brackets.

Date	THC (cells x 10^6)	Lactate (mmol L^{-1})	Glucose (mmol L^{-1})	Ca^{++} (mmol L^{-1})	Mg^{++} (mmol L^{-1})	K^+ (mmol L^{-1})
June '97	nd	1.1± 0.2 (7)[a]	0.4 ± 0.1 (7)[c]	12.9 ± 0.3 (7)[d]	10.1 ± 0.2 (7)[e]	9.8 ± 0.4 (7)[g]
July '97	4.43 ± 0.54 (7)	0.4 ± 0.2 (10)[b]	0.3 ± 0.1 (10)[c]	13.7 ± 0.5 (10)[d]	12.1 ± 0.3 (10)[f]	9.7 ± 0.4 (9)[g]
March '98*	nd	0.7 ± 0.2 (5)[a]	0.2 ± 0.0 (5)[c]	14.0 ± 0.2 (5)[d]	9.7 ± 0.1 (5)[e]	11.8 ± 0.5 (5)[h]
Nov. '98	nd	0.9 (1)	0.3 (1)	14.4 (1)	11.8 (1)	10.9 (1)

*Values for dive samples taken from animals captured in pots underwater.

Table 2. Variations in hemolymph parameters in relation to moult stage in free-ranging western rock lobsters *Panulirus cygnus*. All concentrations are given in mmol L^{-1}. Numbers in parentheses are sample sizes. Superscripts indicate values that are significantly different ($p < 0.05$, ANOVA).

Stage	Lactate	Glucose	Ca^{++}	Mg^{++}	K^+
AB (n=4)	0.3 ± 0.2a	0.3 ± 0.0b	12.4 ± 0.2 c	12.3 ± 0.6e	10.6 ± 0.9 g
C (n=23)	0.7 ± 0.1 a	0.3 ± 0.0b	13.6 ± 0.2c	11.0 ± 0.3 e	10.3 ± 0.3 g
D_o (n=1)	0.2	0.4	14.5	11.4	11.3
D_1'(n=1)	0.2	0.2	14.1	11.7	9.8
D_1" (n=1)	0.4	0.3	16.2	14.1	11.1
D_1"' (n=1,2)	0.8 ± 0.6 (2)	0.5 ± 0.3 (2)	17.0 (1)	14.0 (1)	10.0 ± 1.3 (2)
D_2	nd	nd	nd	nd	nd
D_{3-4}(n=2,3)	0.1± 0.0a (2)	0.1± 0.0b (2)	15.6 ± 0.8d (3)	16.0 ± 0.4f (3)	11.2 ± 0.3g (3)

7.1.3 Experiment 1: Single and repetitive sampling of western rock lobsters held in a recirculating seawater system for 42 days

In this trial, lobsters were purchased from a commercial supplier and placed in a recirculating seawater aquarium at Geraldton TAFE. One group of lobsters was identified with numerals painted on their carapace for the repetitive sampling experiment. The remaining lobsters were sampled only once during the recovery period.

7.1.3.1 Repetitive sampling

Repetitive sampling of lobsters, at frequencies ranging from daily to weekly, had no noticeable or significant effect on hemolymph chemistry of these lobsters though the repeated handling led to a higher incidence of leg-loss. Nevertheless, with care, this protocol may be particularly useful when assessing changes in hemolymph protein level. Hemolymph protein level differed considerably between individual lobsters, complicating the process of statistical testing. However, repetitive sampling confirmed that each lobster had a relatively stable 'baseline' of hemolymph protein, allowing rapid or slow deviations in that baseline to be followed with a high degree of precision within individuals.

The data were subjected to ANCOVA analysis, with a view to identify the relative effects of the factor "Type of sampling" and of the covariate "Acclimation period" for the variables surveyed. The results found that, for each sampling time, no significant differences could be associated with the type of sampling.

Furthermore, the data obtained from captive animals that were sampled repetitively (serial group) was subjected to ANCOVA analysis, with a view to identify a possible difference between "Subjects," after taking into account the effect of the covariate "Period of Acclimation". The analysis revealed a significant effect ($p<0.001$) of the factor "Subject", only for the variable hemolymph protein. For each of the 14 specimens sampled, a comparable and progressive decrease in protein concentration was recorded and the respective differences identified between specimens, at time 0, were maintained throughout the experimental period.

7.1.3.2 Acclimation effects

The lobsters in this initial Geraldton study only reached a stable baseline with respect to protein and hemocytes (Figure 1). In other respects, fluctuations in parameters indicated that under these conditions the lobsters did not reach the 'baseline' levels expected of resting, undisturbed lobsters. This inability to reach baseline in a multiple use laboratory area, required a re-appraisal of approach using the more controlled holding regime, complete with opaque tent, described in section 7.1.4. The causes of the fluctuations in lactate and glucose (Figure 2) in particular are unknown, but it is unlikely that hypoxia is responsible, as water quality was checked regularly. However, the results could be explained if the lobsters were regularly disturbed and tail-flapped when inexperienced students serviced the tank or if people visiting the room regularly examined the contents of the tank. Similar findings have been reported in *Panulirus cygnus* held in shallow storage tanks without shelters, when compared to animals kept in deep tanks equipped with plastic baskets as shelters (Spanoghe 1996). When the trial was repeated under closer supervision at Fremantle a far more acceptable result ensued (below). Nevertheless, the data generated here confirm that lobsters can be maintained in captivity for an extended period of time and that in doing so, acclimation is accompanied by changes in their physiology.

The changes in values recorded over the acclimation period were found to be significant ($p < 0.05$) for all of the variables measured, with the exception of the glucose titre. For some of the variables, such

as lactate and hemocyte count, pronounced changes occurred during the first 24 to 48 hours of acclimation, accounting for the levels of significance detected. Limited changes were recorded over subsequent periods (Figure 1 and 2).

For other variables such as glucose, a wide ranges of values was recorded thoughout the experimental period, failing to reach a stable value even after 42 days of captivity. Hemolymph glucose concentration in particular was low on arrival but thereafter was very variable during the 42 day holding period (Figure 2). Hemolymph protein (Figure 3) showed an initial rapid decline in the first 2 days of captivity. Hemolymph protein continued to decline gradually during the period of captivity. At the time of introduction to captivity, hemolymph magnesium was at baseline. After 2 days in captivity, magnesium was significantly reduced (Figure 3) and these low levels of magnesium were maintained for the duration of the captive period. Hemolymph calcium showed a similar pattern of change to that seen in magnesium (Figure 3). Total hemolymph cell (hemocyte) count (THC) was significantly elevated upon arrival at the holding facility, but had returned to baseline levels within 2 days. No further changes occurred during the 42 day period (Figure 1). The vigour of the lobsters was low on arrival (VI = 2 or 3). VI of lobsters recovered within one day. During the remainder of the captive period most lobsters were considered to be strong (VI = 4 or 5) (Figure 4).

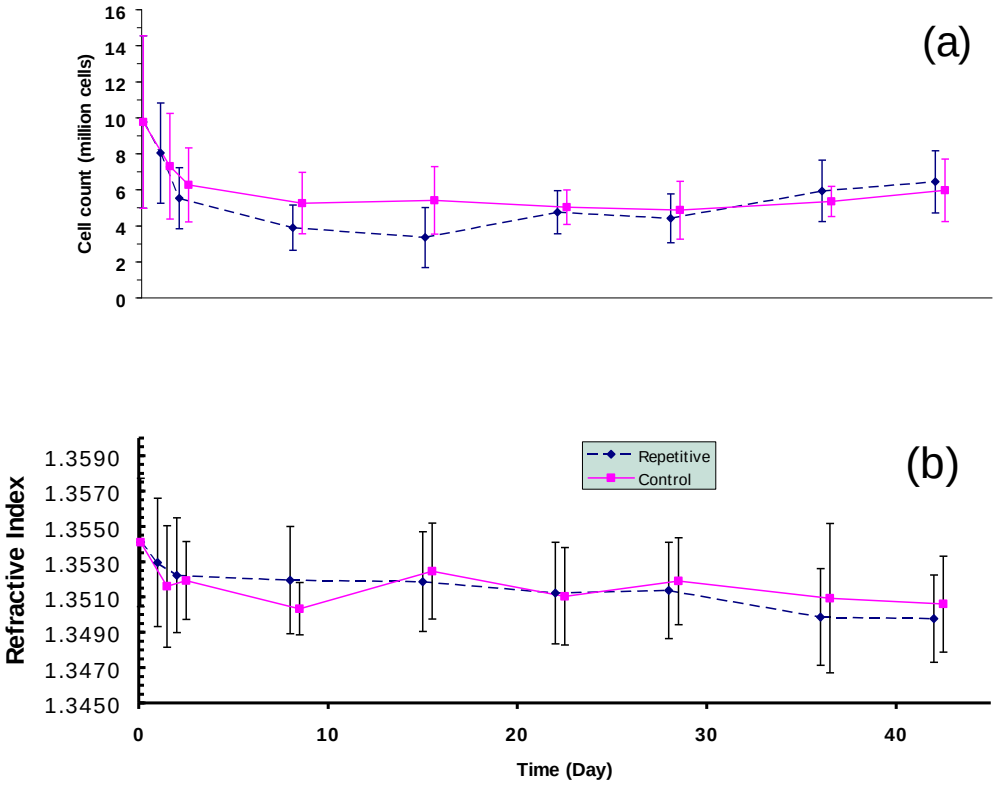


Figure 1. Hematology of western rock lobsters *Panulirus cygnus*, stored in a recirculating seawater system for 42 days (a) total hemocyte count and (b) hemolymph refractive index. Each point represents average ± SE. 'Repetitive' indicates data from a group of the same 12 lobsters sampled at each time during the experiment. 'Control' indicates data from different groups of lobsters (n=15 each time) sampled at each time during the trial.

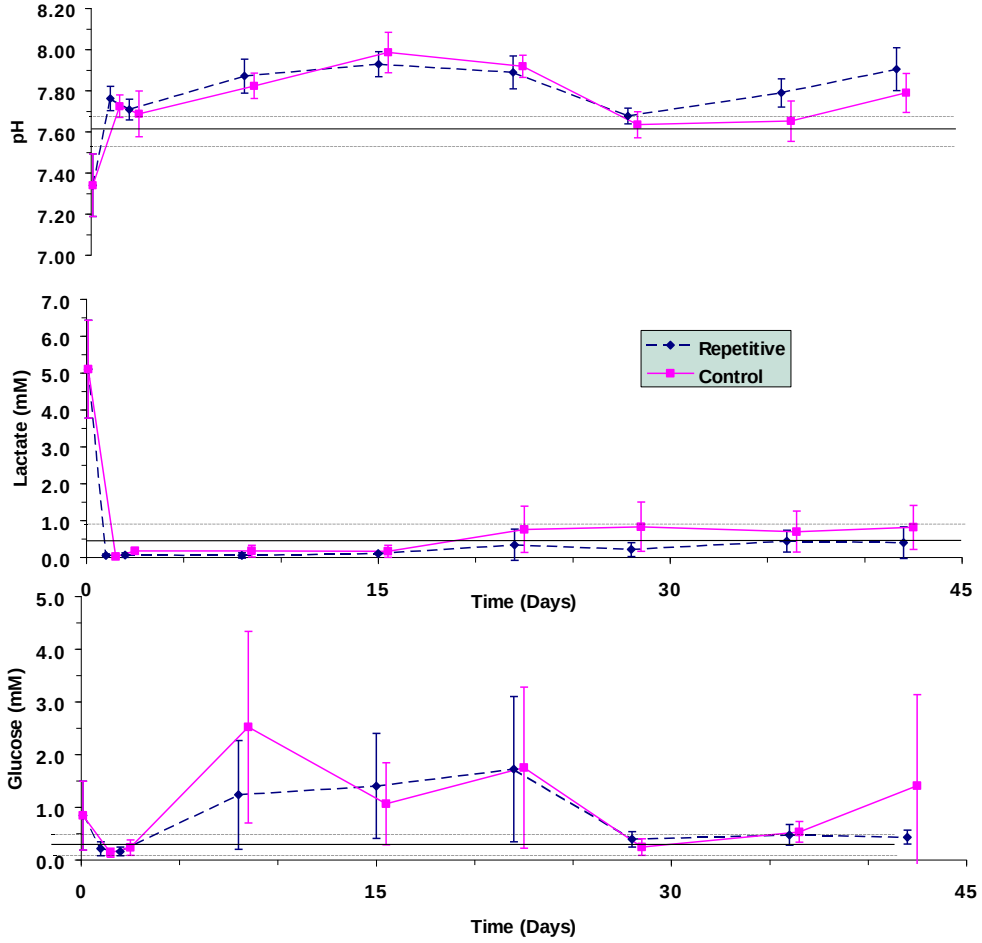


Figure 2. Hemolymph pH, lactate and glucose concentration (average ± SE) of of western rock lobsters *Panulirus cygnus*, stored in a recirculating seawater system for 42 days. For explanation of figure, refer to Figure 1.

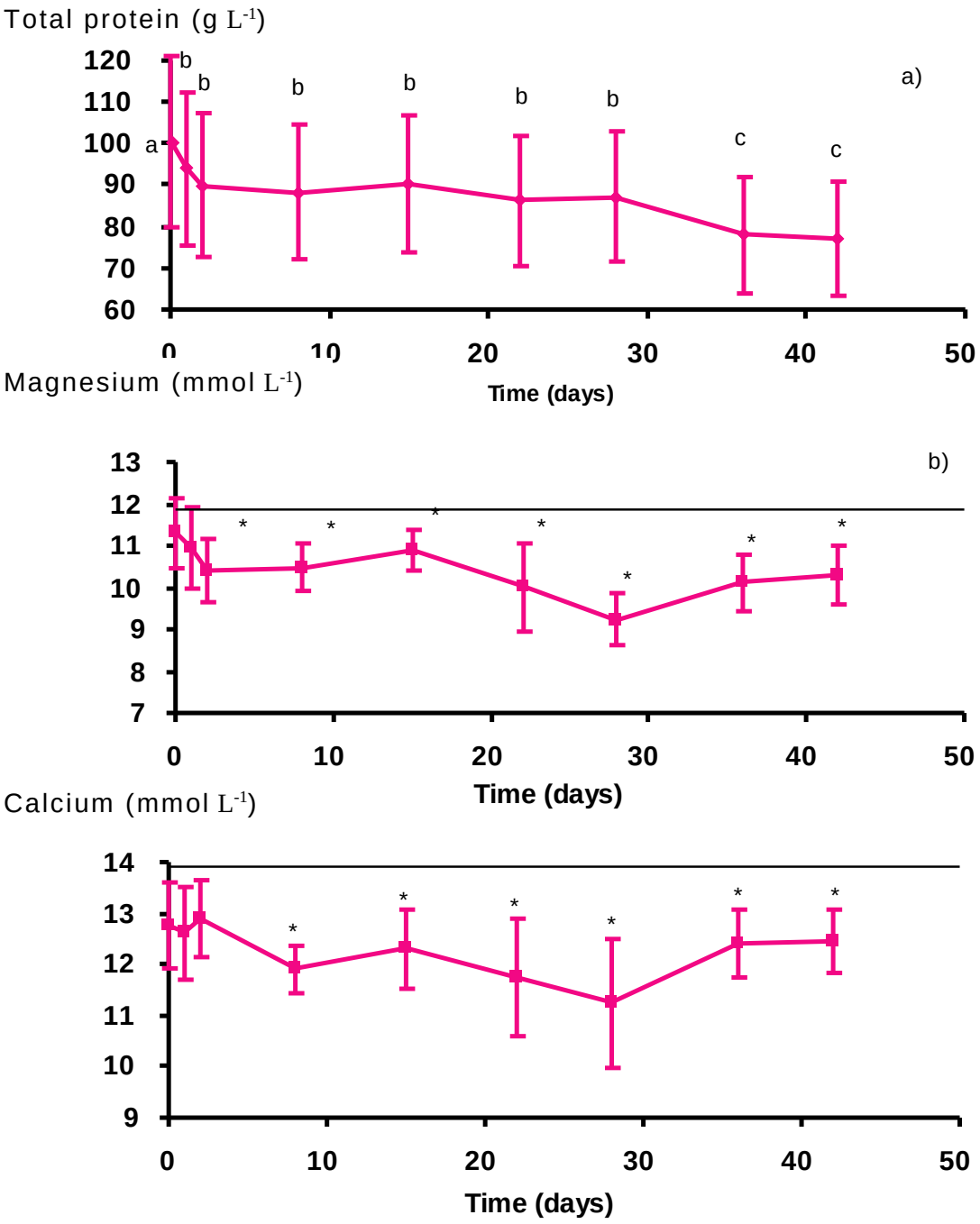


Figure 3. Variation in a) hemolymph total protein (g L^{-1}) , b) hemolymph magnesium (mmol L^{-1}) and c) hemolymph calcium (mmol L^{-1}) concentrations in western rock lobsters *Panulirus cygnus* during a 42 day period of captivity. Note that in a) averages are presented for repeatedly sampled animals (N = 12) and that common subscripts indicate averages that are not statistically different (P > 0.05). In b) and c), * indicates averages for singly-sampled lobsters are significantly different (p < 0.05) from baseline values. For magnesium and calcium, mean baseline values are shown by horizontal black lines. N = 15, all data presented as means ± S.D.

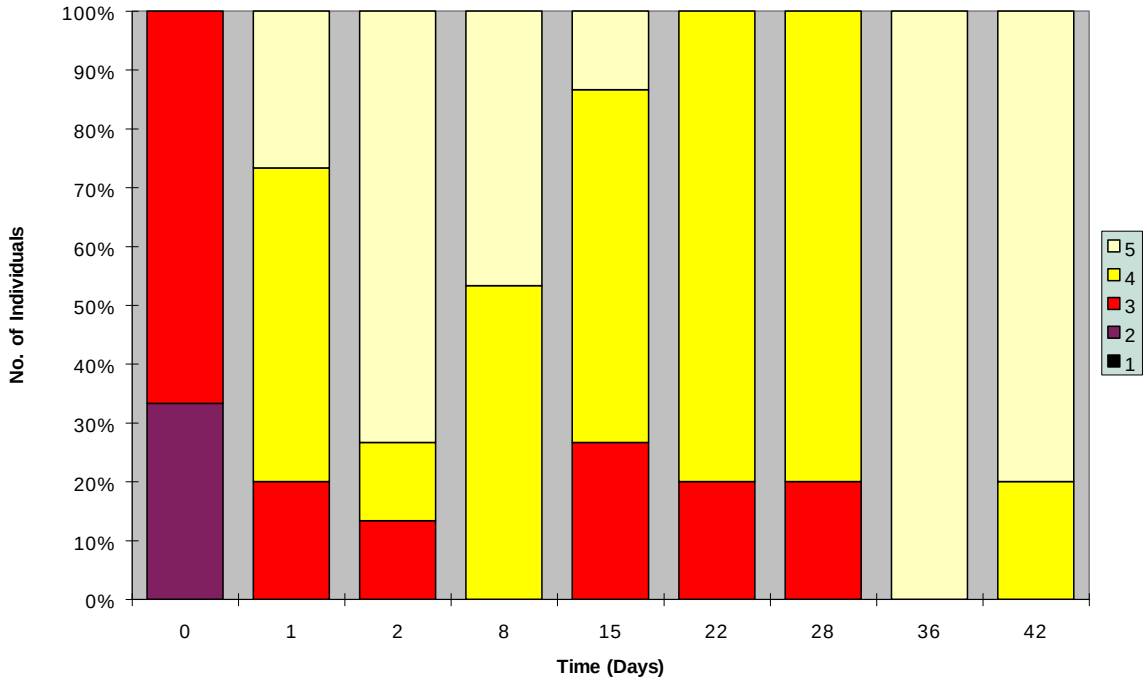


Figure 4. The percentages of each vigour category (1 to 5) in the samples (n=15) of western rock lobsters *Panulirus cygnus,* stored in a recirculating seawater system for 42 days.

7.1.4 Experiment 2: Acclimation and physiological responses of lobsters to captivity- (sub-objective 1)

This study was conducted in preparation for the laboratory emersion experiments conducted in a later section (Section 7.3) and involved a recirculating seawater system constructed specifically for these trials. Apart from studying the lobsters during recovery in captivity, we were also interested to see how quickly a group of lobsters reached baseline upon arrival in the facility, as a guide for planning later experiments.

7.1.4.1 Protein in hemolymph

Total hemolymph protein was found to be very variable between animals, with overall values ranging between 15 and 169.3 g L^{-1}. No statistically significant changes in hemolymph protein level were observed in lobsters during this experiment, no doubt in part due to the wide variation in this parameter. The data would pass without comment were it not for the fact that the trends seen in mean protein concentration of inter-moult lobsters (pre- and post-molt lobsters were excluded from the analysis) clearly mirror those in the hemolymph pH, calcium and magnesium data, pointing to the action of a broad fundamental process.

The average values of total protein reported here (42.0 ± 6.9 to 83.5 ± 11.6 g L^{-1}) are almost half those reported in a previous study in intermoult *P. cygnus* (Dall 1974b), with 116.4 ± 4.5 g L^{-1}. Nevertheless, total protein concentration values comparable to the ones reported in this study have been identified in free ranging intermoult lobsters sampled by SCUBA (see section 7.1.2.2.1).

Feeding may account for the discrepancy between the values published here and Dall's data. In this study, lobsters were fed a minimal "conservative" diet, somewhat different from Dall's animals which were fed *ad libitum.* In another paper, that author reported a fall in hemolymph protein concentration followed by a gradual rise and recovery in captivity (Dall 1975), a report that is difficult to interpret because of the lack of comparative data, but which probably supports the idea that lobsters in captivity are getting access to more food than in the wild. Additional work investigating the effects of diet on the hemolymph protein of crustacean (Stewart *et al.* 1967) supports the views that the diet directly affects muscle and serum protein concentration.

7.1.4.2 Hemolymph pH

The hemolymph pH stabilised within 24 hours of introduction to captivity and limited changes were recorded during the subsequent 22 days. Significant ($p < 0.05$) changes in pH were recorded over the first 24 hours of acclimation, with a rapid rise from 7.42 ± 0.03 to 7.72 ± 0.02, and also after day 22, where a significant decline ($p<0.05$) was recorded to reach 7.23 ± 0.04 at day 39. During the following period (day 39 to day 43), a significant rise in pH was measured, to reach 7.52 ± 0.03. No significant changes were measured during subsequent periods. Note that this period of depressed hemolymph pH corresponded to a period of elevated protein, calcium and magnesium level.

In an earlier study of the recovery of *P. cygnus* in commercial holding tanks, (Spanoghe 1996), periods of up to 24 hours were required for the hemolymph pH to reach a stable value, after which it would remain unchanged for at least 72 hours. The reason why pH recorded a decline from days 22 and 43 remains unclear since no changes could be identified with regard to environmental conditions. Hemolymph lactate level did not increase during this period (section 7.1.4.3). However, it must be noted that during this period, a larger proportion of animals (50%) in the tank were identified as being in premoult condition. These pre-moult lobsters were not included in the preparation of the figures and in the statistical analysis, yet it might well be that some animals classified as 'intermoult' were already going through the beginning of some part of the pre-moult process.

7.1.4.3 Lactate in hemolymph

After the initial high values on arrival, values for hemolymph lactate concentration were found to be consistently low throughout the captivity period and are consistent with those reported in resting crustaceans (Albert and Ellington 1985; Phillips *et al.* 1977).

A highly significant change in lactate concentration ($p < 0.001$) was identified over the acclimation period, but this was due to the initial value for hemolymph lactate recorded for the lobsters, upon delivery to research facilities (Time 0). The concentration of lactate in the hemolymph of lobsters, decreasing from a high value of 5.90 ± 0.75 mmol L^{-1} (Time 0) to reach 0.16 ± 0.03 g L^{-1}. No significant changes were measured during subsequent periods. This elevated level at the start no doubt reflects the combined effects of handling, emersion and general disturbance on lobsters delivered to the laboratory ((Spanoghe and Bourne 1999), see also section 7.2).

7.1.4.4 Glucose in hemolymph

Glucose concentration in the hemolymph of these lobsters was generally low and no statistically significant changes were observed ($p = 0.07$). Nevertheless the peak of mean glucose concentration, beginning at day 8, is of concern, coming as it does when the lobsters could be assumed to have 'recovered.'

Over the period of acclimation, the changes recorded for the variable hemolymph glucose were found to be non-significant with a wide variability recorded between specimens of a same group, at sampling days 8 and 15. Fig 1b reveals that after a moderate decline from 0.7 ± 0.1 to 0.6 ± 0.1 mmol L^{-1} recorded between day 0 and day 4, respectively, a dramatic rise was recorded after eight days of acclimation, to reach a mean value of 4.5 ± 1.9 mmol L^{-1}. This was followed by a progressive decrease over the next two periods, to reach 0.6 ± 0.1 mmol L^{-1} after 29 days captivity, not significantly different from values recorded during subsequent periods.

With exception for the samples taken at days eight and fifteen, hemolymph glucose concentrations in undisturbed lobsters were low (0.26 - 0.91 mmol L^{-1}). These values are consistent with published records (Dall 1975; Telford 1974) and samples obtained from free-ranging lobsters on the seafloor, (section 7.1.2.2.3). While a number of factors such as stress, handling and disturbance are known to influence the levels of circulating glucose, it is difficult to explain why a peak of concentration was recorded after eight days of captivity. Of the variables studied, only circulating magnesium recorded a comparable rise at the same period (Figure 5, see section 7.1.4.5), but a second peak in magnesium concentration, recorded around day 39, was not matched by a corresponding peak in glucose concentration. When comparing the trends identified for glucose concentration with other data such as the moon phase or the water quality data, it appears that high nitrite concentration was recorded from day eight to day fifteen, with a peak value of 14 mg L^{-1}, corresponding to the establishment of the nitrifying bacterial population in the biological filter.

The glucose concentration did not record high levels throughout the whole of the period during which nitrite concentration was elevated. However, it is possible that the peak in glucose titre recorded was part of an acute stress response of crustacean to the presence of toxic substance dissolved in the water, (Telford 1974). Whether the levels recorded for nitrite ions were toxic to *P.cygnus* remains unclear. The high salinity in marine conditions is expected to ameliorate the toxicity of nitrite (Ary and Poirrier 1989).

Alternatively, the increase in glucose concentration could possibly reflect an increase in lobster activity, corresponding to the establishment of a dominance hierarchy and associated competition for preferred sites, in lobsters placed in a new environment and under crowded conditions, (Chittleborough 1974; Telford 1968). With reference to the widely accepted significance of an elevated glucose titre in crustacean, it can be hypothesized that in this case it took about 3 weeks for

lobsters to recover for previous stresses of capture, transport and handling and to acclimatise to captivity.

7.1.4.5 Serum electrolytes (Calcium potassium magnesium).

All electrolytes measured changed significantly during lobster acclimation. Generally, calcium and magnesium levels were correlated, falling gradually after arrival and rising to a peak at day 39 (cf. Protein, section 7.1.4.1) before falling thereafter. The exception was the peak in magnesium level at day 8, coinciding with the apparent hyperglycaemia mentioned above. Potassium level in contrast fell dramatically upon arrival and rose slowly thereafter, eventually returning to close to the initial level by day 50.

The changes in serum concentrations for the three ions studied were found to be significant ($p<0.05$) for magnesium, and very significant ($p< 0.01$) for calcium and potassium. After an initial decrease recorded over the first four days of acclimation, from 13.8 ± 0.4 to 12.9 ± 0.4 mmol L^{-1}, serum calcium rose progressively to reach, on day 8 the value of 13.7 ± 0.5 mmol L^{-1} (Figure 5d). On day 39, serum calcium recorded a value of 15.7 ± 0.5 mmol L^{-1} significantly different ($p<0.05$) from values recorded during the first 15 days, and on the 57th day.

An increase in total calcium is to be expected in crustacean species entering premoult and most of this rise is accounted for by a rise in bound or complexed calcium (Greenaway 1985). In this study, a close relationship between hemolymph protein and serum calcium concentrations ($r^2= 0.49$) was identified, supporting this view. With exception for the rise recorded at day 39, values for serum calcium were found to be slightly more elevated than values previously reported in *Panulirus cygnus* (Dall 1974a) with 12.6 mmol L^{-1}. When considering the acclimatized animals, i.e., sampled after day 29, this trend was further confirmed, with a measured value for serum calcium of 14.4 ± 0.2 mmol L^{-1}.

Conversely, serum magnesium and potassium values measured in these "acclimatised" lobsters (10.4 ± 0.3 and 11.1 ± 0.2 mmol L^{-1}, respectively) were found to be lower than published reports (Dall 1974a) with 13.5 ± 0.30 and 12.2 ± 0.4 mmol L^{-1}, for magnesium and potassium respectively. The reason for this discrepancy remains unknown.

The changes observed for serum magnesium (Figure 5) were characterised by two peaks identified at day 8 (13.20 ± 1.50 mmol L^{-1}) and at day 39 (12.14 ± 0.90 mmol L^{-1}). The change in concentration recorded at day 8 was found to be significantly different ($p<0.05$) from that recorded at other periods (mean value of 10.87 ± 0.19 mmol L^{-1}), with exception for day 15 and 39.

Serum potassium showed a different pattern of change to that shown by the other two ions, a response perhaps linked primarily to the rapid recovery in hemolymph pH in lobsters in arrival (Figure 5). This parameter recorded a marked and significant decline in concentration ($p<0.05$) during the first day of acclimation, from 12.1 ± 0.5 to 9.3 ± 0.2 mmol L^{-1}, to remain almost unchanged up to the 4th day. Serum potassium then rose steadily to reach 10. 8 ± 0.5 mmol L^{-1} after 15 days. No further changes were identified up to 39 days, after which time the concentration rose again to reach a peak value of 11.8 ± 0.2 mmol L^{-1} after 50 days, significantly different from the values recorded between day 1 and day eight. The last period was characterised by a return of serum potassium to a value close to 11 mmol L^{-1}.

A fall in serum potassium level coinciding with a rise in pH may be related to a reversal of the detrimental effects of low pH on cellular potassium regulation. The potassium may have re-entered cells during the recovery process. It could be argued the initial, 'tantalising' dip in hemolymph protein level suggests a slight dilution of the hemolymph. Perhaps the lobsters are imbibing large amounts of seawater- except if this were the case, then the magnesium level does not rise accordingly.

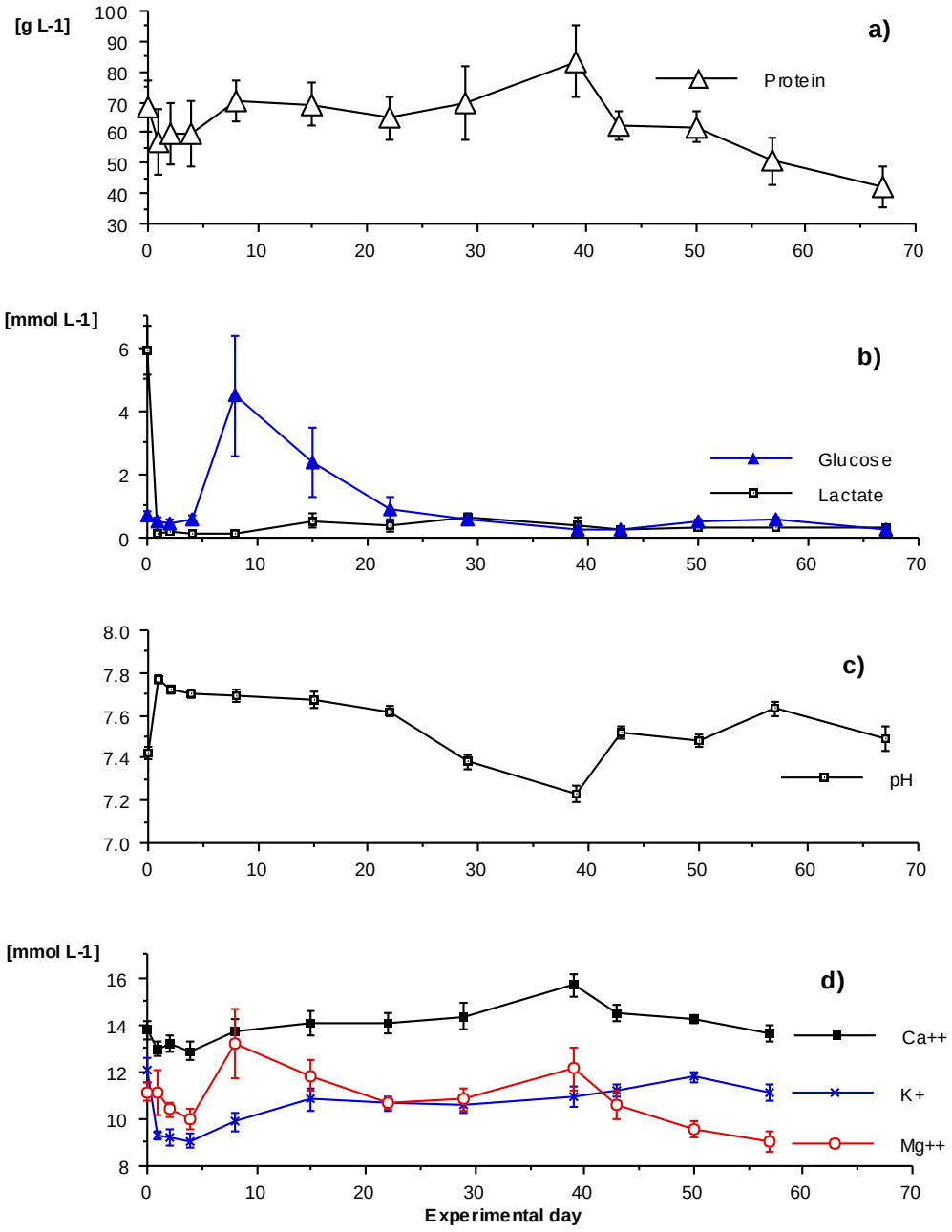


Figure 5. Average values (± SE.) of physiological variables in hemolymph of western rock lobsters *Panulirus cygnus* during a 67 days period of acclimation to captivity. Values include protein, lactate, glucose, calcium, potassium and magnesium concentrations and pH.

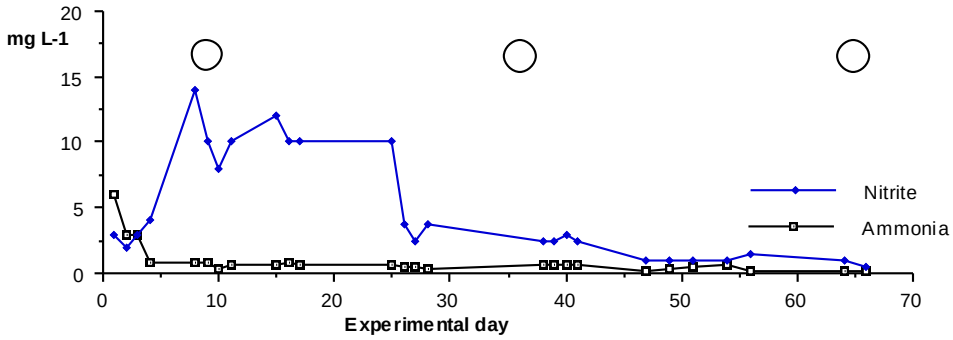


Figure 6. Water quality and moon phase data over a 67 days period of acclimation of western rock lobsters *Panulirus cygnus* to captivity. Ammonia and nitrite concentration are expressed as mg L^{-1} . The full moon is presented as a circle.

7.2 Responses during post-harvest handling (Sub-objective 2)

7.2.1 Summary

Having established baseline levels for a number of parameters in the hemolymph of lobsters, the next step is to see which ones change during the kind of handling that can be meted out during day to day handling of rock lobsters.
The catches of three boats were examined in January, April and June 1997 and December 1998 (for details refer to methods section 6.3). Lobsters were sampled…

1. immediately upon pot retrieval, (*Pot*)
2. upon arrival at the processing factory jetty (*Jetty*),
3. after 'sham' grading (45 min air exposure with disturbance) (*Belt*)
4. after 24h storage in live holding tanks in the factory (*24h*)
5. after 48h storage in live holding tanks in the factory (*48h*)

Initial samples drawn from lobsters as they emerged from the pot (and also baseline values obtained by dive sampling, see section 7.1) showed that levels of several parameters in the hemolymph clearly changed when rock lobsters were handled and stored after harvest. Perhaps not surprisingly, the parameters which changed (our preliminary list of stress indicators) reinforce the idea that stress during lobster handling is centred around periods when the lobsters are out of water. Note that in order to illustrate the effects of handling with disturbing factory routine, a period of 'sham' grading of these lobsters has been adopted. The lobster have not been graded, so any changes reflect the true changes in the sample as a whole, not those associated with removing 'rejected' lobsters from contention. Lobsters are not well adapted for respiring in air, and the symptoms described here, of a temporary rise in hemolymph lactate and glucose level, associated with disturbances in hemolymph electrolytes, are largely what we would expect of a marine crustacean in air (for a more complete discussion of this see section 7.3). This can only be worsened by the lobsters having elevated metabolic rates due to elevated air temperatures, handling disturbance and the fact that they may have recently fed on bait.
The sector of the industry being studied here, lobsters from boats delivering directly to the live factory without an intervening period of land or sea transport, receive treatment that represents the current best practice. Accordingly, the magnitude of the responses picked up here were not alarming in comparison with similar studies in the literature, (Whiteley and Taylor 1992). The changes were also temporary; lobsters in the factory tanks appeared to return to baseline levels for these parameters within about a day of harvest. This apparent lack of any longer-term or chronic effects, particularly in 'classical' stress indicators like glucose was encouraging, though probably to be expected given the excellent prognosis expected of 'jetty crays.' If glucose level is normal however, it does not follow necessarily that the lobsters are therefore not stressed. Perhaps the one cautionary note here was the apparent 'rise' in vigour in the factory compared to levels in lobsters leaving the pots. While pot capture may alter lobster behaviour (and physiology), it is also conceivable that the readiness of these lobsters to resist handling is associated not with 'recovery' but with a heightened aggressive or defensive state associated with captivity. Further examination of the effect of pot capture upon the behaviour and physiology of lobsters could be undertaken to discern whether or not this occurs.
This work serves to emphasise that up to a certain level, stress is not necessarily a problem for harvested lobsters. The key point is that some lobsters may be stressed too much and weaken or die during storage. The indicators that highlight this can be found in two ways. Firstly, bringing lobsters into the laboratory allows us to stress them in air under controlled conditions, bringing a broader array of tests to bear upon then to further examine why these changes occur and which if any could be considered serious (Section 7.3). Secondly and most importantly, returning to the factory with an

augmented or refined list of indicators and establishing whether there is something about lobsters that later weaken which sets them apart early during handling (see Section 7.4 and later).
The remainder of section 7.2 discusses in detail these changes during harvest on a parameter by parameter basis as well as accounting for possible influences or boat or season effects.
As this study occurred in collaboration with the companion project FRDC 96/344 there are also immune system results for the lobsters sampled in this trial. This data is not presented in this report and the reader should refer to the corresponding section of the other report.

7.2.2 Changes after harvest

In this study, the level of hemolymph parameters in lobsters leaving the pot was taken as the closest sampling point we have to that of a normal, undisturbed lobsters. References to results from underwater sampling of free-ranging lobsters (Section 7.1) suggested that, for the majority of parameters, this assumption appeared to be valid. However, the levels other parameters, such as glucose and magnesium (below), appeared to be altered by the process of capture in pots, though this of course could also be due to an effect of recent feeding on bait.

7.2.2.1 pH

Hemolymph pH tended to rise when lobsters were stored on the boats and then fall during the subsequent period of sham grading (Figure 7a). The initial rise thus offset the 'fall' in hemolymph pH when the lobsters were kept briefly in air and brought this parameter back down to baseline levels. The reason for the general rise in pH on the boats could be a side effect of the disturbance and handling prior to entering the tanks on the boats. Elevated rates of gill ventilation in disturbed lobsters may strip carbon dioxide more effectively from the hemolymph (McMahon *et al.* 1978), and this may be expressed here as a rise in pH or 'alkalosis' during recovery.
Mean baseline values of hemolymph pH ranged from 7.46 to 7.79. Changes in pH after harvest were somewhat variable. On 2 of 4 occasions values of hemolymph pH at the jetty were significantly higher ($P < 0.05$) than baseline. On the other 2 occasions, pH was similar to values measured after pot retrieval. After sham-grading hemolymph pH tended to be lower than at the *jetty* sample, and on 3 of 4 occasions hemolymph pH at the time of sham grading was significantly ($P < 0.05$) lower than baseline. After 24 h and 48 h in factory live tanks, pH was either similar to or greater than baseline.

7.2.2.2 Lactate

Lactate was significantly elevated ($P < 0.05$) following sham grading, when lobsters were held out of water for 45min. Otherwise, hemolymph lactate concentration was low (approx. 1.0 mmol L^{-1}) in animals sampled immediately following pot retrieval (Figure 7b) and there was no significant variation in baseline values between trips. This value agrees well with hemolymph lactate concentrations measured in *P. cygnus* sampled underwater by SCUBA divers (section 7.1.2.2) but is somewhat higher than resting lactate concentrations in animals held under laboratory conditions for extended periods (typically approx. 0.3 mmol L^{-1}) (sections 7.1.4 and 7.3.3).
Similar differences in basal lactate between free-ranging and captive decapods have been reported in *Callinectes sapidus* (Lowery and Tate 1986), and *Carcinus maenas* (Houlihan and Mathers 1985). Such differences probably reflect higher levels of routine activity in free-ranging animals and also perhaps a reduction in the capacity to generate lactate after long periods of relative inactivity.
It is noteworthy that animals arriving at the factory jetty generally showed baseline concentrations of lactate. By and large, lactate concentrations were similar to baseline ($P > 0.05$) in animals arriving at the factory jetty, with the exception of December when lactate was significantly elevated ($P < 0.05$) above "pot" levels. This suggests either that sorting of the pot catch following pot retrieval generally has minimal impact on anaerobic metabolism or that the animals recover in onboard live tanks from the disturbance of sorting in air. The latter seems more probable given the intensity of burst activity

(i.e. tail-flipping) exhibited by lobsters during onboard sorting. The provision of effective onboard holding tanks seems prudent given the insult the animals endure when graded.
After 24 h in factory tanks, hemolymph lactate concentrations had usually returned to baseline (*pot* sample) values ($P > 0.05$). In December, however lactate concentrations after 24 h in factory tanks were significantly lower ($P < 0.05$) than baseline and remained so until the end of the experiment (48 h in tank).
Data from laboratory experiments (section 7.3.3) show that similar levels of circulating lactate could be cleared very rapidly from the hemolymph, within a few hours of submersion.

7.2.2.3 Glucose

Glucose concentration in the hemolymph tended to rise during handling/storage on board the boat from the relatively low level seen in lobsters emerging from the pot. The rising trend was maintained during sham-grading. However, lobsters that had been in captivity in the factory for at least a day showed baseline levels of glucose.
Hemolymph glucose concentrations were low (generally < 0.5 mmol L^{-1}) in freshly caught lobsters, with the concentration in June being significantly lower ($P < 0.05$) than in April and December. Typically decapod crustaceans exhibit a hyperglycaemia in response to stressful stimuli. This response is hormonally mediated and it is likely that there is a useful window of time for sampling animals before the hormonal response can be mounted. Thus we are confident that the recorded values are representative of "true" baseline and indeed the values reported here agree very well with those recorded from free-ranging lobsters sampled underwater (section 7.1.2.2.3).
With the exception of June, glucose concentrations were significantly elevated ($P < 0.05$) upon reaching the jetty, and were further elevated following sham grading on all occasions (Figure 7c). Peak mean values of between 1.5 and 2.5 mmol L^{-1} were recorded. As mentioned above, a rise in hemolymph glucose concentration is a generalised stress response in crustaceans and this points to the release of a diabetagenic factor, such as crustacean hyperglycaemic hormone, during post-harvest handling. A rise in hemolymph glucose concentration was also reported in tropical rock lobsters (*Panulirus ornatus*) arriving on a mother ship from a catching dinghy in Torres Strait, (Paterson *et al.* 1997b).
In all cases, hemolymph glucose was restored to baseline concentrations ($P > 0.05$) within 24 h of being placed in factory tanks. The recovery may have happened relatively quickly. When 'stressed' lobsters were re-submerged in the laboratory, their glucose levels returned to baseline in a few hours (Section 7.3.5). Glucose concentrations generally remained at baseline after 48 h in factory tanks, except during April when glucose concentration was significantly elevated ($P < 0.05$). This holding environment shows little evidence of lingering or chronic stress, though at this point we cannot explain this one instance of a slight elevation in glucose level in tanked lobsters. However, we can't use the absence of a single symptom to argue that the lobsters are therefore not stressed. Recently, it has been demonstrated that crowded shrimp (*Penaeus monodon*) do not exhibit hyperglycaemia (Hall and van Ham 1998).

7.2.2.4 Hemolymph ions

Significant changes in the ionic composition of hemolymph occurred during post-harvest handling of western rock lobsters. The patterns of change in potassium and magnesium differed suggesting that dehydration or fluid shifts between internal compartments were not the primary determinants of the observed changes. Each ion will be considered separately.

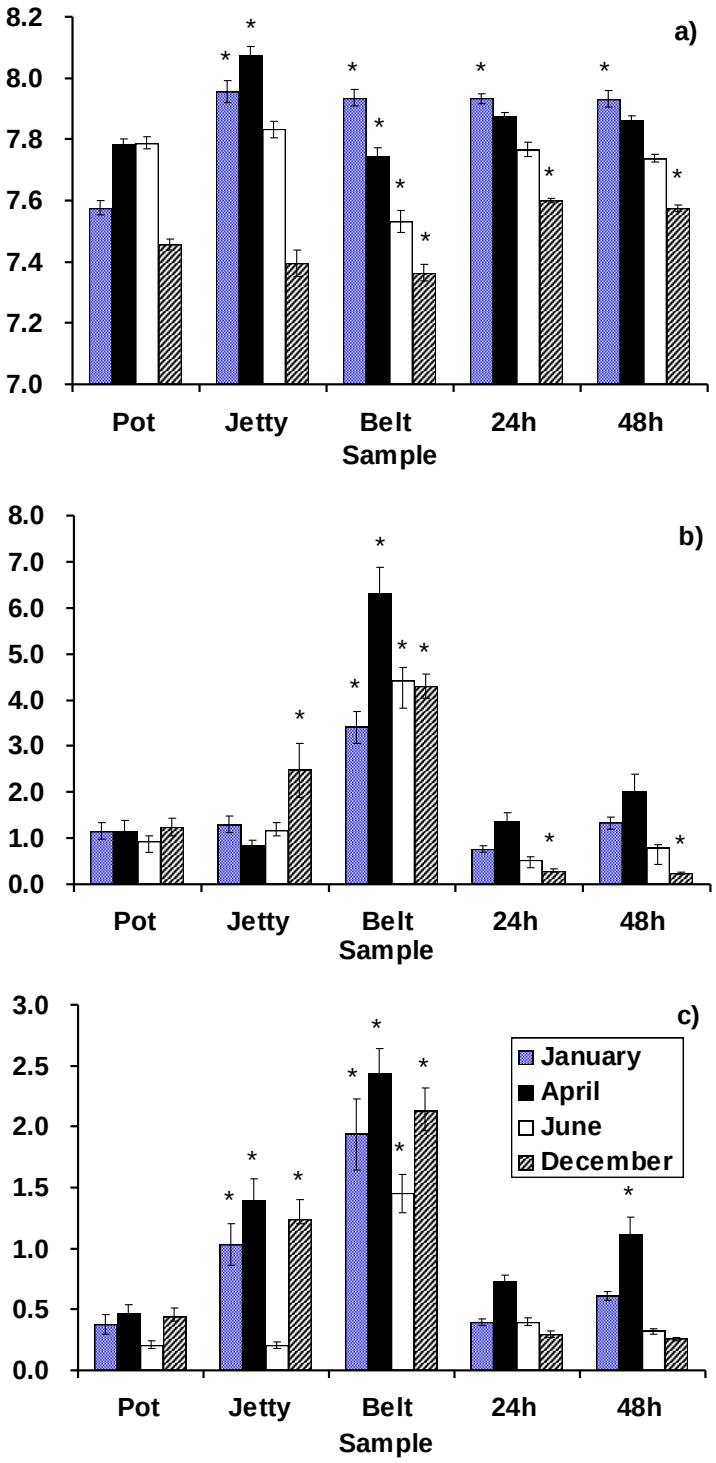


Figure 7. Post-harvest changes in a) venous hemolymph pH, and concentrations of b) lactate (mmol L^{-1}) and c) glucose (mmol L^{-1}) in western rock lobsters *Panulirus cygnus* during different stages of post-harvest handling (see text). Each bar is the average (± SE) for 15 lobsters. Asterix (*) indicates value is significantly different ($P < 0.05$) from respective "*pot*" values.

7.2.2.4.1 Calcium

Hemolymph calcium concentrations varied relatively little during the post-harvest process (Figure 8a). This was surprising. Increased calcium concentrations are often associated with periods of extracellular acidosis, and the lobsters studied here showed elevated lactic acid levels. The high concentrations of lactate measured following sham grading suggest that the lobsters would be acidotic at this time. However, the pH fluctuations were not major and there was no indication that exoskeletal $CaCO_3$ was entering the hemolymph.
Baseline hemolymph calcium concentrations varied between 13.0 and 14.5 mmol L^{-1} and there were significant differences ($P < 0.05$) between field trips, with the concentrations in January and April being significantly higher than in June and December. Apart from minor deviations, the concentration of calcium in the hemolymph remained relatively constant throughout the post-harvest handling chain and there were no consistent trends apparent (Figure 8).

7.2.2.4.2 Magnesium

Unlike calcium, hemolymph magnesium concentration increased following sham-grading. (Figure 8b). Baseline values of magnesium varied between 10.5 and 11.5 mmol L^{-1} and was significantly higher ($P < 0.05$) in January than at any other time. This difference tended to persist throughout the post-harvest process. A seasonal high in hemolymph magnesium level could be related to the moult cycle of lobsters (Mercaldo Allen 1991). With the exception of January, magnesium was significantly higher ($P < 0.05$) than baseline following sham-grading and tended to be lower than baseline during storage in factory tanks.
The significant rise in magnesium concentration is difficult to explain because this ion is seldom measured in physiological studies. There are a number of possible explanations for the observed rise:

1) movement of magnesium into the extracellular fluid from the intracellular space, which may result from the depletion of ATP with the resultant liberation of free magnesium (Burton 1980),
2) cessation or impairment of antennal gland function in the face of continued uptake from sea water in the gut or gill chambers (Malley 1977b), and
3) release of magnesium in the form of magnesium carbonate from the exoskeleton.

The rise in magnesium occurred in rock lobsters kept in air and must reflect an accumulation or re-distribution of magnesium within the lobster's body. Dehydration is a possible contributing factor, but as stated above, there is no evidence for it in the profiles of other ions. European lobster, *Homarus gammarus*, arriving in poor condition at a wholesale market also had elevated levels of magnesium in the hemolymph following dry transport (Whiteley and Taylor 1992). This was explained as a loss of regulatory capacity in the stressed animals, however the weak lobsters concerned were also in late pre-molt, (Whiteley and Taylor 1992). Pre-moult *H. americanus* and *P cygnus*, (see section 7.1.2.4) have elevated hemolymph magnesium concentration (Mercaldo Allen 1991). However, most lobsters harvested here were in intermoult and certainly, the proportion of various moult stages didn't vary during post-harvest handling. For further discussion of the effects of moult stage on magnesium and other parameters (see page 23).

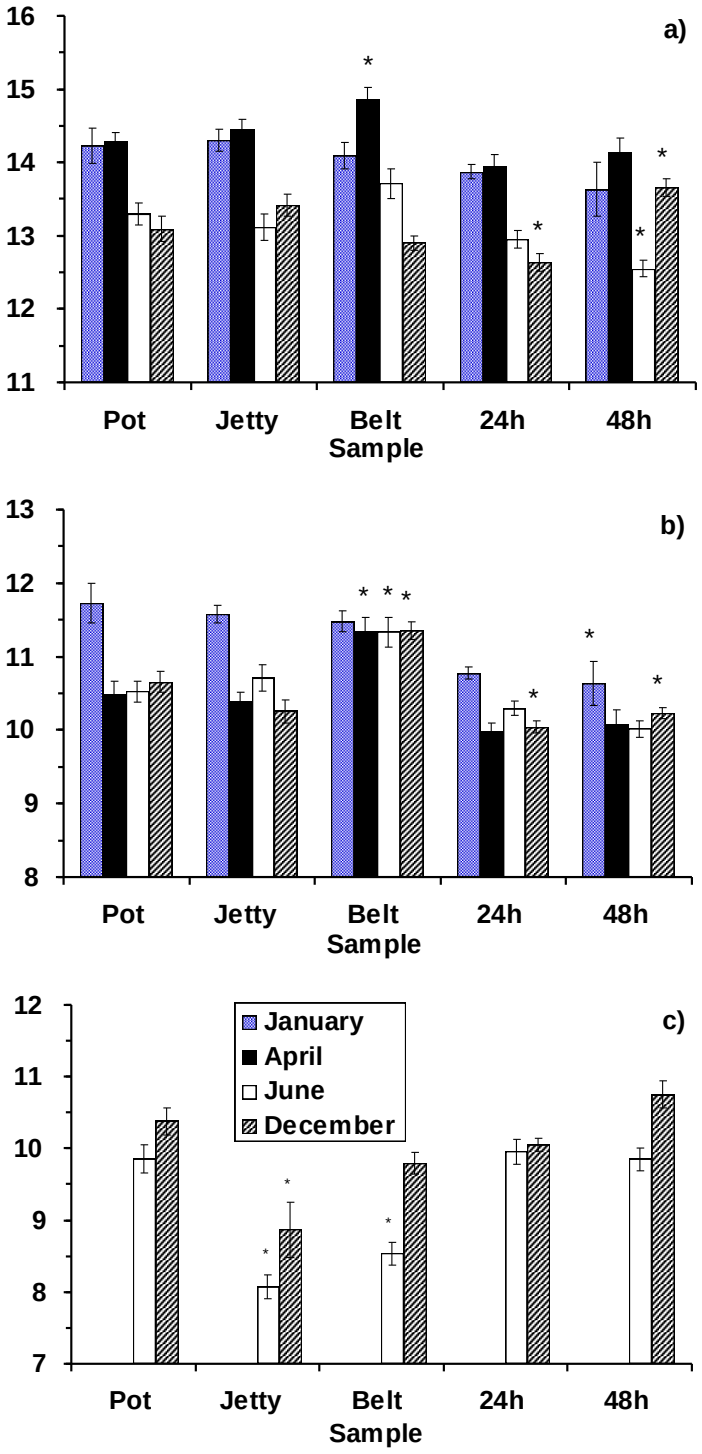


Figure 8. Changes in hemolymph concentrations (mmol L^{-1}) of a) calcium, b) magnesium and c) potassium in western rock lobsters *Panulirus cygnus* during different stages of post-harvest handling (see text). For further explanation of figure, see caption of Figure 7.

7.2.2.4.3 Potassium

Our attention in this study was originally focussed upon only calcium and magnesium. Since changes in magnesium were seen in the first couple of trips, attention was then given to potassium levels as well. This meant that hemolymph potassium concentration was determined for the June 1997 samples and December 1998 only (Figure 8c). Baseline potassium concentrations in the 2 months were similar (approx. 10.0 mmol L^{-1}). Potassium was significantly lower ($P < 0.05$) upon arrival at the factory jetty and, apart from being significantly lower ($P < 0.05$) after sham grading in June only, was not different to baseline values throughout the remainder of the post-harvest chain.

Clearly, no case can be put that there are broad shifts in ionic level when lobsters are kept out of water. If individual ions show different patterns of changes in concentration, then the reasons must be specific to those ions and not to general processes such as dehydration.

7.2.2.5 Vigour index

The vigour index (VI) values for animals that had been held in onboard storage tanks were similar to those in animals immediately after pot retrieval (Figure 9). Examination of the vigour index data shows that lobsters became less vigorous (that is, VI decreased) during handling after capture, reaching a low following sham grading when a large proportion of VI = 2 and 3 lobsters were observed. After 24 - 48 h in factory tanks, the proportion of animals classed as 5 on the VI scale was greater than at any prior sample time, including the baseline (pot) sample. This "excited" state may not be a baseline state at all, but rather may indicate heightened levels of disturbance brought on by the conditions of storage in the factory.

7.2.2.6 Protein

Hemolymph protein varied considerably between individual lobsters (60 - 130+ g L^{-1}). This variation was not entirely explained by typical moult stage categories as most lobsters were in inter-moult stage C. The frequency distribution of protein concentration was skewed toward the lower end of the range. The proportion of lobsters with hemolymph protein levels greater than 134 g L^{-1} (top of the scale of the refractometer being used) was higher in April (45%) than it was in June (24%). These high values are probably pre-molt lobsters, though as indicated previously, nutritional condition is also likely to influence hemolymph protein level. These effects of moult stage and nutrition have been discussed above (pages 23 and 31).

7.2.3 Boat/time effects

Significant differences were noted between the different boats and between different times of the year for a number of parameters. All variables, with the exception of magnesium, differed significantly between months ($p < 0.05$). All variables, with the exception of lactate, were significantly different between boats ($p < 0.05$). We have already alluded to the possibility that moulting related processes could explain some of the variation in hemolymph parameters observed from month to month, though other seasonal factors (eg. ambient air temperature) could play a role. Differences between boats are also to be expected because of the wide variation in factors such as boat design, experience of the fishers and distance of grounds from the factory.

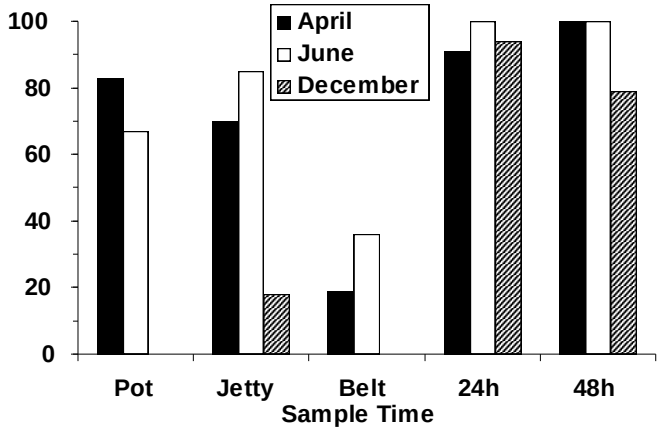


Figure 9. Changes in the proportion of "strong" (i.e. vigour index ≥ 4; see main text for details) individual western rock lobsters *Panulirus cygnus* during different stages of post-harvest handling (see text).

7.3 Responses to controlled emersion (Sub-objective 3)

7.3.1 Summary

Measurements in the field (reported above) showed that periods when lobsters were stored and handled in air caused a number of disturbances to their hemolymph physiology. As a number of things were going on under those circumstances, it was difficult to say exactly what was causing the changes. Probably the major reason for bringing lobsters into the lab and experimenting on them is that we can control the type of stress that the lobsters undergo and secondly that we can do more assays with the resources at hand. But this isn't done in isolation. The whole point of doing this experiment was to better understand the responses shown by lobsters in latter work, for example the factory-based experiments (sections 7.4 - 7.6). In this experiment we studied the physiological changes when seawater was drained without disturbance from around lobsters for 24h (at 20°C and approx. 100% relative humidity). We also studied the changes in those parameters after the seawater was returned for up to 2 days of recovery (for details see methods in section 6.4). Groups of lobsters were sampled at pre-determined times, 0, 3, 12 and 24h after emersion, and 1, 3, 8, 12, 24, and 48 hours after re-submersion. Because sampling may have disturbed other lobsters in the room, the experimental design involved sampling a parallel set of submerged controls for each time that emersed treatment lobsters were sampled. The methods are described in more detail in section 6.4.

The results show that when seawater was drained from around lobsters, without disturbance, in the laboratory, hemolymph oxygen level fell precipitously. The lobsters consequently showed all the classical signs of asphyxiation, accumulating carbon dioxide and lactate acid in their hemolymph, with both changes contributing to a dramatic fall in hemolymph pH. The accumulation of lactate was accompanied by a rise in the circulating concentration of glucose, a source of "energy" for metabolism and the tail muscle itself showed stable energy levels (in terms of ATP concentration) during this time. Levels of two electrolytes in the hemolymph, magnesium and potassium, increased. However hemolymph protein concentration did not change, indicating that the high humidity was preventing dehydration. When the laboratory tanks were refilled with seawater, the hemolymph oxygen level became very high for a short period and the hemolymph lactate, glucose and electrolyte levels abruptly returned to baseline levels within 3 hours and thereafter remained constant.

Lobsters handled this stress (being out of water for 24 hours at ambient temperature) surprisingly well, and recovered rapidly when re-submerged. The same parameters that changed in our earlier field trials changed here, but furthermore we can see that deviations in these parameters, while associated with an extended period of oxygen deprivation, do not necessarily signify stress of sufficient magnitude to kill lobsters. Energy levels in the tissue were sustained. Stress clearly happens, but it is not necessarily going to kill lobsters.

A practical corollary of this is associated with the revelation that lobsters do not appear to be substantially oxygenating their hemolymph when kept out of water at ambient temperature. If this is because the gill filaments are too wet and matted together, then it may be misguided to spray seawater onto lobsters in order to help them to endure in air. On the contrary, spraying seawater may have the opposite effect, that of further suffocating the lobsters. This point will be picked up further in sections 7.5 and 7.6, where we evaluate whether spraying seawater of lobsters actually helps them to live longer out of water.

The remainder of this section examines the results on a parameter by parameter basis, examining the effects of emersion on…

- gases and pH of the hemolymph, section 7.3.2
- lactate and glucose levels, sections 7.3.3 to 7.3.5
- electrolytes in the serum, section 7.3.6
- hemolymph protein and hemocyanin section 7.3.7
- muscle adenylate levels, section 7.3.8, and
- lobster liveliness or 'vigour,' section 7.3.9.

A brief glossary of the terms and units used in this chapter is presented in Table 3. As this study occurred in collaboration with the companion project FRDC 96/344 there are also immune system results for the lobsters sampled in this trial. This data is not presented here in this report and the reader should refer to the corresponding section of the other report.

Table 3. Glossary of technical terms and abbreviations.

Word, symbol or phrase	Explanation
AEC	Adenylate energy charge, an index of cellular energy available in the form of adenosine triphosphate (ATP) and adenosine diphosphate (ADP).
A-V difference	The difference in dissolved oxygen level between hemolymph entering the gill and hemolymph leaving the gill. When applied to the oxygen dissociation curve of the respiratory pigment, the A-V difference determines how much oxygen the pigment binds as the hemolymph passes by the gills
branchial	Branchiae = gill. Branchial. See pre- and post-branchial
carbon dioxide content	Carbon dioxide dissolves readily in solution, so much of the gas in hemolymph is actually present as bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions rather than as the free gas CO_2, so a measure of 'carbon dioxide content' is necessary in order to assess respiration. The dissociated forms of carbon dioxide are involved in buffering of hemolymph pH.
$CvCO_2$	Abbreviation of venous carbon dioxide content (above),
emersion	Leaving the water (antonym of 'immersion')
$PvCO_2$	Venous carbon dioxide partial pressure (see torr). See also Total venous carbon dioxide content
$Pa0_2$	Arterial oxygen partial pressure (see torr)
$Pv0_2$	Venous oxygen partial pressure (see torr)
pHa	Arterial pH
pHv	Venous pH
Post-branchial hemolymph	Hemolymph leaving the gills, usually rich in oxygen, and bound for the heart. Equivalent to 'arterial' in vertebrate circulation. Equivalent to 'arterial' in vertebrate blood circulation, hence usually abbreviated as PaO_2
Pre-branchial hemolymph	De-oxygenated hemolymph flowing from amongst the body tissues and entering the gills. Equivalent to 'venous' in vertebrate blood circulation, hence usually abbreviated as PvO_2
torr	A unit of barometric pressure, equivalent to one millimetre of mercury (Hg) and sometimes written as mmHg. When equilibrated to the atmosphere or medium, all gases present in a solution such as hemolymph have a total pressure that adds up to 1 atmosphere or 760 torr. In an equilibrated solution, oxygen contributes a "partial pressure" equivalent to its typical atmospheric composition (around 21% of 760 torr) or just under 160 torr. Of course, levels in the hemolymph are usually much lower than this ideal.

7.3.2 Hemolymph gases and pH

7.3.2.1 Oxygen

The gills of a lobster are adapted to extract oxygen from seawater and will not function as well in air. The hundreds of minute filaments that comprise the surface for gas exchange are well bouyed up in the water but collapse and matt together in air.

Prior to emersion, values of pre- and post-branchial oxygen partial pressure (P_vO_2 and P_aO_2) were stable at approximately 18-19 torr and 30 - 50 torr, respectively. One hour after exposure to air, post-branchial or "arterial" oxygen partial pressure (P_aO_2) fell significantly ($p < 0.05$) compared to pre-emersion values, but not compared to the control ($p > 0.05$) (Figure 10). A marked depression of P_aO_2 appears to be a generalised response of aquatic decapods to air exposure (DeFur and McMahon 1984; Morris and Oliver 1999; Whiteley and Taylor 1992). The P_aO_2 remained at similar levels for the duration of the 24 h emersion period. One hour after emersion, the pre-branchial ("venous") oxygen partial pressure (P_vO_2) declined significantly ($p < 0.05$) compared to controls, but had recovered to control levels by 3 h of air exposure. As a consequence of the relative decline in P_aO_2 in air, the change in oxygen partial pressure as hemolymph traversed the gill was reduced to a mere 2-3 torr. Immediately following reimmersion, both P_aO_2 and P_vO_2 showed a significant increase ($p < 0.05$) compared to the controls. By 3 h after reimmersion P_vO_2 had returned to control levels, but P_aO_2 remained elevated for between 4 - 8 h after reimmersion.

Reoxygenation of hemolymph at the gills was severely limited in air, despite the much higher concentration of oxygen in air than water. Presumably, in the absence of a substantial venous reserve, hemocyanin plays a more significant role in delivering oxygen to the tissues, with any shortfall being met through anaerobic processes. From this perspective it would be informative to examine changes in hemolymph oxygen content during air exposure and reimmersion.

Immediately following reimmersion, P_ao_2 increased greatly, and now the change in oxygen partial pressure as hemolymph traversed the gill was quite marked. The elevated values of P_ao_2 presumably contribute to repayment of an oxygen debt accrued during the exposure period. The presence of an oxygen debt was indicated by the accumulation in the hemolymph of lactate during exposure to air (section 7.3.3). In a similar study, oxygen consumption in *J. edwardsii* following 8 h of exposure was elevated for up to 8 h following reimmersion (Taylor and Waldron 1997). In addition, ventilation rates in *Cancer productus* were elevated for several hours following a period of air exposure (DeFur and McMahon 1984). These observations correspond well with those of the present study and help to explain the relative hemolymph alkalosis occuring in *P. cygnus* following air exposure (see Figure 12).

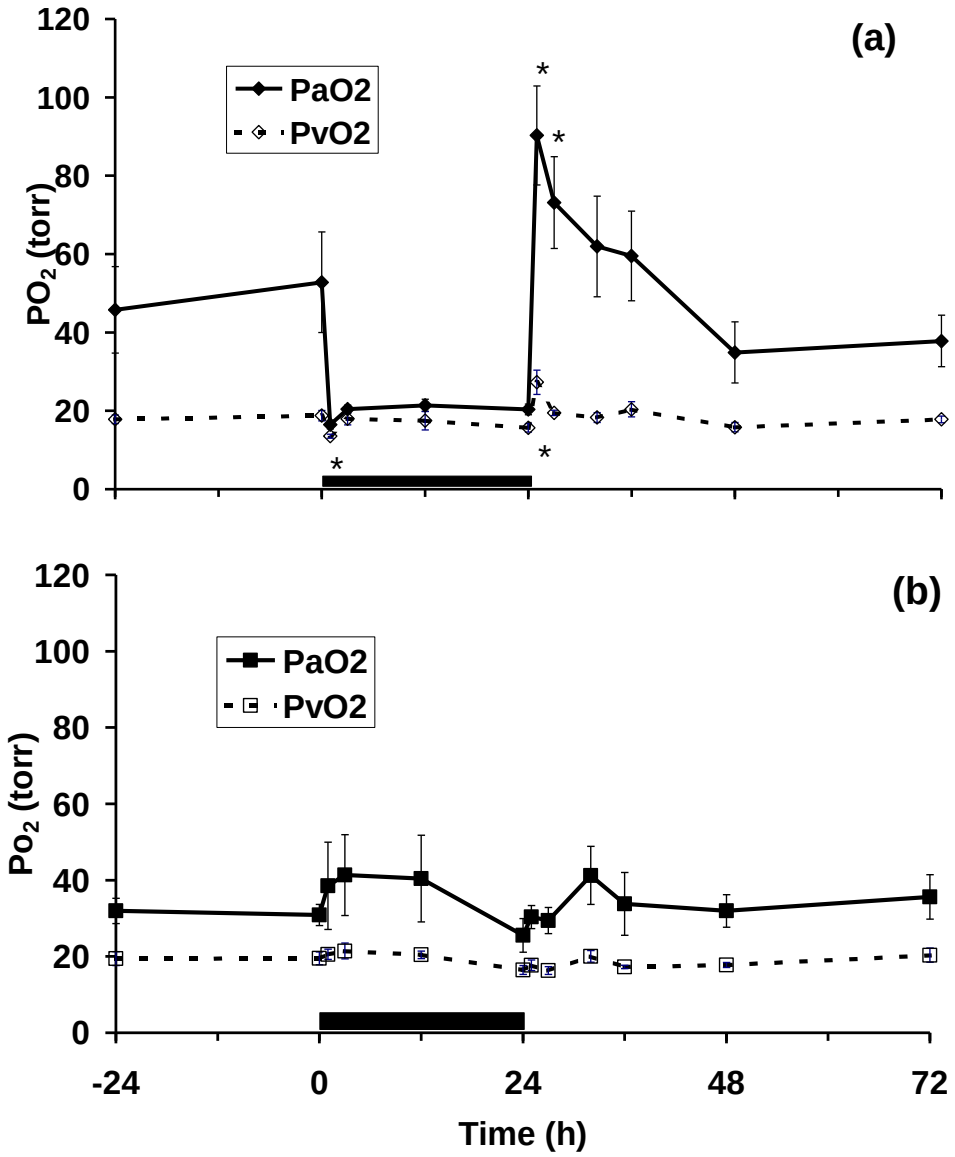


Figure 10. Changes in a) pre- and post-branchial Po_2 in western rock lobsters *Panulirus cygnus* during a 24 h period of exposure to air (horizontal black bar) followed by 48 h of reimmersion. b) Pre- and post-branchial Po2 in control animals during the experiment. Each point is the average (±SE) of 12-16 lobsters

7.3.2.2 Carbon dioxide and pH

The reduced capacity of the lobster's gills to function in air impedes not only oxygen uptake, but also the excretion of carbon dioxide. Total pre-branchial CO_2 content (C_vCO_2) showed a rapid increase during emersion and was significantly higher than control values ($p < 0.05$) after 1 h and 24 h of exposure to air (Figure 11a). Following reimmersion C_vCO_2 showed a general trend to decrease below control levels and was significantly depressed ($p < 0.05$) 8 h and 24 h following emersion. Total bicarbonate ion (HCO_3^-) content showed a similar pattern of variation to C_vCO_2 (Figure 11b).

Immediately following emersion calculated P_vCO_2 began to rise and was significantly elevated above control levels ($p < 0.05$) after 1 h of exposure to air (Figure 11c). P_vCO_2 increased steadily throughout the emersion period and showed no sign of reaching a maximum within the 24 h emersion period. P_vCO_2 remained significantly elevated ($p < 0.05$) after 1 h of reimmersion, but for the 24 h thereafter tended to be lower than control values. After 48 h of reimmersion, P_vCO_2 had returned to control values.

Prior to emersion, pre-branchial pH (pH_v) was approximately 7.65 - 7.70 (Figure 12). Following emersion pH_v began to fall and was significantly depressed ($p < 0.05$) within 1 h. Pre-branchial pH continued to drop during the emersion period and reached a minimum of 7.24 ± 0.06 after 24 h emersion. Pre-branchial pH began to recover immediately following reimmersion. One hour after reimmersion pH_v was still significantly lower ($p < 0.05$) than that in control animals but by 3 h had recovered to control levels. Despite a lack of statistical significance, following reimmersion pH_v tended to remain at lower values than in control animals. There was no significant difference between pre- and post-branchial pH ($p > 0.05$).

The accumulation of carbon dioxide resulted in the development of a respiratory acidosis, although the acidosis clearly had a metabolic component, as evidenced by the accumulation of lactic acid in the hemolymph (below). The resultant acidosis can be compensated for by a rise in HCO_3. Indeed, a general rise in HCO_3 was observed in *P. cygnus* following exposure to air. Normally when submerged, HCO_3 can be transported across the gills via a Cl^-/HCO_3 exchange (Wheatly and Henry 1992), when in air however, this source of buffering is unavailable. Alternatively HCO_3 may be mobilised as $CaCO_3$ and $MgCO_3$ from the exoskeleton, producing a concomitant increase in both hemolymph calcium and HCO_3. However no continuous accumulation in calcium or HCO_3 ions was found here, and any compensatory mechanisms failed to counteract the large acidosis during air exposure (Figure 12).

Hemolymph pH_v and P_vCO_2 tended to be higher and lower, respectively than in control animals, for at least 24 h after reimmersion. This suggests the development of a respiratory alkalosis following reimmersion, probably due to persistently high rates of ventilation. It also implies that full recovery took somewhat longer than suggested by values of P_aO_2.

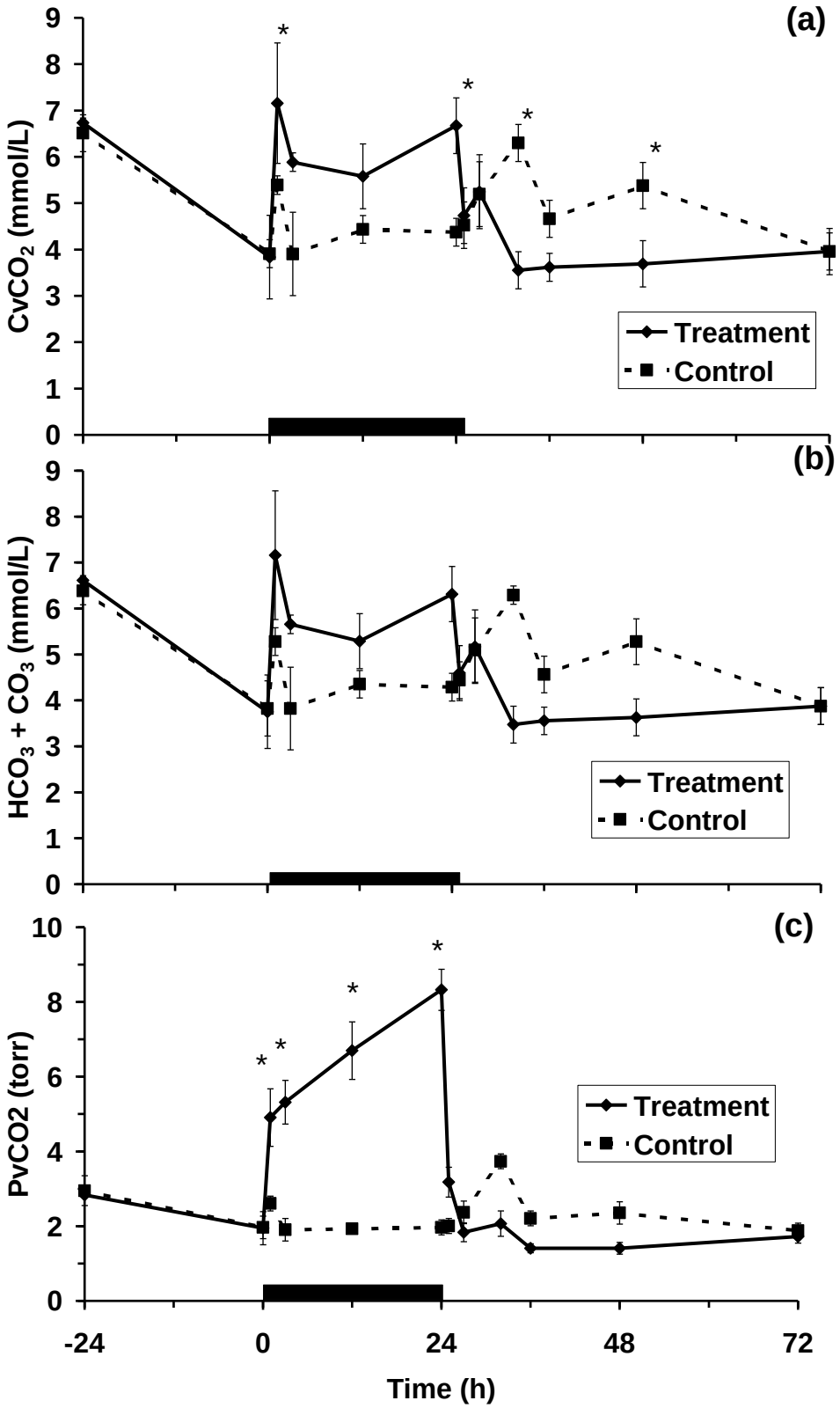

(a)
CvCO2 (mmol/L)
Treatment
Control
(b)
HCO3 + CO3 (mmol/L)
Treatment
Control
(c)
PvCO2 (torr)
Treatment
Control
-24
0
24
48
72
Time (h)

Figure 11. (preceding page) Effect of emersion on carbon dioxide levels in western rock lobsters *Panulirus cygnus*. (a) Changes in measured total carbon dioxide content ($CvCO_2$); (b) calculated levels of bicarbonate and carbonate ions (HCO_3^- and CO_3^{2-}) and (c) calculated carbon dioxide partial pressure ($PvCO_2$) during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.

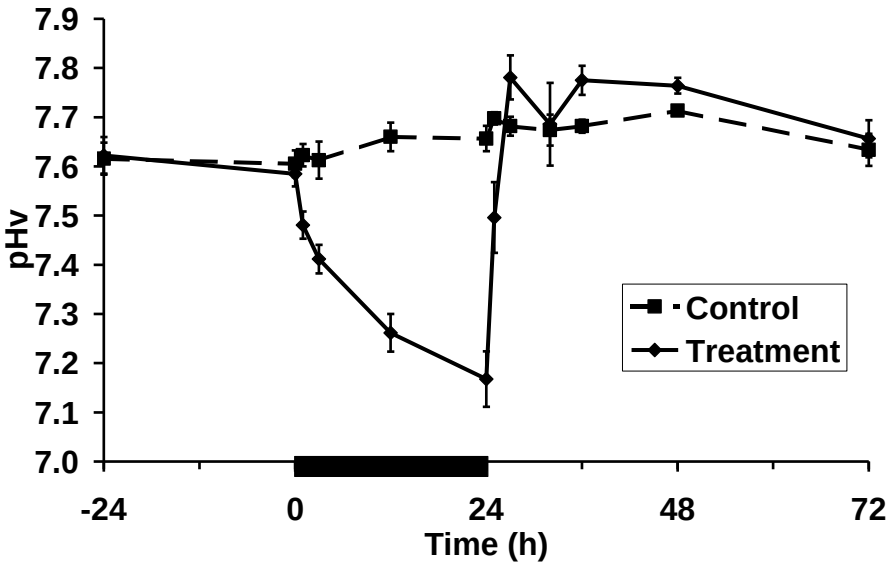


Figure 12. Changes in venous hemolymph pH (pHv) levels in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.

7.3.3 Hemolymph Lactate

The levels of lactate in the hemolymph of the lobsters before seawater was withdrawn from the tanks were quite low (0.3 - 0.5 mmol L^{-1}). This is somewhat lower than values we have measured in free-ranging *P. cygnus* sampled underwater by SCUBA divers (see section 7.1.2.2.2). Similar differences in basal lactate concentrations between free-ranging and captive decapods have been reported in *Callinectes sapidus* (Lowery and Tate 1986), and *Carcinus maenas* (Houlihan and Mathers 1985). These differences probably reflect higher levels of routine activity in free-ranging animals and also perhaps a reduction in the capacity to generate lactate after long periods of relative inactivity whilst in captivity.

Exposure to air resulted in a progressive increase in hemolymph lactate, so that after 12 h lactate was significantly elevated ($P < 0.05$) above control values (Figure 13). The rise in hemolymph lactate concentration report here in *P. cygnus* in response to air exposure is very similar to that reported for other lobsters, including other spiny lobsters (*Jasus edwardsii*) (Morris and Oliver 1999; Taylor and Waldron 1997) and clawed lobsters (eg *Homarus gammarus* (Whiteley and Taylor 1990)). In decapod crustaceans, lactate enters the hemolymph from the tissues by simple diffusion (Kinsey and Ellington 1996).

Following reimmersion, hemolymph lactate concentration decreased rapidly and had returned to a value similar ($P > 0.05$) to that in control lobsters within 3 h of reimmersion.

7.3.4 Hemolymph versus tissue lactate

The data presented in Figure 14 show muscle lactate concentrations and hemolymph lactate concentrations from a number of lobsters used in the experiment. The diagonal line indicates the line of equimolar tissue and hemolymph lactate concentrations. Most of the points lie below this line, indicating tissue lactate concentrations exceeding hemolymph values, as is expected. However a number of the points lie above the line indicating hemolymph lactate concentrations exceeding those in the muscle. This suggests either that…

a) the abdominal muscle is not the primary source of lactate appearing in the hemolymph or,
b) the lactate concentration may not be uniform throughout the muscle and the single biopsy taken was not representative of the average muscle lactate concentration.

In lobsters the abdominal muscles are large and it may be prudent to take multiple biopsies from each animal for determination of an average lactate concentration.

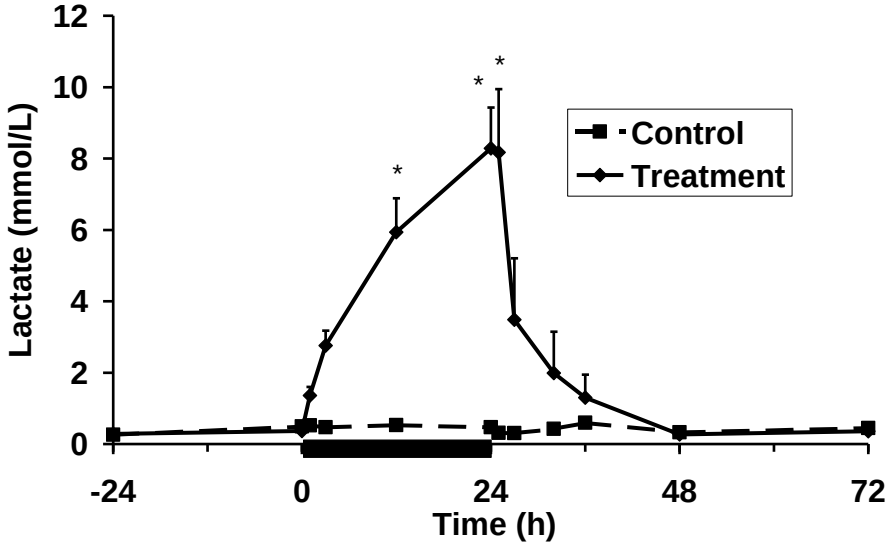


Figure 13. Changes in hemolymph lactate concentration in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.

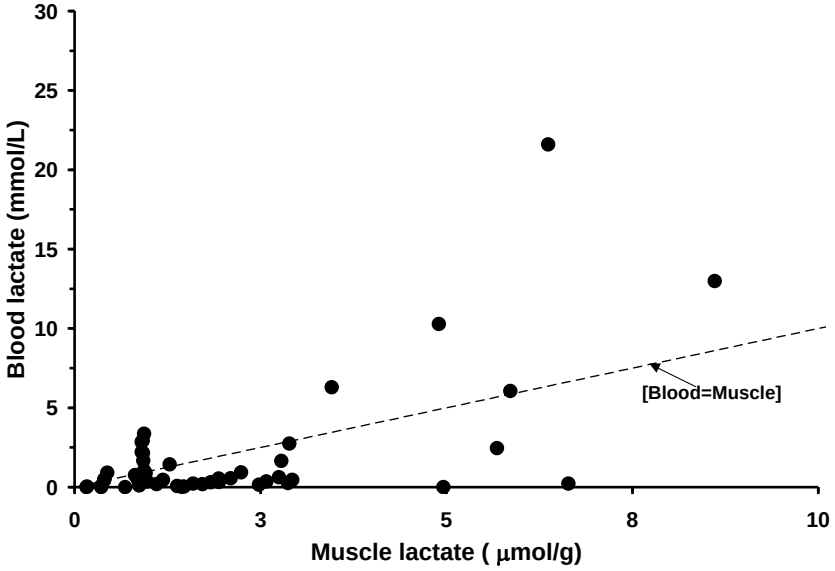


Figure 14. Comparison of measurements of hemolymph and muscle lactate concentrations in western rock lobsters *Panulirus cygnus* during the experiment.

7.3.5 Glucose

Along the lines of the rise in lactate level, the glucose concentration began to increase immediately following exposure to air and was significantly ($P < 0.05$) elevated within 12 h (Figure 15). Mean glucose concentration continued to increase throughout the exposure period peaking at 3.6 ± 0.7 mmol L^{-1} after 24h. Following reimmersion, glucose concentration declined rapidly and was restored to control values within 3 h ($P > 0.05$).

Hemolymph glucose level is regulated by hormones, though the details of the process are poorly known. Eyestalk ablation retards hyperglycaemia and metabolism in emersed crayfish (Santos and Keller 1993). It seems reasonable to assume that the hemolymph glucose level is ramped up (by breakdown of glycogen reserves?) to release substrates to supply the anaerobic glycolysis (lactate production) that must sustain tissue metabolism. This correlation between rising glucose and lactate concentrations doesn't necessarily mean that lobsters with high lactate levels necessarily have high glucose levels. Later stress trials (sections 7.4 and later) will show that glucose level was effectively independent of lactate concentration. There is probably a limit to how much glucose is available to flush into circulation, while lactate accumulates and probably remains in circulation until conditions favour its removal.

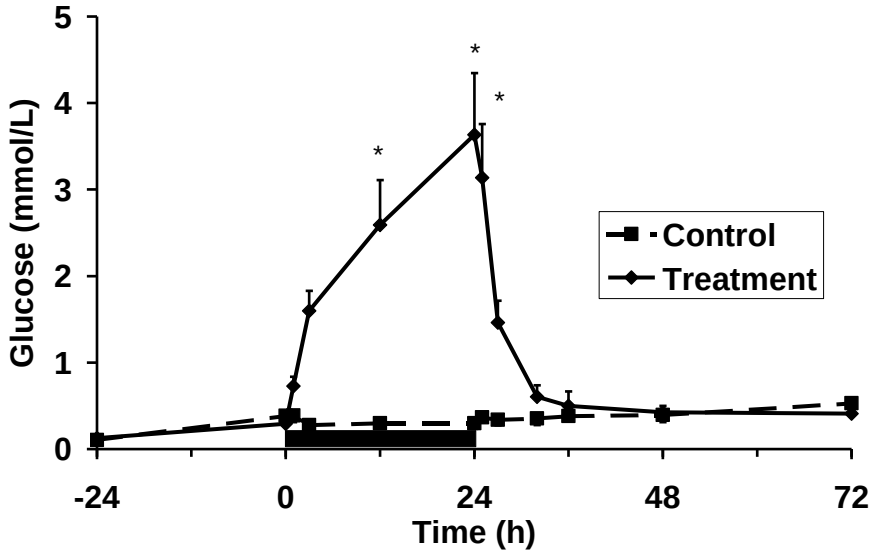


Figure 15. Changes in hemolymph glucose concentration in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.

7.3.6 Serum electrolytes

Levels of a number of electrolytes are regulated in the hemolymph of western rock lobsters. The active transport of electrolytes across cell membranes requires energy, and if cellular energy levels are compromised by stress, then changes in levels of electrolytes may follow. There is also a more prosaic reason for monitoring electrolytes following stress. The exoskeleton or 'shell' of many crustaceans contains high levels of calcium and magnesium carbonates, and entry of these compounds into solution when the animal's are in air is thought to play a major role in countering changes in acidity in the animal's tissues and hemolymph.

7.3.6.1 Calcium

Serum calcium concentration was not significantly affected by exposure to air (Figure 16). In earlier work, a brief period of exposure to air at ambient temperature also had no significant impact on hemolymph calcium levels (section 7.2.2.4.1). Yet, increased hemolymph calcium is commonly associated with acidosis in crustaceans. Increased calcium concentration apparently arises from the mobilisation of carbonate buffers from the exoskeleton (DeFur *et al.* 1980). However in *P. cygnus*, changes in serum calcium during air exposure did not parallel the changes in pH, indicating the two parameters were not closely related. In addition, hemolymph pH continued to decline throughout the period of air exposure suggesting that any mobilisation of exoskeletal ions was ineffectual at buffering the acidosis. This is consistent with findings that mobilisation of exoskeletal buffers during hypercapnic acidosis (that is, acidosis brought about by high dissolved carbon dioxide levels) in the blue crab *Callinectes sapidus* could only account for a very small fraction of the observed compensation (Cameron 1985). The suggestion is that the contribution of exoskeletal buffers to acid-base regulation is more important in terrestrial species than in aquatic species (Wheatly and Henry 1992). The latter being able to access an external pool of base via gill ionic exchanges.

7.3.6.2 Magnesium

Unlike calcium, serum magnesium rose steadily throughout the emersion period and was significantly elevated ($p < 0.05$) after 12 h of air exposure (Figure 16). This is consistent with earlier observations during post-harvest handling of lobsters (Section 7.2.2.4.2). Following re-immersion, magnesium concentration had recovered to control levels within 3h.

Baseline levels of magnesium prior to air exposure were somewhat lower than those reported by Dall (1974a), but compare well to values measured in free-ranging intermoult *P. cygnus* sampled in the wild by SCUBA divers (Section 7.1.2.2.4). The magnesium concentrations in *P. cygnus* are among the lowest reported for any decapod species (Tentori and Lockwood 1990) but are consistent with the general correlation in decapods between extracellular magnesium and activity (Morritt and Spicer 1993). *P. cygnus* is a very active species, undertaking large daily foraging trips and large seasonal migrations (Jernakoff *et al.* 1987).

The dynamics of serum calcium and magnesium were quite different suggesting other sources and/or mechanisms of regulation. The increase in serum magnesium during air exposure closely parallelled changes in hemolymph pH, suggesting a possible relationship between the two. Interestingly, previous workers have identified an association between elevated hemolymph magnesium concentration and severe acidosis in *Homarus gammarus* that arrived at market in poor condition following commercial transport, (Whiteley and Taylor 1992).

There are essentially two possible sources of magnesium entering the extracellular fluid, either from inside or 'outside' of the organism. The intracellular concentration of free magnesium in invertebrates appears to be regulated at much lower concentrations than in the extracellular fluid (Ashley and Ellory 1972; Burt and Kleps 1992; Doumen and Ellington 1992). Still, there is a substantial pool of magnesium bound to various intracellular constituents, and in particular ATP (Ashley and Ellory 1972; Brinley *et al.* 1977; Doumen and Ellington 1992). The proportion of ATP bound to magnesium

in muscle of the horseshoe crab *Limulus polyphemus* decreased under conditions of anoxia and acidosis but the concentration of free magnesium remained unchanged (Doumen and Ellington 1992). These authors suggested the presence of an outwardly directed magnesium transport system for maintaining intracellular magnesium concentration. In the present study no decrease in ATP in abdominal muscle was recorded (see section 7.3.8) suggesting, at least in this tissue, intracellular magnesium was not liberated through ATP depletion.

The second possible source of magnesium during air exposure is the fluid in the gut, technically 'outside' of the animal. Magnesium levels are determined by a balance between uptake at sites such as the gut and excretion by the antennal glands (Malley 1977a; Malley 1977b). The magnesium concentration in seawater is approx. 5.5 times greater than in the hemolymph. Any seawater that is ingested prior to air exposure provides a source for the influx of ions into the hemolymph. Assuming no effect of air exposure on magnesium uptake across the gut, increased extracellular magnesium could only occur if antennal gland function was impaired. Indeed, urine production has been shown to decrease during air exposure of semi-terrestrial and intertidal decapods (Harris and Santos 1993) and freshwater decapods (Tyler-Jones and Taylor 1986). Presumably this is a strategy to conserve water and ions during air exposure. Whether a wholly sub-tidal species, such as *P. cygnus*, utilises a similar stategy remains to be determined.

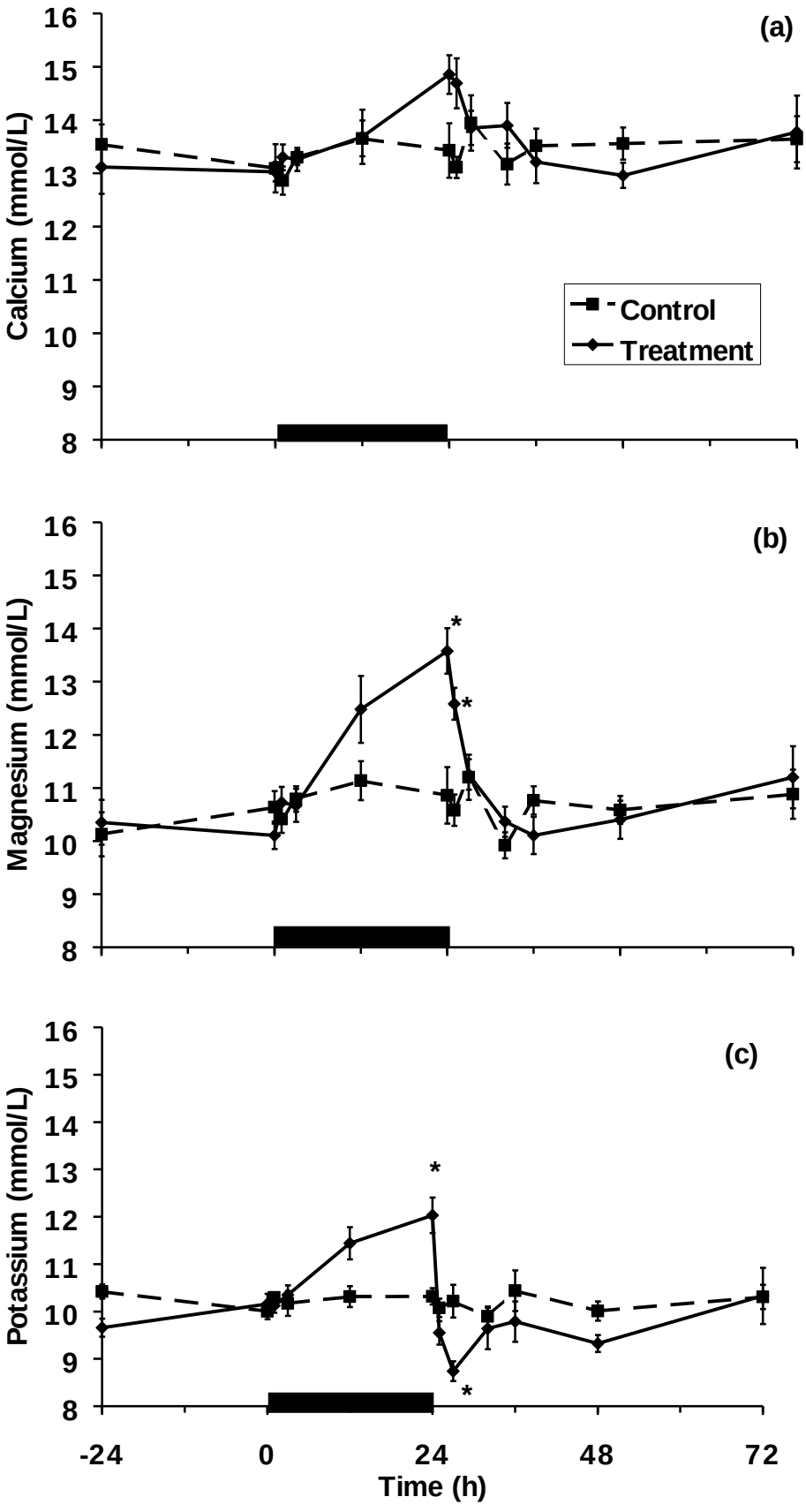
7.3.6.3 Potassium

Serum potassium increased during emersion and was significantly elevated ($p < 0.05$) after 24 h (Figure 16). Following re-immersion, potassium levels returned to control levels within 1 h, but then continued to fall, reaching a value after 3 h that was significantly lower ($p < 0.05$) than in controls. Potassium had returned to control levels by 8 h after re-immersion.

In control and recovered *P. cygnus* serum potassium concentrations were similar to those reported previously (Dall 1974a). These values also agreed well with those recorded from free-ranging lobsters in the wild, (section 7.1.2.2.4). Serum potassium concentration increased during exposure to air, but not immediately. This was not consistent with measurements made during the earlier sham grading experiment (section 7.2.2.4.3). There, the rise in serum potassium level may have been masked by a previous fall in concentration during on-board handling.

The emersion experiment also revealed a falling serum potassium level. Unlike magnesium, potassium showed a complex response upon reimmersion, along the lines of an "over-correction," (a sharp fall in potassium concentration to below baseline) followed by a return to equilibrium. Remember that a fall and a protracted rise in serum potassium level occurred when recently caught lobsters were returned to seawater, during the captive acclimation studies (section 7.1.4.5). The most probable source and sink for these apparent movements of potassium ions was the intracellular compartment. These changes may reflect adjustments in cellular ionic regulation. In *Nephrops norvegicus* the total concentration of potassium in the muscle is actively regulation to around 77 times higher than in the hemolymph (Robertson 1961). However it is also possible that some potassium may have entered the hemolymph from the gut (Malley 1977a).

Figure 16. (following page) Variation in serum concentrations of a) calcium, b) magnesium and c) potassium in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.


(a)
Calcium (mmol/L)
16
15
14
13
12
11
10
9
8
Control
Treatment
(b)
Magnesium (mmol/L)
16
15
14
13
12
11
10
9
8
*
*
(c)
Potassium (mmol/L)
16
15
14
13
12
11
10
9
8
*
*
-24
0
24
48
72
Time (h)

7.3.7 Protein and hemocyanin

In previous field and acclimation work we had already established that protein level in the hemolymph varied considerably from lobster to lobster. Usually the bulk of the protein in circulation is the oxygen-carrying pigment oxy-hemocyanin, which gives the characteristic blue-grey tint to crustacean hemolymph. Here, we were concerned to see what happened to the gross level of protein in the hemolymph during emersion as well as trying to interpret what the hemocyanin was doing.

Total hemolymph protein was indeed quite variable between animals, ranging from 37-162 g L^{-1}. This range spans the data reported for fed and starved *P. cygnus* by previous studies (Dall 1974b). After accounting for significant differences ($p < 0.05$) in protein between replicates of the experiment, (presumably reflecting changing 'condition' of lobsters within the fishery), aerial exposure was shown to have no significant effect ($p > 0.05$) on hemolymph total protein (Figure 17). Relatively constant levels of protein during emersion and recovery suggest that no appreciable shifts of fluid into, or out of, the extracellular compartment occurred that might explain the general rise in the concentration of other hemolymph constituents toward the end of the emersion period.

The contribution of hemocyanin to the total protein level was also of interest. There was a significant though not tight relationship between the size of the absorbance peak of hemocyanin (measured at 340nm) and the total protein content of the hemolymph (Figure 18). Perhaps this isn't so surprising since hemocyanin is probably the major protein present in the samples, though the amount of variation for a given total protein level is considerable. A further point to note from the figure is that the proportion of hemocyanin in the hemolymph does not appear to keep in step as the protein content rises. On average, a lobster with 60g L^{-1} protein in its hemolymph has a hemocyanin absorbance of 0.509 units, however a lobster with twice as much protein in its hemolymph (120g L^{-1}) does not show twice the Hcy absorbance (only 0.869 units). The implication here is that much of the protein in low protein samples is hemocyanin, whereas other proteins may be displacing hemocyanin as the total protein level rises. It should be remembered that in lobsters and other crustaceans, protein concentration is linked to ecdysis and can rise simply by the same amount of protein circulating within a reduced hemolymph volume in late intermoult animals.

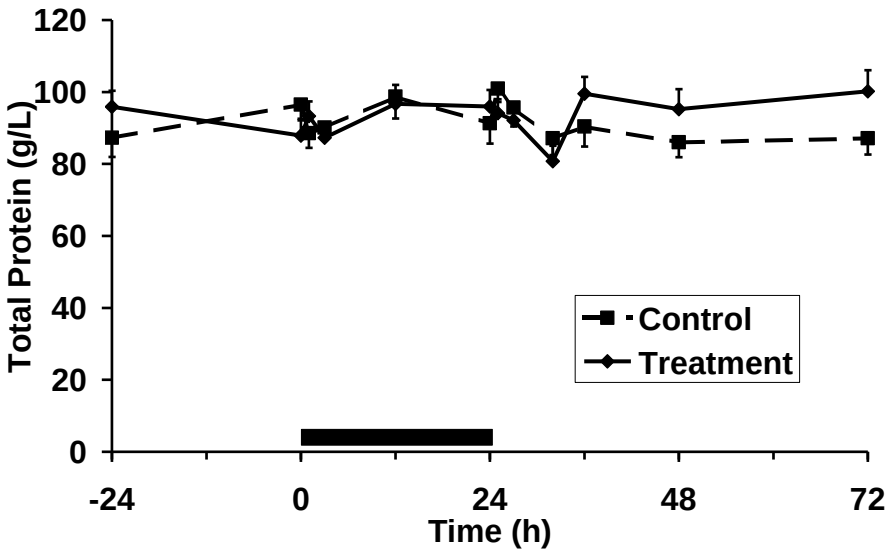


Figure 17. Hemolymph protein concentration in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.

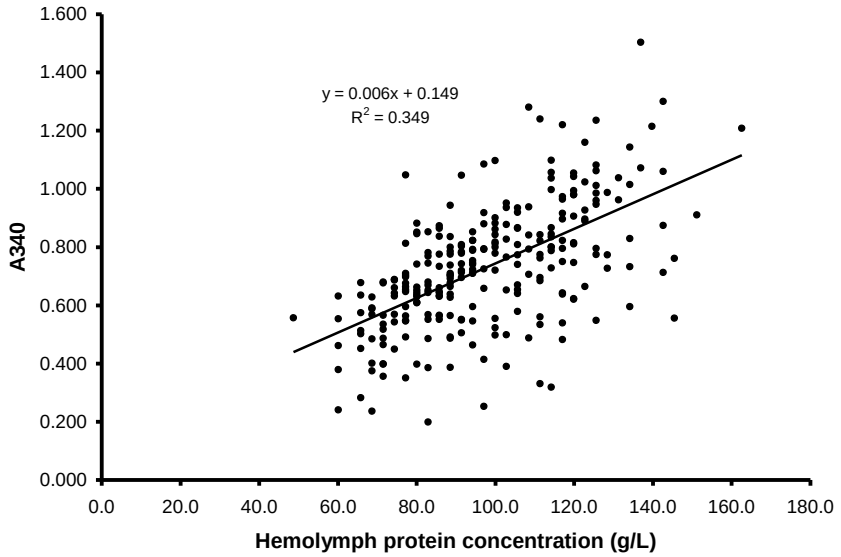


Figure 18. The relationship between the absorbance of oxy-hemocyanin (at 340 nm) and the total protein concentration in the hemolymph of western rock lobsters *Panulirus cygnus*.

7.3.8 Tissue Adenylates

In *P. cygnus*, the combination of aerobic and anaerobic metabolism may have produced sufficient ATP to meet the animal's requirements during air exposure, at least, there was no reduction in adenylate energy charge (AEC) of abdominal muscle (Figure 20). Though of course other muscles and tissues may have been more severely effected, these may explain the loss in vigour observed (below).

Concentrations of ATP, ADP and AMP in the abdominal muscle of control lobsters were 8.31 ± 0.25, 4.45 ± 0.31 and 0.27 ± 0.07 mmol g^{-1} (n = 38), respectively. The mean value of AEC in submerged control lobsters was 0.82 ± 0.01. Twenty four hours of air exposure had no significant effect ($P > 0.05$) on values of AEC of the abdominal muscle.

The values of AEC we report here are within the range (0.8 - 1.0) suggested to occur under optimal physiological conditions (Ivanovici 1980), which is perhaps surprising as one would not consider storage in air to be an optimal environment for this species. Of course, it could be argued that 'functional' anaerobiosis from extreme tail-flip exercise is a normal physiological 'condition' of the abdominal musculature in tailed decapods and under the circumstances prevailing here, this could allow the abdominal muscle to sustain AEC during the functional anaerobiosis occasioned by emersion.

7.3.9 Lobster Vigour

The proportion of strong lobsters (VI ≥4) in control groups varied between 78 - 100 % (Figure 21). The proportion of strong lobsters was significantly reduced ($P < 0.05$) after 3 h of exposure to air and remained so until 1 h after reimmersion. Between 3 and 8 h after reimmersion the proportions of strong lobsters in treament groups was not significantly different ($P > 0.05$) from those of control groups. However, between 12 and 48 h following reimmersion, the proportions of strong lobsters were again significantly lower than in controls ($P < 0.05$).

These apparent variations in vigour during and following air exposure did not correspond to variations in AEC. The general depression in vigour observed after air exposure may have resulted from the direct effects of prolonged hypoxia on the central nervous system of the animals, rather than peripheral effects on the muscles themselves.

The major difference between 'strong' lobsters and less vigorous lobsters is in the tail-flip behaviour. A grade 3 lobster will move its appendages and can still maintain its abdominal posture, whereas strong lobsters (VI≥4) flick their abdomen vigourously when handled. Of course, at level 5 they also resist manipulation actively.

Emersion is also known to retard the tail-flip response in other lobsters, and this vulnerability is a major problem with release of emersed individuals into the wild (Brown and Caputi 1983; van der Meeren 1991; Vermeer 1987). At present the reason for this lingering behavioural effect on the lobsters in this experiment is not known, as the physiological parameters measured in the stressed lobsters had recovered to control levels.

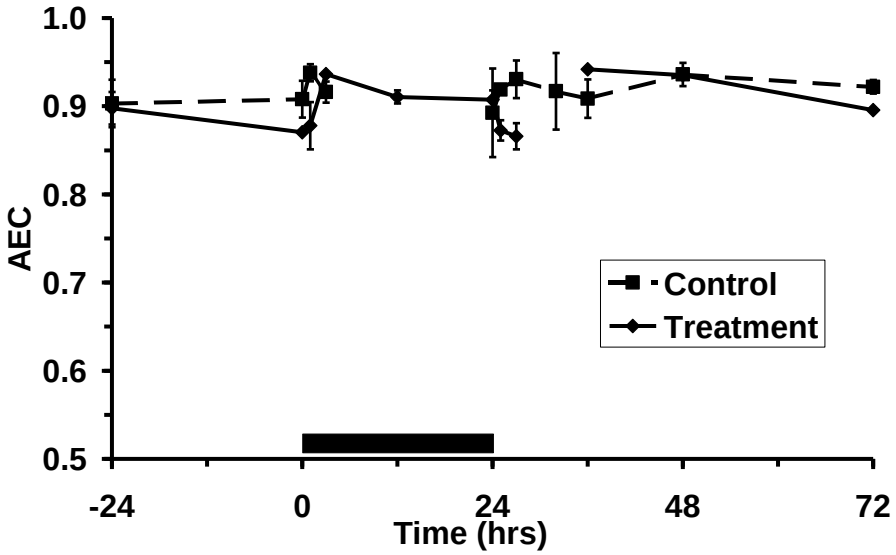


Figure 20. Changes in muscle adenylate energy charge (AEC) in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each point is the average (±SE) of 12-16 lobsters.

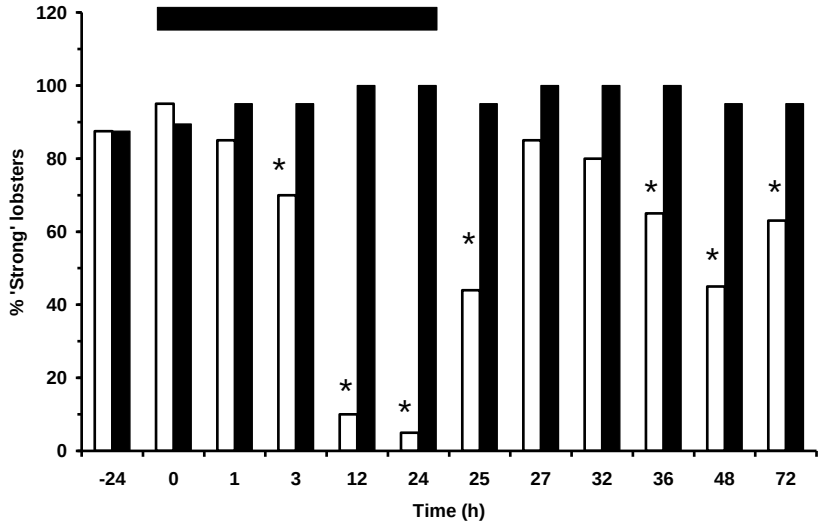


Figure 21. Changes in proportion of "strong" lobsters (vigour index, VI >= 4) in western rock lobsters *Panulirus cygnus* during 24 h of air exposure (horizontal black bar) followed by 48 h of reimmersion. Each bar is based on 12-16 lobsters. Asterisk (*) indicates that the treatment proportion (white bar) is significantly different from that of the corresponding control (black bar).

7.4 Validation of indicators (pilot study, March 1998) (subobjective 4)

7.4.1 Summary

Storing and transporting lobsters out of water clearly has a major impact on the hemolymph chemistry, as indicated in the preceding section 7.3. The next question is whether this matters in a practical context. Do the lobsters that weaken and die in factories show unusual levels of these parameters at some point before they die?

To establish the relationship between stress indicators and mortality we subjected lobsters to a simulated period of spray truck transport, summarised in Figure 22 (for detailed methods see section 6.5). A grader at the factory collaborating in the study then separated the lobsters as rejected or accepted for live export holding. Hemolymph samples were taken from equal numbers of accepted and rejected lobsters. Following sampling, the lobsters were fitted with identifying tags and then placed in a tank at the factory. Their fate was followed for a week until a simulated live export pack-out was conducted with the survivors. Lobsters were assigned to a number of groups on the basis of whether they were removed dead from the tanks ('D'), removed weak from the tank ('W') and at the simulated load-out, whether they were rejected because of legloss/injury ('LL'), rejected because they were weak ('LW') or acceptable for loadout ('LA').

A total of 348 lobsters were hemolymph sampled after being stressed in two replicate trials of the spray experiment. During the following week, about 21% of these lobsters were removed from the factory tank because they were either dead or weak (D or W). This degree of loss is exceptionally high, no doubt partly because we ensured that many rejected lobsters were included in the sample and perhaps also because of the extra handling and possible injury caused by the sampling method. A complete account of the fate of the lobsters and the grades given is found in section 7.4.2.

We analysed the hemolymph samples that had been taken from the lobsters leaving the spray truck module and found that even then there were already significant differences between the groups (D, W, LL, LW and LA) in levels of a number of parameters in the hemolymph, namely lactate, magnesium, potassium and protein (see section 7.4.3).

The association between early changes in these stress indicators and later losses of lobsters in the tank was relatively strong. Some groups did not differ significantly, so these were pooled to produce two groups 'tank rejects' (D+W) and or 'not tank rejects' or in simpler terms 'survivors' (LL+LW+LA). Using a statistical technique called discriminant analysis, four hemolymph tests could correctly classify 83% of the lobsters. This was done by comparing the results of each lobster to the average results for the groups 'tank rejects' or 'not tank rejects' and judging whether or not the lobster was likely to belong to one or the other group, (see section 7.4.4). The analysis classified tank rejects or 'not tank rejects' with roughly equal accuracy.

The four stress indicators, ranked here in order from strongest to weakest by their ability to discriminate between the groups, were:

$$[\text{Magnesium} > \text{Potassium} > \text{Calcium} > \sqrt{\text{Lactate}}]$$

It should be stated clearly that we are not proposing that measurement of these stress indicators will ever replace on-the-spot commercial grading. Nevertheless, it is interesting to see how this result compares to the grading the lobsters received leaving the spray truck. The single grader who assisted the study correctly graded 67% of lobsters, that is, correctly rejected tank rejects and accepted not tank reject lobsters (section 7.4.2.2). In keeping with the grader's basic objective, of filling the live factory tanks with vigorous lobsters, 90% of accepted lobsters were 'not tank rejects,' but only 38% of rejected lobsters were tank rejects, that is, became dead or weak in the tanks. This figure is of course only indicative, and because of the extreme conditions of the experiment it cannot be said to apply to routine commercial grading of lobsters.

We have shown that certain stress indicators are associated with losses of lobsters, and that, via discriminant analysis, this association is sufficiently strong that the indicators provide a means of

predicting the fate of lobsters following a stress treatment. These stress indicators will be particularly pertinent when assessing the responses of lobsters to alternative handling regimes.

But so far, all we've done is 'predict' an observed outcome using the very same lobsters used to develop the discriminant analysis. In order to get a better idea of its repeatability and predictive scope, we now have to use the discriminant equations (the derviation of which is detailed below) to predict the fate of other lobsters. This was one of the objectives of the additional factory-based experiments conducted in November 1998 and March 1999 (see section 7.5 and beyond). Another task is to simplify the sampling process by reducing the number of hemolymph tests required from each lobster to the bare minimum while at the same time, seeking hemolymph constituents that better discriminate between lobsters.

The remainder of this section details…

- the breakdown of tagged lobsters into their respective groups (section 7.4.2),
- the physiological differences between the groups (section 7.4.3) and
- the use of discriminant analysis to classify the lobsters (section 7.4.4).

Due to the size of the statistical analysis involved, the issue of predicting using the equations from this trial with data collected in later trials will be left until section 7.7.

As this study occurred in collaboration with the companion project FRDC 96/344 there are also some immune system results for the lobsters sampled in this trial. This data is not presented in sections 7.4.2 to 7.4.3 of this report and the reader should refer to the corresponding section of the other report. However, in the interests of completeness, all available data for each lobster has been included in the discriminant analysis (section 7.4.4).

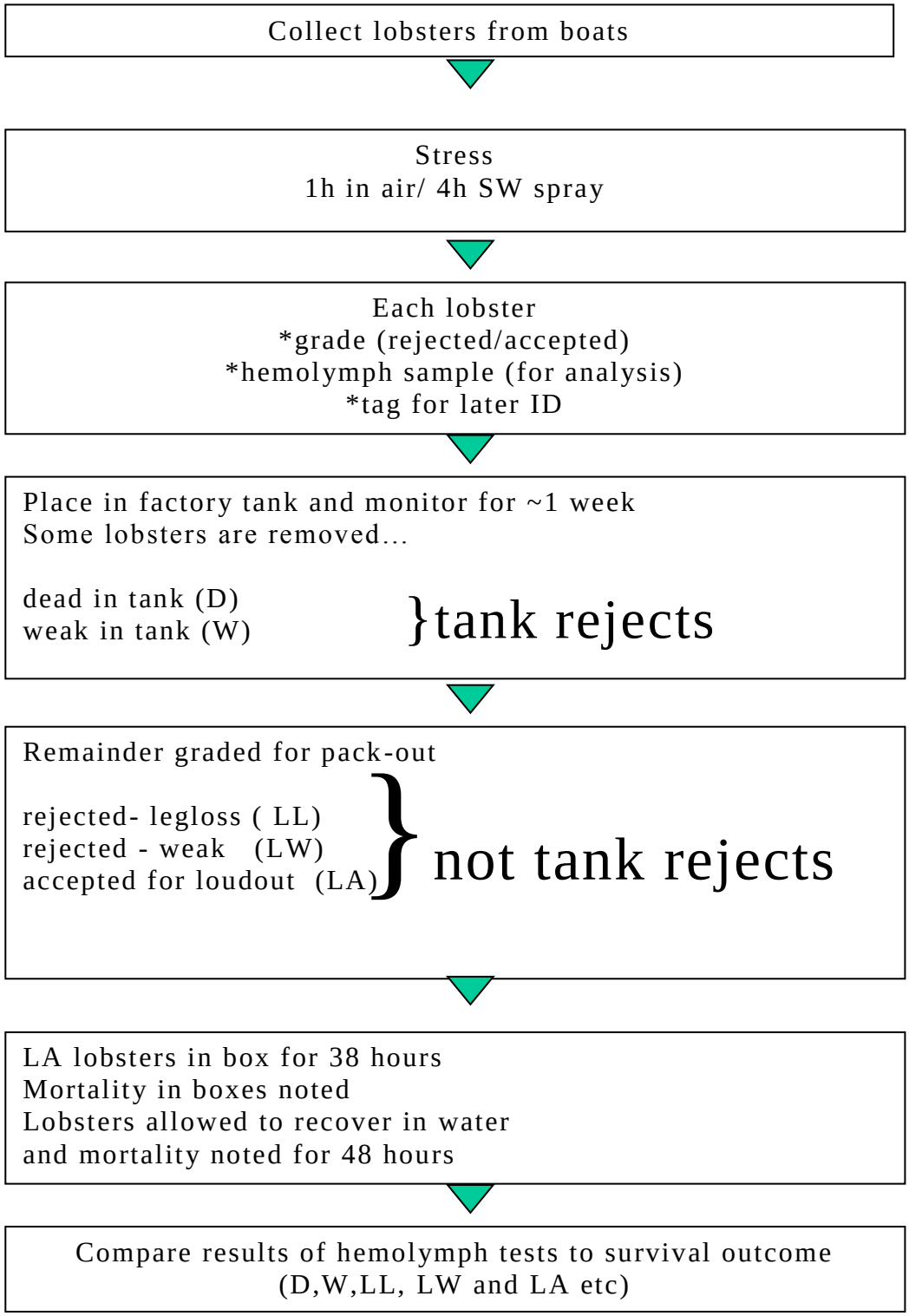


Figure 22. Flow chart of experiment

7.4.2 How many tank rejects?

7.4.2.1 Missing tags

Of the 348 lobsters tagged, 17 tags were recorded as lost. No later record was found following loadout, unpacking and so forth for a further 24 tags due to transcription errors, confused numbers and misadventure. Thus, the fate of 12% of the lobsters sampled (Table 4) could not be tallied against the parameters measured in their hemolymph parameters.

Table 4. Numbers of western rock lobsters *Panulirus cygnus* packed and those not packed following each of two spray truck experiments during the March 1998 factory trial.

	Trial 1	Trial 2	Total
No. tagged	198	150	348
No. tank rejects dead+weak (%)	36 (18.2)	37 (24.7)	73 (21.0)
No. not packed - weak (%)	55 (27.8)	21 (14.0)	76 (21.8)
No. not packed - leg loss (%)	29 (14.6)	16 (10.7)	45 (12.9)
No. accepted for loadout (%)	59 (29.8)	54 (36.0)	113 (32.5)
No. of tags unaccounted for (%)	19 (9.6)	22 (14.7)	41 (11.8)

7.4.2.2 Grading and later mortality

An experienced factory grader was asked to assess lobsters coming from each experimental trial (Table 5), as a preliminary means of assessing the efficacy of grading practices versus likely stress indicators. A more detailed breakdown for each trial is given in Table 6 and Table 7. It is clear that lobsters accepted by the grader were largely 'fit' for storing in the factory in that they did not die or weaken in the tank. The grader is accepting lobsters that will not die or weaken in the tank in proportions better than would be expected due to chance alone (χ^2=19.04 P<<0.001). If we broaden the focus to include the question of whether the accepted lobsters will be found acceptable for loadout, it is clear that the grader is still doing considerably better than could be done by selecting lobsters at random (χ^2=19.81 P<<0.001). However, a high proportion of accepted lobsters in this experiment later prove unfit for loadout (due to legloss, weakening etc) (Table 6). This confirms that the initial grading step is efficacious in keeping suspect lobsters out of the factory, but at least under the conditions of this trial, does not necessarily extend to predicting future suitability for live export. The quality of accepted lobsters comes at a price, since some of the lobsters rejected by the grader turned out to be acceptable for factory storage and for loadout (Table 6). Of course this is only really an issue in cases of low supply or high demand for live lobsters. A similar situation arose following the next day's trial (Table 7), when the grader was picking lobsters fit for factory storage (χ^2=16.77 P<<0.001). and those later graded fit for loadout (χ^2=19.81 P<<0.001) at a rate higher than that of chance alone, though again, in this experiment the grader's ability to let fit lobsters into the factory comes at a cost in terms of a sizeable group of "false negatives."

7.4.2.3 Losses during tank storage

Collectively, during the two replicates, 21% of the 348 lobsters tagged later had to be removed from the tank in the 7 to 8 days (Table 8). The lobsters did not 'fail' at a constant rate. According to the times recorded when weak or dead lobsters were found following the first spray truck trial (Trial 1, 29/3/98), most of the lobsters that died or weakened did so in the first 100 hours in the tank (Figure 23a). Following the second trial (Trial 2, 30/3/98), the spray truck module was set to a higher temperature because of the lethargy observed in lobsters taken from the truck in the colder trial the

day before. It was felt that the lethargy of the lobsters may make it harder for the grader to assess the lobsters. Most of the deaths in the tanks were now found in the first 50 hours following sampling (Figure 23b), and the weak lobsters were largely found between 50 and 100 hours of sampling.

Table 5. Grading of western rock lobsters *Panulirus cygnus* versus outcome of storing and loadout during the March 1998 factory trial.

Stage	Outcome	Trial 1		Trial 2	
		Reject	Accept	Reject	Accept
Tank	Dead	21	2	20	6
	Weak	9	4	9	2
Loadout	No. not packed, weak	30	25	10	11
	No. not packed, legloss	15	14	8	8
	No. acceptable for packing	16	43	17	37
Totals		91	88	64	64

Table 6. Contingency table for efficacy of grading to predict whether western rock lobsters *Panulirus cygnus* are suitable for tanking and for loadout during the first trial during the March 1998 factory trial.

		Grade		
		Reject	Accept	Total
Tank				
	Unfit	30	6	36
	Fit	61	82	143
	Total	91	88	179
Loadout (including tank)				
	Unfit	75	45	120
	Fit	16	43	59
	Total	91	88	179

7.4.2.4 Losses during and following simulated load-out

Of the 348 lobsters sampled and tagged on the two days of the trials, 21% had been rejected from the tanks before loadout (Table 4 and Table 5). When graded for simulated export, a further 35% of the lobsters were rejected, either because they were too weak to export (21.8% of tagged lobsters) or had lost an unacceptably high number of legs (12.9% of tagged lobsters, Table 4 and Table 5). These lobsters were not packed. Of those accepted for load-out, a small percentage could not actually be packed into cartons, not being enough to fill a complete carton, and of those actually packed, most survived simulated transport. For trial 1, 59 lobsters were graded as acceptable (Table 4 and Table 5), but 6 surplus lobsters remained after filling cartons. A total of 51 of the 53 lobsters packed actually survived more than 48 hours following re-tanking (one lobster died in the box). For the second trial, 54 lobsters were graded as acceptable (Table 4 and Table 5), but two surplus lobsters were not packed. There were no mortalities in the boxes, but a total of 46 of the 52 lobsters packed, survived more than 48 hours following re-tanking.

Table 7. Contingency table for efficacy of grading to predict whether western rock lobsters *Panulirus cygnus* are suitable for tanking and for loadout during the second trial during the March 1998 factory trial.

		Grade		
		Reject	Accept	Total
Tank				
	Unfit	29	8	37
	Fit	35	56	91
	Total	64	64	128
Loadout (including tank)				
	Unfit	47	27	74
	Fit	17	37	54
	Total	64	64	128

Table 8. Numbers of western rock lobsters *Panulirus cygnus* found dead or weak in the tank for seven-eight days following each of two spray truck experiments during the March 1998 factory trial.

	Trial 1	Trial 2	Total
No. tagged	198	150	348
No. found dead (%)	23 (11.6)	26 (17.3)	49 (14.1)
No. found weak (%)	13 (6.6)	11 (7.3)	24 (6.9)
No. dead+weak (%)	36 (18.2)	37 (24.7)	73 (21.0)

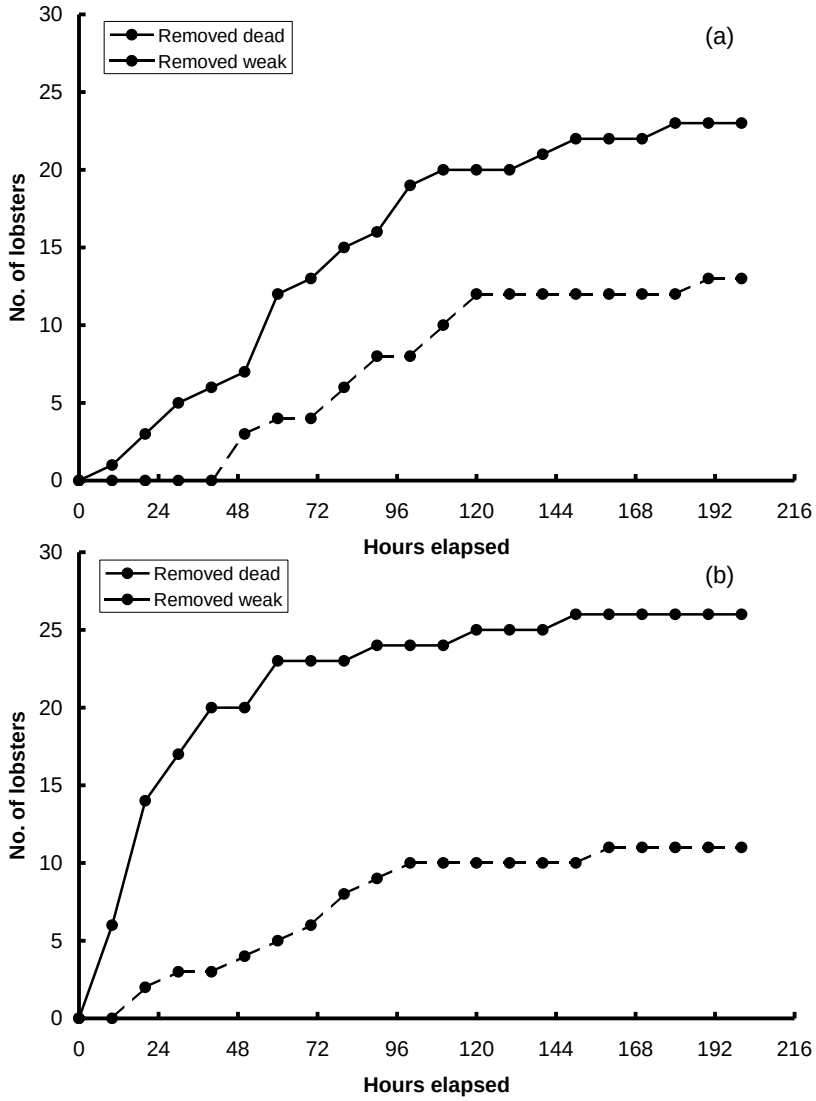


Figure 23. Accumulated rejection of dead and weak western rock lobsters *Panulirus cygnus* of the 198 lobsters sampled in (a) the first trial and (b) the second trial during the March 1998 factory trial. Calculated according to time elapsed between sampling the lobsters and the time factory staff removed them from the tank and recorded their particulars on the tally sheet

7.4.3 Immediately after the stress treatment, do lobsters that will die later differ physiologically from those that will survive?

The lobsters that later died or weakened in the tanks differed significantly when sampled emerging from the stress treatment.

Lactate, potassium, magnesium and protein concentration in the hemolymph of lobsters immediately after leaving the spray truck differed significantly between the groups of lobsters which had different fates in the factory (Table 9). The lobsters that were later found dead in the tank had a higher average lactate concentration than other lobsters when removed from the truck. When sampled from the truck, the lobsters that were later found dead or found weak in the tank had a higher average potassium concentration than those that survived until load-out. Conversely, the group of lobsters later found good enough to load-out originally had, upon leaving the spray truck just over a week before, a higher protein and lower magnesium concentration in the hemolymph than those which were found dead in tank, weak in tank or weak at load-out.

The lobsters later rejected with leg-loss at load-out could apparently be either 'weak' or 'strong' at load-out judging by the fact that this group had an intermediate average protein level when originally sampled emerging from the spray truck.

Table 9. Physiological variables and grading of western rock lobsters *Panulirus cygnus*. Lobsters sampled immediately after treatment during the March 1998 factory trial and categorised according to the outcome of storage in tank and grading for loadout in terms of death, weakness, leg-loss and packing.

	Results from lobsters according to the outcome of storage (mean±std)				
Parameter	**'D' Died in tank**	**'W' Weak in tank**	**'LW' Weak at packing**	**'LL' Leg-loss at packing**	**'LA' Packed-out**
Protein	60.4±13.1a	58.9±20.7a	59.4±15.8a	63.4±17.3a	67.1±16.6b
Glucose	2.2±2.1	2.6±1.9	2.7±2.0	2.4±1.6	2.6±1.6
Lactate	7.1±3.0a	5.3±4.0b	3.9±2.8b	5.5±4.0b	4.7±2.8b
K^+	20.9±4.4a	22.2±8.2a	19.1±3.6b	19.4±2.4b	18.7±2.9b
Ca^{2+}	14.6±2.7	15.2±2.1	15.4±1.7	15.4±1.9	15.1±1.8
Mg^{2+}	14.9±2.7a	15.0±2.8a	14.1±2.2a	13.6±1.7b	12.7±2.0c
grader					
No. rejected	41	18	40	23	33
No. accepted	8	6	36	22	80

Concentrations in mmol L^{-1} except for Protein g L^{-1}. In each row, averages with similar postscripts are not significantly different at 5%.

Potassium, lactates, glucose and calcium occurred at higher levels than found in resting undisturbed lobsters. The lobsters emerging from the truck simulation were, by definition, all 'stressed' but they did not all die.

Some parameters such as glucose and calcium, seem to be generally elevated, although the variation for glucose was quite wide, with some surprisingly low values. It could be argued that there was something seriously wrong with a lobster emerging from the truck simulation with a 'baseline' level of glucose but an elevated hemolymph lactate concentration. Calcium and glucose may have risen early and reached some kind of limit or 'ceiling' that prevented continual accumulation. These parameters may indicate that the lobsters are stressed, however we must look to other parameters to find an expression of the different degrees of stress which presumably explain morbidity and death of lobsters.

The results of this factory trial were very encouraging. A number of hemolymph parameters had some value as predictors of future lobster condition, and some, such as hemolymph protein and magnesium level seemed to describe the internal physiological status of a lobster and its fate better than grading itself can. Some discussion is warranted about what these indicators tell us about the lobsters.

7.4.3.1 Lactate

Lactate is an end-product of anaerobic metabolism. It is clear that the build up of large amounts of lactate, and associated internal acidosis, is complicit in the rejection of lobsters arriving at a factory. These lobsters will appear exhausted (or fatigued), and the results confirm that rejecting these fatigued lobsters stops a number of compromised lobsters, which will likely die, from being tanked.

7.4.3.2 Potassium

The concentration of potassium (K^+) is normally tightly regulated in the hemolymph of lobsters and other animals (Dall 1974a). It is present at high concentrations inside cells and the gradient of K^+ either side of cell membranes is a key mechanism involved in nerve transmission and muscular contraction. Hemolymph potassium level increases as the ion leaks from the cells in response to factors such as tissue oxygen deprivation, extreme acidosis or fatigue and cell damage. It is likely that this movement of ions in turn contributes to the weakness or lethargy observed in 'stressed' lobsters. All lobsters sampled, even those that survived, had unusually high levels of potassium in their hemolymph, with the highest levels occurring in those lobsters which were later rejected as dead or weak from the tank. High levels of potassium were also reported in hemolymph of tropical rock lobsters stored on a mother ship in Torres Strait (Paterson *et al.* 1997b). As reported earlier, long periods of emersion increased the potassium level, (see section 7.3.6.3).

7.4.3.3 Magnesium

Magnesium (Mg^{2+}) is found in a relatively large amount in seawater. Most marine organisms (even western rock lobsters) regulate their hemolymph magnesium at much lower levels than found in seawater because this ion interferes which nerve function (Dall 1974a). This regulation appears to become less effective when crustaceans are stressed, though at this stage we have little evidence to explain why this happened in lobsters in a spray truck. Hemolymph magnesium level increased when lobsters were stored out of water (the reader is directed to the emersion experiment, section 7.3.6.2, for further discussion) and this symptom has been associated with *Homarus gammarus* weakened by commercial transport (Whiteley and Taylor 1992). The antennal gland is the excretory organ that is responsible for both hemolymph volume regulation and magnesium regulation (Malley 1977b). In freshwater crayfish it has been demonstrated that urine production is curtailed during emersion, (Tyler-Jones and Taylor 1986).

7.4.3.4 Protein

Protein level in the hemolymph varies considerably between individual intermolt lobsters, which may therefore reflect differences in their condition or nutritional status. We have no overwhelming evidence yet to show that hemolymph protein concentration actually changes in response to stress, though we aren't going to rule this out because some lobsters appeared to 'swell-up' during the spray truck treatment as if their hemolymph volume was changing under unusual circumstances. There is much anecdotal evidence that 'swelling' does occur in stressed lobsters (Wayne Hosking personal communication). Another explanation could be that lobsters caught by particular vessels had different protein levels because of differences in lobster populations being fished and differences between these boats in on-board handling lead to downstream effects on the survival of the lobsters. It is important to establish in future trials what is happening here. Long-term holding of lobsters without feeding

them, a practice which is becoming increasingly common for a number of species, will reduce their hemolymph protein concentration (for example, see section 7.1.3).

7.4.4 Discriminant analysis

Two stepwise discriminant analyses are presented below. Firstly, one examines four of the groups used to tally the fate of the lobsters above (D,W, LW and LA in Table 9), but does not use the lobsters rejected for injury (LL), because we were not trying to predict damage. Again, the outcome of the simulated loadout has been ignored, with most of the lobsters suitable for loadout actually surviving the challenge. In the second analysis, the groups are pooled into the two groups 'tank rejects' and 'not tank rejects' (survivors) used for the later studies (sections 7.5 and beyond).

7.4.4.1 Stepwise discriminant analysis – 4 groups (dead, weak, weak at packing and accepted for packing)

There are clearly significant differences between groups for average levels of individual variables. At this point it is worth reiterating that these differences are not characteristics of lobsters sampled when they were found dead in the tank or acceptable for loadout. These results show, for example, that the lobsters that later died in the tank (D), when sampled emerging from the original stress treatment, had hemolymph characteristics which were already significantly different from the lobsters that later proved to be the 'not tank rejects' (LW and LA). Discriminant function analysis uses the results of all of the hemolymph tests to describe the differences between the groups in such a way that predictions can be made about lobsters based simply on their hemolymph characteristics emerging from the stress treatment.

7.4.4.1.1 Stepwise selection of parameters

Five variables remained in the discriminant model after step-wise selection of variables (Table 10).

Table 10. Summary of stepwise discriminant function analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial.

Variable to Enter	Step	F to enter	DF	entry p-level	No. of vars. in model	Wilks' Lambda (Λ)	F value	DF	p-level
Mg	1	14.92	3,234	0.000	1	0.84	14.92	3,234	0.000
√Lactate	2	9.74	3,233	0.000	2	0.75	12.26	6,466	0.000
Protein	3	7.37	3,232	0.000	3	0.68	10.73	9,565	0.000
K	4	6.47	3, 231	0.000	4	0.63	9.79	12,611	0.000
Ca	5	6.27	3,230	0.000	5	0.58	9.22	15,635	0.000

The first summary table (Table 10) gives the F-to-enter as each parameter is added to the model and the associated degrees of freedom and the p-value. It also shows the incremental improvement (i.e. decrease) in Wilk's Lambda after each variable is added to the model, as well as the corresponding F-value for that value of Wilks' Lambda, degrees of freedom and associated probability.

The Wilks' Lambda (Λ) for this model was 0.581 (approximate F (15,635)=9.215 p<< 0.001). Selection was based upon F-to-enter = 2, where the F statistic tests the additional discrimination introduced by a particular variable after taking into account the discrimination already achieved by variables previously included in the model, (Klecka 1980). The variables Mg, √Lactate (i.e. the square root of lactate concentration), Protein, K, and Ca were selected. The variables √Glucose and % granulocytes (abbreviated in the table to "% granul.") remained outside of the model, (Table 10 and Table 11).

Table 11. Summary of variables in and not in the model following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial. N=238. Lobsters with missing data for any parameter excluded from the analysis.

	Wilks' Lambda if Removed (Λ)	Partial Lambda of variable	F to remove (3,229)	p-level	Tolerance	1-Toler. (R^2)
Variables in the model						
Mg	0.701	0.829	15.838	0.000	0.743	0.257
√Lactate	0.657	0.884	10.088	0.000	0.899	0.101
Protein	0.615	0.944	4.515	0.004	0.860	0.140
K	0.658	0.882	10.208	0.000	0.842	0.158
Ca	0.628	0.924	6.267	0.000	0.632	0.368
Variables not in the model						
%granul.	0.577	0.994	0.491	0.689	0.992	0.008
√Glucose	0.567	0.976	1.912	0.128	0.910	0.090

7.4.4.1.2 Contribution of each variable

With such a large number of parameters, it is not surprising that they differ in the degree of discriminating power they provide (Table 11). Variables like Mg and √Lactate and K cannot be removed from the model without a considerable loss of discrimination, judging by the major rise in Wilks' Lambda observed.

Table 11 also shows that the order in which variables enter the model in the stepwise process (Table 10) does not necessarily reflect their discriminative power. The F-to-remove statistic in Table 11 is calculated for each variable's 'partial lambda' and can be used to rank the discriminating power of each variable. Note that while Protein entered the model before K did, the Wilks' Lambda rise upon removal and the partial lambda contributed by the variable alone suggest that K contributes more to discriminating between lobsters than Protein does. Also presented in Table 11, 'tolerance' is a multiple correlation test, that would return a value close to zero if a particular variable was a linear combination of one or more other variables in the model. This is undesirable. Fortunately, no variable failed the tolerance test.

7.4.4.1.3 The discriminant functions

The first two of the three discriminant functions (DF1 and DF2) derived from the data account for the bulk of the differentiation between the groups judging by the eigenvalues or 'lambdas' and the canonical correlation coefficients (R) of these functions (Table 12 and Table 13). The larger either the eigenvalue or the canonical correlation is, the greater is the discriminating power of a particular function.

Statistical testing confirms that the third discriminant function (DF3) contributes nothing to discrimination between the groups (Table 13). The eigenvalue for each function is used to calculate a value for Wilk's Lambda, (which was also used above during the stepwise development of the model). This parameter can be used as a measure of 'residual' discrimination present in the data set.

Before the discriminant power provided by individual functions is accounted for (k=0), the value of Wilk's Lambda remains at that for the overall model (0.581, Table 13). The corresponding approximation of the chi-square statistic shows that a significant amount of discrimination exists in the data. If the discrimination provided by the first function is removed (k=1), it is clear that this function doesn't account for all of the discrimination provided by the model, since a significant amount remains. Once the impact of both functions is taken into account (k=2), no significant discriminative capacity remains. The third function is unnecessary.

Table 12. The standarised coefficients for discriminant functions derived for the groups using the five variables remaining following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial.

	Discriminant functions		
Variable	DF1	DF2	DF3
Mg	-0.804	0.297	0.767
√Lactate	-0.511	-0.582	-0.336
Protein	0.253	-0.594	0.739
K	-0.621	-0.269	-0.301
Ca	0.550	0.376	-0.337
Eigenvalue (λ)	0.513	0.137	0.001
Cumulative Proportion	0.788	0.999	1.000

Table 13. Calculations based on the eigenvalue: canonical correlation. Wilks' Lambda and testing the residual discrimination remaining prior to deriving each discriminant function following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial.

Functions derived (k)	Eigen-value (λ)	Canonical Correlation (R)	Wilkes Lambda (Λ)	χ^2	df	p-level
0	0.513	0.582	0.581	126.349	15	P<0.001
1	0.137	0.348	0.879	30.071	8	P<0.001
2	0.001	0.024	0.999	0.138	3	P=0.99

7.4.4.1.4 Group differentiation

Together, DF1 and DF2 explain the differences between groups. The D lobsters are generally well differentiated from the LW and LA groups, judging by the squared Mahalanobis distances between the groups (Table 14), though there is less distinction between D and W and in turn between W and LW, the latter of which returns a relatively high p-value. This is a point of concern. It appears that the W group was less distinct, an outcome that will have ramifications below when attempting to classify lobsters into this group.

DF1 itself is largely responsible for separating the the D and LA groups, while the W and LW groups are discriminated by both DF1 and DF2. This is clear when the mean discriminant function scores for each group, sometimes called the 'group centroids' are plotted against the DF1 and DF2 axes (Figure 24).

Table 14. Squared Mahalanobis distances between groups following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial.

Squared Mahalanobis Distances				
RESULT	D	W	LW	LA
D	0	0.934	2.580	3.481
W	0.934	0	0.796	2.434
LW	2.580	0.796	0	0.777
LA	3.481	2.434	0.777	0
F statistics				
RESULT	D	W	LW	LA
D		2.44	13.55	21.09
W	2.44		2.32	7.67
LW	13.55	2.32		6.25
LA	21.09	7.67	6.25	
p-levels				
RESULT	D	W	LW	LA
D	--	0.04	0.00	0.00
W	0.04	--	0.04	0.00
LW	0.00	0.04	--	0.00
LA	0.00	0.00	0.00	--

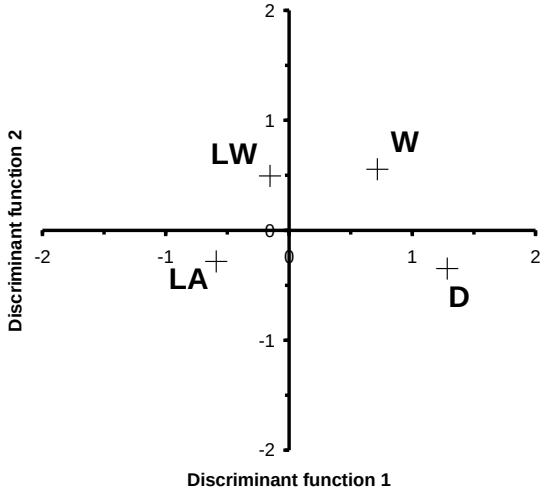


Figure 24. The mean group scores (centroids) of each group of western rock lobsters *Panulirus cygnus* from the March 1998 trial plotted against each discriminant function.

7.4.4.1.5 'Internal' classification of lobsters

By using the values of DF1 and DF2 calculated for individual lobsters it was now possible to attempt to classify the lobsters into the groups simply on the basis of how well their discriminant function scores (based on the original hemolymph tests) resemble those typical of particular groups. The next step after that was to see how well these predictions compare with the observed fate of each lobster. This was the first test, albeit 'internal' to the data used to generate the functions, of how well the discriminating power of the hemolymph test results allowed us to classify/predict whether lobsters will live or die after being stressed.
Classification, based upon the squared Mahalanobis distance between the lobster's position relative to each group centroid was used to produce the classification matrix shown in Table 15.

Table 15. Classification matrix obtained using posterior probabilities calculated from the distance between original cases and group centroids following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial.

Group	Percent Correct	D*	W*	LW*	LA*
D	56.52	26	0	8	12
W	0.00	8	0	10	4
LW	40.00	4	1	28	37
LA	80.19	7	0	14	85
Total	56.97	45	1	60	138

Rather than using only the simple geometrical distance between lobsters and each centroid, these classifications are based upon 'posterior probabilities' derived from these distances, that is, upon the likelihood that lobsters belong to a given group.

The present model only got 57% of classifications correct. A major reason for this was that the observed group W was compromised from the start by appearing to be an 'unlikely' collection of lobsters. The small sample size no doubt contributed to this, but recall also the small between-group distances calculated between W and either D or LW (Table 14). Given this situation, lobsters close to the W centroid were assigned in preference to other groups. It is worth noting that the model correctly classified 80.2% of the 106 lobsters belonging to LA (Table 15), though it considered 138 lobsters in all to belong in LA on the basis of their hemolymph test results.

7.4.4.1.6 Predicting unknown cases

The classification functions emerging from this discriminant analysis are presented in Table 16. These equations can be used to predict group membership of lobsters by applying these coefficients to the hemolymph test results of lobsters. The lobsters can then be assigned the group for which the corresponding classification function returns the highest score.

Table 16 Coefficients for classification functions derived from the discriminating variables following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to groups (D, LA, LW, W) during the March 1998 factory trial.

Variable	D	W	LW	LA
Mg	2.434	2.340	2.053	1.773
√Lactate	5.958	4.988	4.325	4.586
Protein	0.259	0.232	0.251	0.284
K	3.232	2.995	2.722	2.677
Ca	1.237	1.549	1.782	1.782
Constant	-68.405	-64.994	-59.919	-58.626

7.4.4.2 Stepwise discriminant analysis – 2 groups (tank rejects, not tank rejects)

One group used in the preliminary study in March 1998, lobsters removed weak from the tank (W), were poorly discriminated from other groups. The decision about whether a lobster was considered 'dead'; or 'weak' when removed by staff from the tank may be quite arbitrary (eg. dead lobsters may still be "physiologically" alive or weak lobsters could be close to death). Therefore, in the later studies, a broader set of groups, 'tank rejects' (lobsters removed from the tanks, whether dead or weak), 'box mortality' (lobsters that died during and immediately after simulated transport) and 'survivors' was used. In order to simplify the process of predicting forward from these March 1998 equations, the discriminant analysis of this data was repeated using only two groups, 'tank rejects' (D+W) and 'not tank rejects' (LW+LL+LA) (Table 17). Again, only a couple of lobsters failed the loadout challenge so the box mortality category was not used here.

Table 17. Summary of stepwise discriminant function analysis of western rock lobsters *Panulirus cygnus* sampled according to pooled groups (Tank rejects, Not tank rejects) from the March 1998 factory trial.

Variable to Enter	Step	F to enter	df	p-level	No. of vars. in model	Wilks' Lambda (Λ)	F value	df	p-level
Mg	1	28.74	1,277	0.000	1	0.906	28.74	1,277	0.000
Ca	2	18.23	1,276	0.000	2	0.850	24.38	2,276	0.000
K	3	26.96	1,275	0.000	3	0.774	26.77	3,275	0.000
√Lactate	4	19.57	1,274	0.000	4	0.722	26.33	4,274	0.000

This time, only four variables remained following the stepwise procedure, though the variables selected were different to those seen when 4 groups were used. However, pooling groups that may nevertheless differ physiologically into these broader categories more in keeping with commercial practice has also led to a loss of discrimination. The new groups are less coherent, as shown by the higher though nevertheless significant value for Wilks' Lambda for this model (Λ=0.722, $F_{4,274}$ = 26.33 P<<0.001). The variables chosen, in order of selection, were Mg, Ca, K, and √Lactate. However, by considering the F-to-remove statistic, it is clear that though K was added to the model after Ca, it actually provides more discrimination than Ca, (Table 18).

Table 18. Summary of variables in the model following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled according to pooled groups (Tank rejects, Not tank rejects) after the March 1998 factory trial. N=279. Lobsters with missing data for any parameter excluded from the analysis.

	Wilks' Lambda if removed (Λ)	Partial Lambda of variable	F to remove (1,274)	p-level	Tolerance	1-Toler. (R^2)
Mg	0.855	0.845	50.154	0.000	0.760	0.240
Ca	0.801	0.902	29.893	0.000	0.695	0.305
K	0.814	0.887	34.766	0.000	0.854	0.146
√Lactate	0.774	0.933	19.568	0.000	0.954	0.046

With only two groups to distinguish, only one function is derived and the calculations based on the eigenvalue are only summarised here. The canonical correlation coefficient for this function is 0.527 and it accounts for a significant amount of discrimination between groups (Wilks Lambda= 0.722, chi-square = 89.44, df=4, p<0.001). Separation between the groups was also significant (Squared Mahalanobis distance= 2.15, $F_{4,274}$ = 26.17, P<<0.001). The discriminant function is presented in Table 19.

Table 19. The standarised coefficients for the discriminant function derived for the two groups (Tank rejects, Not tank rejects) using the four variables remaining following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled during the March 1998 factory trial.

Variable	Discriminant function
Mg	0.856
Ca	-0.714
K	0.689
√Lactate	0.502
Eigenvalue	0.384
Cumulative Proportion	1.000

Despite the evident loss of discrimination when pooling similar though not completely identical groups together, by absorbing the statistically insignificant 'W' group from the earlier analysis into 'tank-rejects,' the model is now marginally better able to account for group membership of lobsters using the hemolymph test results, getting 83.3% of classifications correct (Table 20).

Table 20. Using discriminant function analysis to predict group membership for the two groups (Tank rejects, Not tank rejects) using the four variables remaining following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled during the March 1998 factory trial.

		Predicted classification		
	Percent	Tank reject	Not tank reject	Total
Observed	Correct			
Tank reject	39.71	27	41	68
Not tank reject	96.80	7	212	219
Total	83.28	34	253	287

In fact it correctly classifies virtually all (97%) of the lobsters that were known 'not tank rejects,' but in doing this, it still predicts incorrectly that some known tank rejects are 'probably' 'not tank rejects' judging by their hemolymph characteristics.

Classification functions suitable for predicting group membership of lobsters in later experiments are given in Table 21.

Table 21. Coefficients for classification functions derived from the discriminating variables following stepwise analysis of lobsters sampled according to the two groups (Tank rejects, Not tank rejects) using the four variables remaining following stepwise analysis of western rock lobsters *Panulirus cygnus* sampled during the March 1998 factory trial.

	Classification functions	
Variable	Tank reject	Not tank reject
Mg	2.242	1.680
Ca	1.942	2.482
K	3.140	2.641
√Lactate	6.431	5.461
Constant	-60.863	-51.473

7.5 Validation of indicators including a test of alternative environments- November 1998 (sub-objectives 4 and 5)

7.5.1 Summary

This was the first of the projected replicate trials aimed at confirming that the principle established in the initial trial (section 7.4) was repeatable, and further to see if parameters could be identified that better discriminate between groups. An additional objective was to broaden the range of treatments used, so that lobsters stored under different regimes could be compared.

Apart from the fact that 5 storage treatments were used, the experiment was conducted in a similar manner to the one already described (see summary diagram, Figure 25). Lobsters were stressed for a period, graded by an experienced grader, and their hemolymph was sampled. They were then tagged and returned to factory tanks for a period of observation to identify lobsters that would die in the tanks before those remaining were challenged by a period of simulated export. The method is described in detail in section 6.5.

Of the 293 lobsters sampled during the three replicates, the grader rejected 22% of them. The grader found fewer rejects in the submerged treatments than in the non-submerged treatments. In all, there was 19% mortality in the tanks following the treatments. The lobsters from the submerged treatments showed negligible mortality in the tanks during the subsequent week, whereas the non-submerged ('emersed') treatments produced higher levels of mortality. There was no difference in survival between lobsters stored submerged in flowing or in recirculated seawater, indicating that deterioration in water quality had no impact upon survival. The recirculating seawater spray showed the highest level of mortality.

In the meantime, the hemolymph samples taken from the lobsters were analysed for a broad suite of parameters. This showed that lobsters sampled emerging from the different treatments differed significantly in a number of physiological parameters, again, in accordance with expected changes accompanying storage in air (section 7.3).

Using discriminant analysis, three hemolymph tests could correctly classify 89% of the lobsters sampled as tank rejects or not. But to put this in perspective, the grader correctly predicted the fate of 81% of the lobsters sampled here. The variables are ranked here according to their ability to discriminate between groups.

$$\sqrt{\text{Lactate}} > \log(\text{Lactate dehydrogenase activity}) > \text{Magnesium}$$

Only 2 of these parameters figured in the analysis of the pilot study (section 7.4).

We used a wider range of parameters here to see whether any contribute a greater degree of discrimination to the model. While there were differences in the variables selected for the model given here (only two variables remained from the original model), there has been no quantum improvement in discrimination.

This experiment has confirmed that by using a few hemolymph tests, it was possible to predict whether lobsters would live or die following an imposed stress, with a moderate degree of error,. This is further confirmation of the earlier finding that much of the stress that kills lobsters during post-harvest handling can be detected and monitored using a key group of hemolymph tests.

This is not to say that we expect these tests to be used to grade lobsters in the commercial sense. The potentially small improvement in accuracy brought about by classifying lobsters in this manner does not justify the added complication and expense of performing several hemolymph tests. What this finding does do however, is allow indicators to be put forward as the short list of those key parameters that should be measured during studies of lobster post-harvest handling in order to account for correlates of lobster mortality rather than simply measuring "stress." Any steps taken to reduce the extreme deviations in these parameters during post-harvest handling of lobsters are expected to improve lobster survivorship. Once these major deviations are addressed, then attention may shift to examining whether sub-lethal deviations in these and similar parameters highlight 'stress' that has a

more subtle impact. For example, those impacting on the condition and growth of lobsters during long term storage.

The objective of the next trial (section 7.6) was again to see whether the work was repeatable, how good predictions were using older models and to see how stable the list of indicators was. Due to the size of the statistical analysis involved, the issue of predicting using the equations from one trial onto the data collected in other trials will be left until section 7.7. The fact that mortality was so low in the recirculated submerged treatment and that spraying seawater on emersed lobsters apparently had no beneficial effect on survival also focused attention on repeating the storage environment trials.

The remainder of section 7.5 examines the data from this trial in detail. An account is given of:

- the outcome of grading and subsequent losses in the tanks (section 7.5.2).
- storage treatment effects (section 7.5.4).
- the hemolymph chemistry of the tank rejects versus other lobsters (section 7.5.5)
- the discriminant analysis of the hemolymph test results (section 7.5.6)

As this study occurred in collaboration with the companion project FRDC 96/344 there are also immune system results for the lobsters sampled in this trial. This data is not presented in sections 7.5.2 to 7.5.5 of this report and the reader should refer to the corresponding section of the other report. However, in the interests of completeness, all the data for each lobster has been included in the discriminant analysis (section 7.5.6).

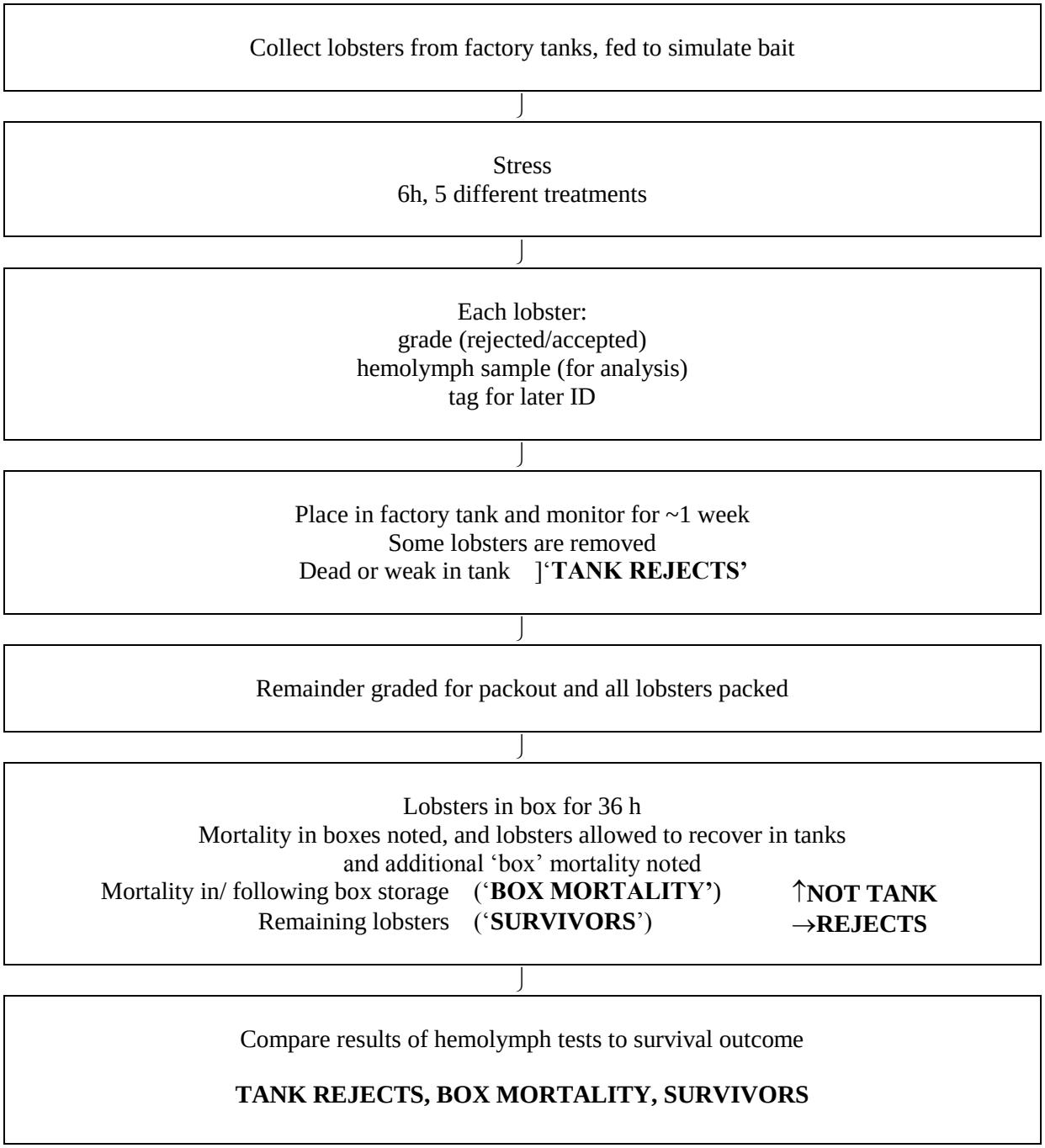


Figure 25. Flowchart outlining the methods used in the November 1998 factory trial. Replicate stress trials were conducted on three consecutive days.

7.5.2 Water quality

Oxygen saturation remained at acceptable levels in the water in both the submerged tanks and in the sumps for the spray treatments during the experiments. Spot samples taken at various times during the three replicates are pooled in Figure 26.

As expected, the water pH was lower in the recirculating treatments than in the flow-through treatments (Figure 27). The treatments receiving free flowing seawater were continually replenished with seawater, whereas the recirculating treatments would accumulate waste acids- carbon dioxide in particular. In the recirculating spray treatment, the water acidified gradually whereas, when lobsters were fully submerged in the recirculating water the pH fell to a new equilibrium immediately from the beginning of the experiment.

Ammonia accumulated in both recirculating seawater treatments at similar rates, reaching levels of 6-9 mg L^{-1} in 6 h (Figure 28). Naturally, flowing seawater did not accumulate wastes. The levels of ammonia reached during this trial were not particularly high. As the survival results indicate, short-term exposure to these levels of ammonia did not seem to be detrimental, whereas long-term exposure to ammonia at these levels would probably be lethal. Apart from the likely fact that much higher levels of of ammonia are probably required to kill lobsters in this time frame, a further mitigating factor is the lowering of the water pH, which reduces the proportion of ammonia present in the toxic un-ionised form.

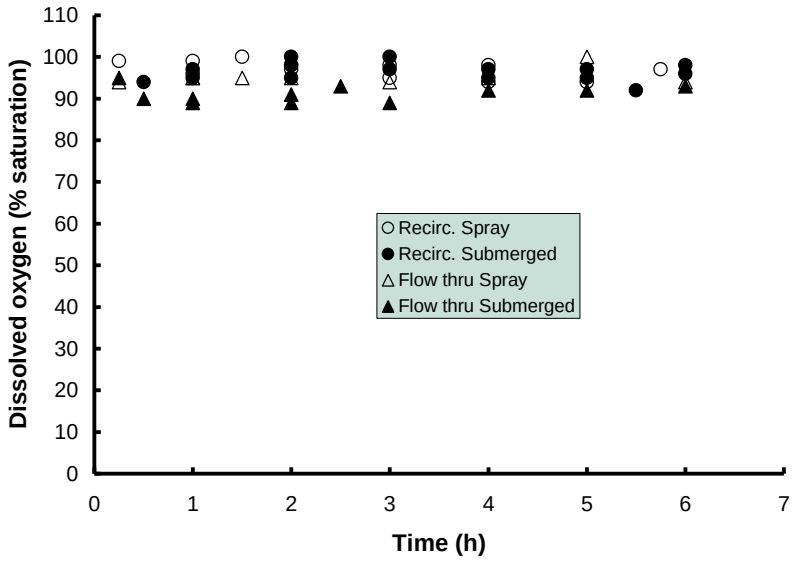


Figure 26. Oxygen saturation measured during the submerged and spray treatments during the November 1998 experiment.

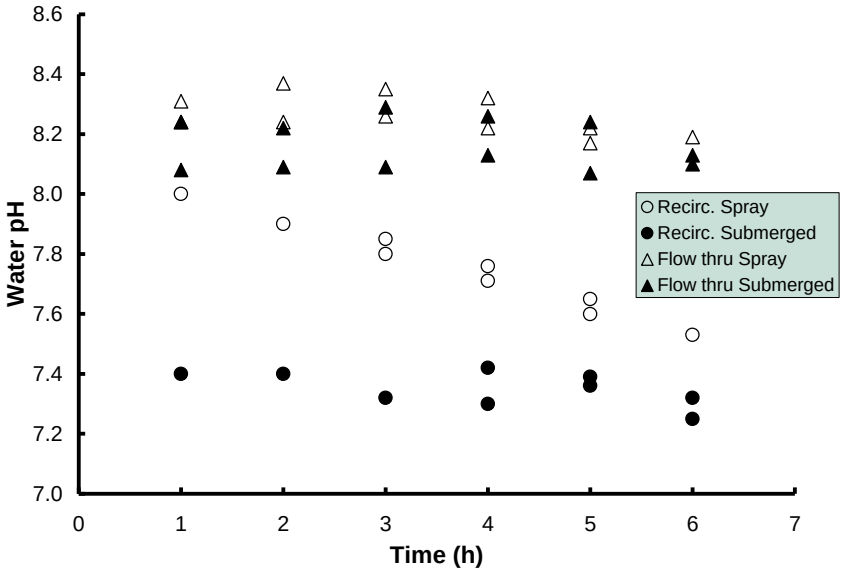


Figure 27. Water pH measured during the submerged and spray treatments during the November 1998 experiment.

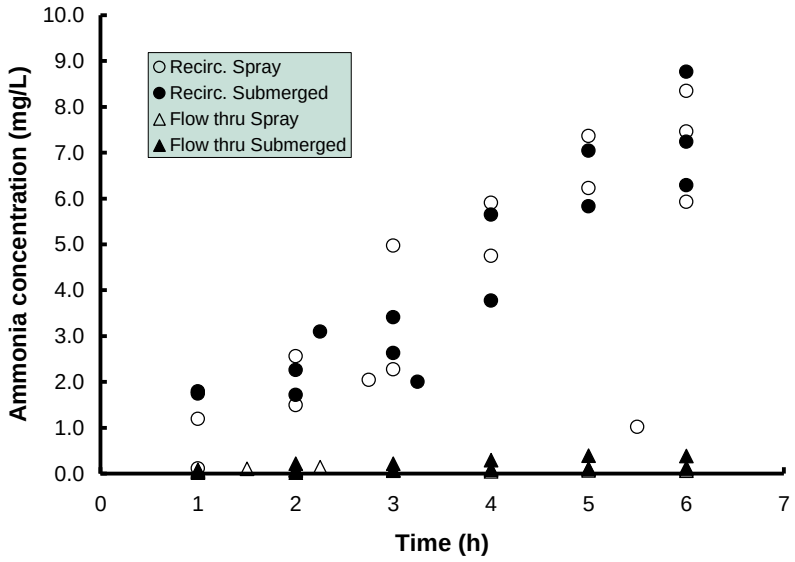


Figure 28. Water ammonia concentration measured during the submerged and spray treatments during the November 1998 experiment.

7.5.3 Grading and losses during storage in tanks

7.5.3.1 Grading

The grader accepted most of the lobsters removed from the submerged treatments (Table 22) but lobsters stored in humid air or seawater spray met with high levels of rejection, particularly the recirculated spray treatment.

Table 22. Number of rejected and accepted lobsters sampled, and tagged during the November 1998 factory-based trial.

Table	Grading result by treatment					
	Flow subm	Recirc subm	Humid air	Flow spray	Recirc spray	Total
Rejected	1	5	14	12	33	65
Accepted	59	54	44	44	27	228
Total	60	59	58	56	60	293

The number of lobsters surviving after a week in the factory tanks and following after the simulated packout and storage showed a broadly similar pattern (Table 23) to that seen in the grader's figures (Table 22), even in the elevated levels of mortality in the recirculated spray treatment (Figure 29). Lobsters that had been stored submerged showed high survival and those that had been kept in air or sprays showed poor survival. Most losses that occurred were lobsters that were rejects in the tanks for the week after the stress treatments, followed by lower numbers of losses during recovery from the export simulation. Very few lobsters died in the export cartons ("Dead in box", Table 23) and this box mortality group ("Dead in box" and "Re-tank reject") was more or less uniform across treatments.

Table 23. Losses of lobsters following tanking and packing in boxes for individual lobsters for each treatment during the November 1998 factory-based trial.

	Treatment Flow subm	Recirc subm	Humid air	Flow spray	Recirc spray	Total
Tank reject	1	0	13	14	29	57
Dead in box	1	0	2	0	3	6
Re-tank reject	6	7	5	4	4	26
Survivor	52	52	38	38	24	204
Total	60	59	58	56	60	293

The grader correctly predicted the fate of 81.6% of the lobsters (Table 24). That is, the grader correctly rejected 34 lobsters that became tank rejects and correctly accepted 205 lobsters that were 'not tank rejects' out of a total of 293 lobsters. The grader accepted lobsters that were not 'tank rejects' more reliably than he rejected 'tank rejects.' Specifically, only 52.3% of rejected lobsters were in fact lost during tank storage, while 89.9% of accepted lobsters (205 out of 228) remained following storage in the tanks though some were lost during and immediately after the simulated loadout. Overall, the grader appeared to be quite strict, screening out large numbers of possibly borderline lobsters in order to ensure a good outcome for the accepted lobsters.

Table 24. Relationship between the grader's figures and the losses following tanking for lobsters and packing in boxes during the November 1998 factory-based trial.

	Rejected	Accepted	Total
Tank reject	34	23	57
Dead in box	2	4	6
Re-tank reject	4	22	26
Survivor	25	179	204
Total	65	228	293

Mortality of untagged lobsters removed from the tanks prior to loadout (estimated at about 14%), was slightly lower than that of the tagged lobsters (20%) indicating a small hemolymph sampling mortality. However, this mortality was not uniform across treatments and it is likely that the lobsters from the stressed treatments were those more susceptible to the effects of sampling. There was no evidence that sampling alone killed lobsters. The 119 lobsters sampled after submerged storage treatments (regardless of whether recirculating or not) showed less than 1% mortality.

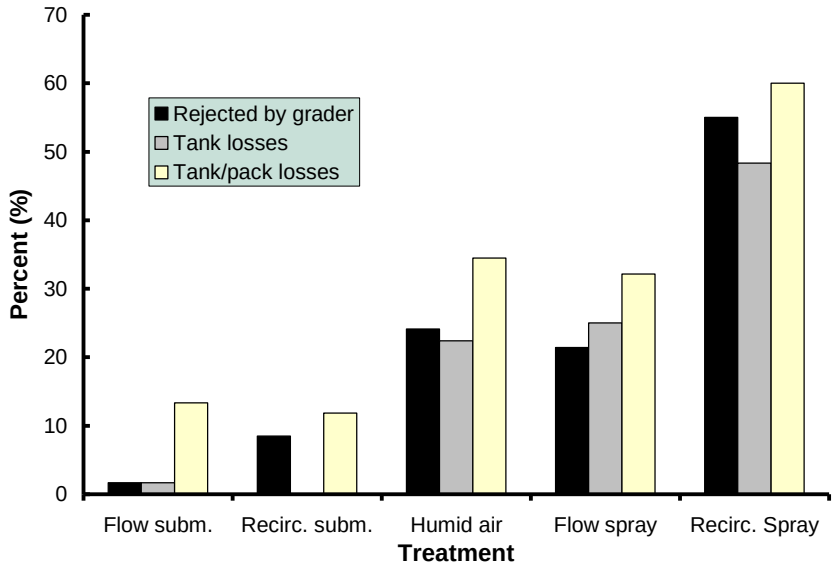


Figure 29. Comparison of rejection by the grader for each treatment, percentage of lobsters removed from the tanks and total losses (from the tanks and following simulated pack-out and storage) during the November 1998 factory-based trial.

7.5.3.2 Physiological differences between rejected and accepted lobsters

Lobsters were seldom rejected following submerged storage so there were insufficient 'reject' samples in these treatments to gain a clear picture of the characteristics of lobsters rejected under these circumstances.

Table 25. Average levels of hemolymph parameters (±SE) for lobsters from each treatment and further split according to the lobsters grade assigned by an experienced human grader during the November 1998 factory-based trial..

	Flow Subm.		Recirc. Subm.		Humid air		Flow spray		Recirc Spray	
	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected
No. of lobsters	59	1	54	5	44	14	44	12	27	33
Calcium*	13.2±0.1 a	15.3	12.7±0.2 a	12.1±0.4	14.5±0.3 b	14.1±0.4	15.9±0.2 c	15.2±0.3	15.5±0.3 bc	16.3±0.4
Chloride*	479.3±3.3 a	480.0	478.1±6.4 a	459.2±10.6	466.6±4.6 ab	465.9±6.1	474.9±3.6 a	470.7±6.8	452.2±5.6 b	456.8±6.1
Glucose *	0.3±0.1 a	0.1	0.3±0.0 a	0.2±0.1	2.2±0.2 b	2.2±0.3	1.9±0.3 b	2.1±0.3	2.1±0.2 b	2.3±0.3
Potassium *	11.5±0.1 a	10.8	11.1±0.1 a	10.4±0.5	13.1±0.1 c	13.4±0.3	12.1±0.1 b	12.9±0.2†	12.5±0.2 b	13.3±0.2†
Lactate*	1.1±0.2 a	0.7	0.9±0.1 a	1.3±0.2	4.9±0.5 b	6.0±1.5	5.1±0.6 b	6.4±1.0	4.9±0.5 b	8.2±0.7†
LDH*	54.8±14.2 a	33.9	54.1±8.7 ab	42.6±15.3	75.6±7.5 b	106.4±46.3	129.7±39.2 ab	60.9±15.9	83.8±24.0 ab	218.7±52.3†
Magnesium *	10.4±0.2 a	9.8	10.9±0.2 a	9.9±0.6	12.9±0.3 b	12.6±0.6	12.4±0.2 b	13.0±0.5	12.6±0.4 b	15.1±0.5†
Sodium *	534.2±4.0 a	531.0	555.9±3.5 b	541.0±7.7	543.7±3.5 ab	550.2±13.2	538.7±5.9 ab	514.6±10.0	526.2±8.5 a	538.3±4.3
Protein ns	83.8±3.2	91.4	79.8±2.4	68.7±5.5	84.6±2.3	78.0±3.3	82.6±3.5	78.8±1.8	91.5±5.2	87.1±4.0

Concentrations in mmol L^{-1} except for Protein g L^{-1}. Parameters that differ significantly between averages of accepted lobsters from each treatment indicated by *, whereas ns indicates not significantly different. † refers to instances where averages for accepted and rejected lobsters differ significantly in a particular treatment group. In each row, averages with the same letter postscript are not significantly different at 5%.

. Only lobsters stored in the spray treatments showed significant differences between grade outcome with respect to hemolymph parameters (Table 25). Remarkably, there were no significant differences in physiological parameters between rejected and accepted lobsters after storage in humid air. In the flow through spray treatment, the accepted and rejected lobsters differed significantly in hemolymph potassium level. For the recirculating spray treatment (the treatment with the highest rejection rate and subsequent losses), the impact was more pronounced, with potassium, lactate, lactate dehydrogenase activity (LDH) and magnesium all differing significantly between accepted and rejected lobsters.

7.5.4 Treatment effects

The different storage treatments significantly influenced the physiology of lobsters that were otherwise graded as acceptable (Table 25). The only hemolymph chemistry parameter that did not differ significantly between treatments was protein content.

The differences seen reflected whether or not the lobsters were submerged. Glucose, lactate, calcium, and magnesium levels were uniformly higher in lobsters from the non-submerged treatments than in the submerged treatments. Similarly, potassium level was also elevated in lobsters stored out of water, with the highest being in the humid air treatment. LDH activity was quite variable, and while the average activity rose in all non-submerged treatments, this was only significantly different between the flow submerged treatment and humid air. Though the latter was not the highest average activity.

The pattern became more treatment specific for other variables. Sodium level was significantly higher in the recirculating submerged treatment than in the recirculating spray and flow submerged treatments.

7.5.5 Hemolymph chemistry of future tank rejects

Having established that lobsters coming from different treatments differed significantly, the next step was to see whether this allowed us to identify lobsters that would later be taken dead or weak from the factory tanks over the following week.

'Future' survivors and tank rejects showed significantly different average levels of a number of hemolymph parameters when removed from the treatment cubicles (Table 26).

Table 26. Average hemolymph chemistry parameters (±SE, sample size in brackets) in western rock lobsters sampled leaving the non-submerged treatments (see text) in the November 1998 factory trial grouped by subsequent outcome during factory storage

	Outcome			
	Tank rejects	Box mortality	Survivor	ANOVA
Protein g L^{-1}	82.9±2.2 (54)	94.1±8.0 (17)	84.3±2.0 (99)	ns
Hcy Abs. A_{340}	0.284±0.012 (54)	0.330±0.038 (17)	0.297±0.009 (93)	ns
Chloride	465.5±4.1 (55)	462.6±6.8 (17)	465.3±2.9 (96)	ns
Sodium	536.6±4.1 (55)	532.5±10.7 (17)	538.4±3.6 (96)	ns
Calcium	15.6±0.3 (55)	16.0±0.5 (17)	15.1±0.2 (96)	ns
Magnesium	14.2±0.3a (55)	12.9±0.4ab (17)	12.5±0.2b (96)	***
Potassium	13.1±0.1a (55)	13.0±0.3ab (17)	12.6±0.1b (96)	**
Glucose	2.3±0.3 (55)	2.5±0.2 (18)	1.9±0.1 (100)	ns
Lactate	8.2±0.6a (55)	5.8±0.6ab (18)	4.4±0.3b (100)	***
LDH, IU L^{-1}	235.9±43.6a (50)	81.8±17.9b (17)	64.3±8.2b (97)	***

Unless indicated otherwise, concentrations in mmol L^{-1} . ns= not significant; ** = P<0.01; *** = P<0.001. In each row, averages with the same letter postscript are not significantly different at 5%.

A preliminary ANOVA performed on all the survivors, box mortality and tank-rejects available, found that the average concentrations of these groups differed significantly with respect to calcium, glucose, potassium, lactate, LDH, and magnesium. But the outcome for calcium and glucose occurred because submerged lobsters had low levels of these parameters (see Table 25), and if all treatments are used in the analysis the submerged lobsters inevitably influence the mean results for 'survivors'. A subsequent analysis, excluding the lobsters from the submerged treatments, removed the differences in mean calcium and glucose concentrations ($P>0.05$), but retained the significant differences for the other parameters (Table 26). Calcium and glucose levels certainly indicate treatment effects, but this apparently does not extend beyond that to predict future mortality in lobsters.
The profile of average lactate and magnesium concentrations in each treatment (pooling all grades, Figure 30) compare well with the proportion of lobsters rejected by the grader and those actually lost from the tanks noted earlier, (Figure 29).

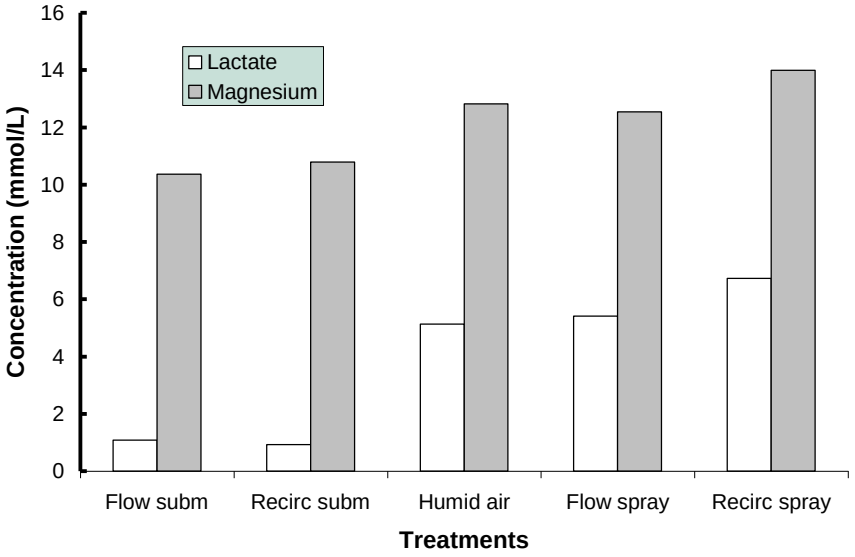


Figure 30. Average hemolymph lactate and magnesium concentrations for each storage treatment in the November 1998 factory trial.

7.5.6 Discriminant analysis

After the prelimnary ANOVA, it was now possible to try to discriminate between lobsters classified as tank rejects and survivors using these physiological differences. However, as noted above, the full data set included many submerged lobsters that were nearly all survivors. It could be argued that including these unstressed lobsters in the analysis biased the outcome. That is, the category 'Survivors' acquires some of the characteristics of these submerged lobsters, particularly with respect to calcium and glucose levels, noted in the ANOVA results above. Fortunately, the discriminant analysis described here discarded glucose and calcium in the step-wise process, selecting instead the parameters that, regardless of treatment, differed significantly between tank rejects and survivors.
Below, the analysis was conducted on all treatments, first using the three basic groups. Then the analysis was repeated, but focusing upon whether or not lobsters were rejected from the tanks. This was done by pooling the small group Box mortality into the group of 'not tank rejects.'
Incidentally, discriminant analyses were also carried out to see whether lobsters could be classified into treatments. The major hassle with this was that there was a lot of similarity between lobsters in certain treatments. Interestingly, when the treatments were pooled into two groups, emersed and submerged, a group of eight variables (with glucose and calcium providing much of the

discrimination) were able to correctly identify the origin of 97% of the samples. As a different set of parameters was obtained for the treatment analysis, this is taken as further evidence that treatment effects have little impact on the classification of lobsters into survival categories in this experiment.

7.5.6.1 stepwise discriminant analysis using all treatments and three groups

When lobsters from all the treatments were analysed, the forward stepwise discriminant analysis, using F-to-enter =2, returned a model using only 3 variables (in order of decreasing power, √Lactate>LogLDH>Mg) with a Wilks' Lambda of 0.637 ($F_{6,344}$= 14.508, ***) (Table 27).

Table 27. Variables in the model following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (and grouped as tank rejects, box mortality and survivors). N=177. Lobsters with missing data excluded.

	Wilks' Lambda if removed (Λ)	Partial Lambda	F-to-remove (2,171)	p-level	Tolerance	1-Tolerance R^2
√Lactate	0.709	0.899	9.708	0.000	0.723	0.277
LogLDH	0.684	0.931	6.422	0.002	0.944	0.056
Mg	0.659	0.966	2.990	0.053	0.749	0.251

The group 'Box mortality' did not separate significantly from Survivors in terms of squared Mahalanobis distances (Table 28), and tests of residual discrimination showed that only the first discriminant function accounted for a statistically significant amount of discrimination (Table 29).

Table 28. Effectiveness of group separation according to squared Mahalanobis distance following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (and grouped as tank rejects, box mortality, survivors).

RESULT	Tank rejects	Box mortality	Survivor
	Squared Mahalanobis distances		
Tank rejects	-	3.036	3.991
Box mortality	3.036	-	0.110
Survivor	3.991	0.110	-
	F-values (DF= 3,172)		
Tank rejects	-	10.44	31.96
Box mortality	10.44	-	0.52
Survivor	31.96	0.52	-
	p-levels		
Tank rejects	--	0.000	0.000
Box mortality	0.000	--	0.671
Survivor	0.000	0.671	--

Table 29. Significance of discriminant functions in order of derivation, following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (and grouped as tank rejects, box mortality, survivors)

Functions derived (k)	Eigen-value (λ)	Canonical correlation (R)	Wilks' Lambda (Λ)	χ^2	df	p-level
0	0.565	0.601	0.637	78.05	6	0.000
1	0.003	0.058	0.997	0.59	2	0.745

This "all treatment" 3 groups model was only able to classify 77% of lobsters correctly into Tank rejects, Box mortality and Survivors (Table 30). A major reason for this was that the small and "improbable" group, 'Box mortality' was overshadowed (mathematically speaking) by its larger near neighbour, Survivors. The reader may recall that a similar problem occurred previously, (see discussion on the 'W' group in the pilot study, Section 7.4.4.1.5). These hemolymph test results, taken a week before packing, cannot discriminate between the lobsters that will or will not die during simulated transport. Pooling the 'Box mortality' lobsters into 'not tank rejects' would seem a practical step, focusing then on lobsters that are tank rejects or otherwise.

Table 30. Re-classification of lobsters using posterior probabilities calculated from the distances between cases and the group centroids, following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (and grouped as tank rejects, box mortality and survivors).

		Predicted classifications		
		Tank rejects	Box mortality	Survivor
Observed				
	% correct			
Tank rejects	62.5	30	0	18
Box mortality	0.0	4	0	24
Survivor	94.5	9	0	154
Total	77.0	43	0	196

7.5.6.2 stepwise discriminant analysis of lobsters from all treatments using two groups (Tank-rejects and Not-tank-rejects).

The classification accuracy increased to 87% (Table 33) when the all treatment analysis for November 98 was repeated using 'tank rejects' and the pooled 'not-tank-rejects' category. The stepwise process arrived at a model requiring the same variables (√Lactate>LogLDH>Mg) with a Wilks' Lambda of 0.643 ($F_{3,137}$=32.07***)(Table 31). The F to remove statistic shows that √Lactate provides the greatest discrimination to the analysis (Table 32). The classification functions are presented in Table 34.

Table 31. Summary of stepwise discriminant function analysis, for lobsters from all treatments sampled during the November 1998 factory trial and grouped according to 2 groups: tank rejects and not-tank-rejects.

Variable to enter	Step	F to enter	df	p-level	vars.	Wilk's Lambda Λ	F value	df	p-level
√Lactate	1	73.76	1,175	0.000	1	0.703	73.76	1,175	0.000
LogLDH	2	11.08	1,174	0.001	2	0.661	44.55	2,174	0.000
Mg	3	4.98	1,173	0.027	3	0.643	32.04	3,173	0.000

Table 32. Variables in the model following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial and grouped according to 2 groups: tank rejects and not-tank-rejects. N=177. Lobsters with missing data excluded.

	Wilks' Lambda (Λ)	Partial Lambda	F-to-remove -1,173	p-level	Tolerance	1-Tolerance R^2
√Lactate	0.715	0.899	19.51	0.000	0.721	0.279
LogLDH	0.691	0.931	12.83	0.000	0.944	0.056
Mg	0.661	0.972	4.98	0.027	0.747	0.253

Table 33. Re-classification of lobsters using posterior probabilities calculated from the distances between cases and the group centroids, following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial and grouped according to 2 groups: tank rejects and not tank rejects.

		Predicted classification	
		Tank Reject	Not Tank Reject
Observed	% Correct		
Tank Reject	60.4	29	19
Not Tank Reject	94.2	11	180
Total	87.4	40	199

Table 34. The classification functions obtained following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial and grouped according to 2 groups: tank rejects and not tank rejects.

	Classification	
Variable	Tank Reject	Not Tank Reject
√Lactate	-1.6643	-3.2878
LogLDH	13.4545	11.322
Mg	4.0699	3.7443
Constant	-41.682	-28.5933

7.6 Validation of indicators including test of alternative environments- March 1999 (sub-objective 4 and 5)

7.6.1 Summary

This was the second of the replicate trials aimed at confirming whether the principle established in the initial trial (section 6.4) was repeatable, and to see if parameters with greater discriminant power could be identified.

The experiment was conducted in a similar manner to the one already described (see Figure 25). Lobsters were stressed for a period, graded by an experienced grader, their hemolymph was sampled, they were then tagged, and returned to factory tanks for a period of observation to identify lobsters that would die in the tanks before those remaining were challenged by a period of simulated export.

Of the 313 lobsters sampled, the grader rejected 46% of them. The grader found fewer rejects in the submerged treatments than in the non-submerged treatments. In all, there was a 21% mortality in the tanks following the treatments.

Submerged storage of lobsters led to no or negligible mortality. While this is not surprising, it is interesting that this apparently also applied to short periods of exposure to deteriorating water quality in a tank of recirculating seawater. The message here is that biological filtration of water during short periods of submerged storage or transport may be unnecessary.

Submerged storage/transport of lobsters may not be possible under many circumstances, making it important to identify the next most effective method of transport. Virtually all of the lobsters that died in the factory tanks came from the non-submerged treatments. The spray treatments in particular produced high mortality and levels of rejection when first graded, and high levels of mortality during later storage in the factory. The lobsters died relatively quickly compared to previous trials, mostly within the first day after the treatment.

The hemolymph samples taken from the lobsters were analysed for a broad suite of parameters. This showed that lobsters sampled on emerging from the different treatments differed significantly in a number of physiological parameters, again, in accordance with expected changes accompanying storage in air (section 7.3).

Using discriminant analysis on lobsters from all treatments, five hemolymph tests could correctly classify 89.6% of the lobsters sampled as tank rejects or not, without any systematic bias toward either category. These five variables are ranked here according to their ability to discriminate between groups.

Clotting time>√Lactate>>%granulocytes>Calcium>√Bactrank

Health related parameters dominated the results, with only lactate and calcium retained from the sets obtained by previous analyses (section 6.4). The clotting time and levels of lactate in the hemolymph explained much of the differences between lobsters that would survive and that those that were lost in tank storage, regardless of treatment. Bactrank is a ranking variable describing the hemolymph bacteria counts. The prominence of immunological variables in this new analysis could be associated with the higher levels of lactate concentration achieved and the faster rate of mortality observed after the treatments. The change may reflect lobsters sampled at a point closer to death.

Despite the variation in the range of stress indicators used to predict between trials, the fact that these predictions are at all possible confirms that there is a large degree of predictability associated with the changes occurring in lobster hemolymph following a stress. In other words, the stress that kills lobsters leaves its mark in their hemolymph before they die, and so the parameters that describe this stress, can be used as markers of lethal stress during lobster handling.

Due to the size of the statistical analysis involved, the issue of prediction using the equations from one trial onto the data collected in other trials will be left until section 7.7.

The remainder of section 7.6 examines the data from this trial in detail. An account is given of:

- water quality changes in the treatments (section 7.6.2).
- the outcome of grading and subsequent losses in the tanks (section 7.6.3).

- storage treatment effects (section 0).
- the hemolymph chemistry of the tank rejects versus other lobsters (section 7.6.5)
- the discriminant analysis of the hemolymph test results (section 7.6.6).

As this study occurred in collaboration with the companion project FRDC 96/344 there are also immune system results for the lobsters sampled in this trial. This data is not presented in sections 7.6.2 to 7.6.5 of this report and the reader should refer to the corresponding section of the other report. However, in the interests of completeness, all the data for each lobster has been included in the discriminant analysis (section 7.6.6).

7.6.2 Changes in water quality

As in the first trial, oxygen saturation remained at acceptable levels in the water in both the submerged tanks of lobsters and in the sumps of the spray treatments (data from the three replicates are pooled in Figure 31). However, this time, the ambient seawater temperature was higher than the air temperature in the room where the storage cabinets were located and for the first couple of hours of the first replicate, air temperature in the humid air treatment was slightly lower than that of the wet treatments. This was corrected after two hours (see Figure 32), and throughout subsequent replicates by aerating a bucket of hot water inside the cabinet.

The water pH and ammonia data showed a different pattern to that seen in the November 1998 trial. With pH, acidification was only seen in the recirculating submerged treatment (Figure 33). Interestingly the water in the recirculating spray system retained a similar pH to that of the seawater supply. The same treatment also showed less ammonia accumulation than that seen in the recirculating submerged treatment (Figure 34), though the later reached levels at 6 h similar to that observed in the earlier trial.

At present it is not clear why the recirculating spray treatment behaved differently this time. Possible contributing factors this time were that a different spray head was used- to increase the spray 'footprint' on the baskets of lobsters, and of course that the ambient temperatures were higher in the current trial. Perhaps this time in the recirculating spray, the wastes were either remaining in the lobsters or were being purged more efficiently from the water this time (eg. perhaps gas stripping occurred while aerating the sump).

The fact that mortality was similar in both flow and recirculating spray treatments (below) and was low in the treatment with the highest ammonia levels suggests that the wastes level in the water under these conditions is not a significant factor in lobster survival.

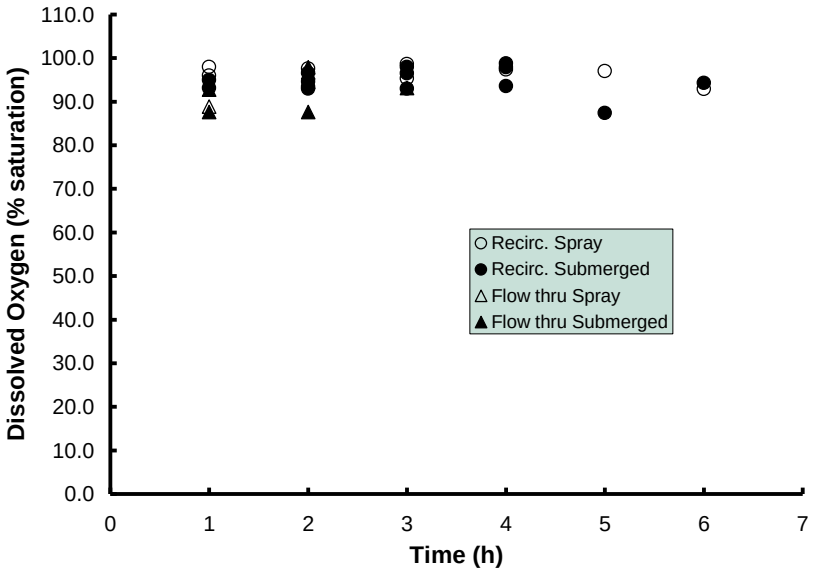


Figure 31. Oxygen saturation measured during the submerged and spray treatments during the March 1999 experiments.

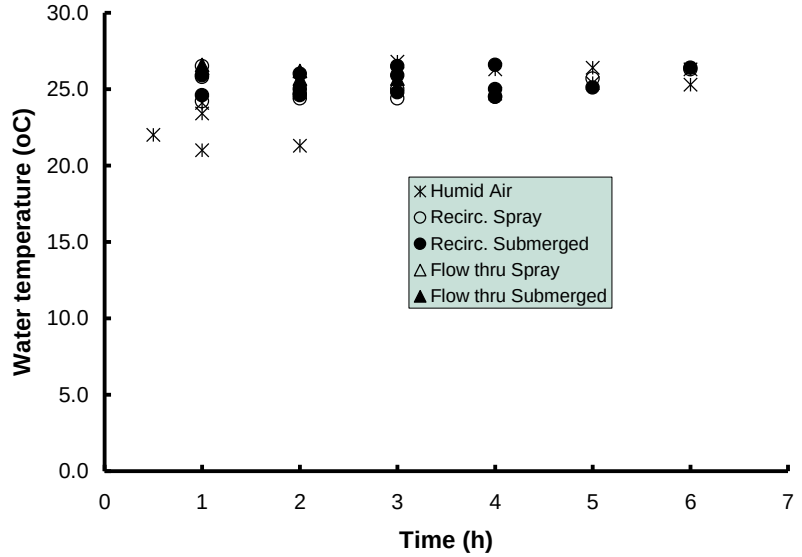


Figure 32. Temperature measured in the submerged, humid air and spray treatments during the March 1999 experiments.

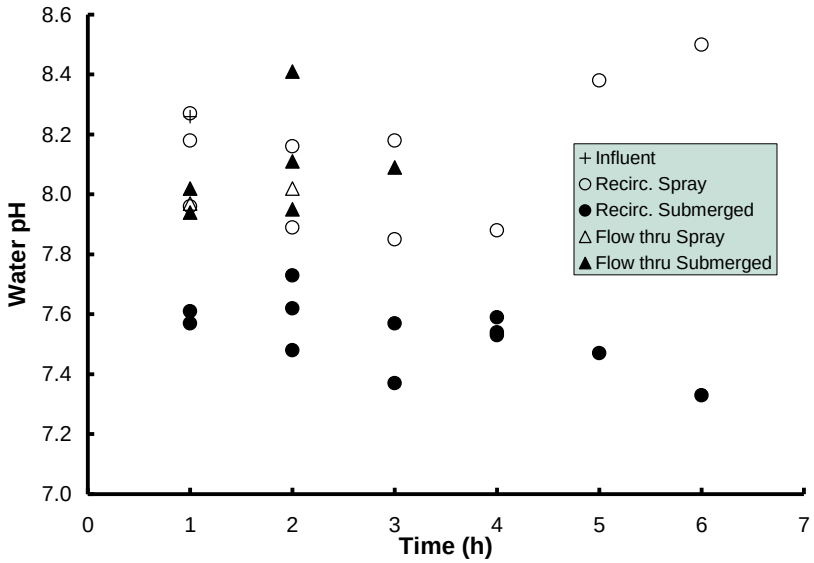


Figure 33. Water pH measured during the submerged and spray treatments during the March 1999 experiments.

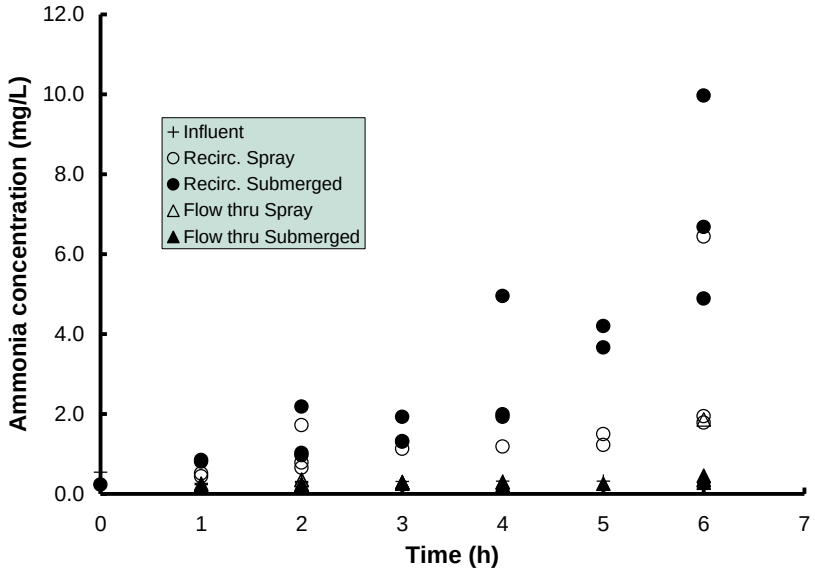


Figure 34. Ammonia concentration measured during the submerged and spray treatments during the March 1999 experiments.

7.6.3 Grading and losses during storage in tanks

7.6.3.1 Grading

These grading figures reflect the condition of all lobsters emerging from the treatments prior to splitting the sample into control and bled groups. The grader accepted most of the lobsters in the submerged treatments (Table 35). A small proportion of these lobsters was rejected as weak, but a major fraction of the rejects were discarded because of leg-loss or injury. Few legs were found in the baskets used so most of this leg-loss apparently preceded the treatments.

Table 35. Grading result when assessing the lobsters leaving the treatments in the March 1999 factory-based trial.

	Storage treatments				
	Flow subm	Recirc subm	Humid air	Flow spray	Recirc spray
Dead	0	0	1	20	19
Weak	8	18	81	81	100
Legloss	9	6	3	4	4
Accepted	122	116	50	33	18
Totals	139	140	135	138	141

The result was poorer for the non-submerged treatments, with a higher proportion of weak lobsters, and a corresponding decline in accepted lobsters. Of interest though is the large number of lobsters that were graded 'dead' emerging from the spray treatments, a situation which hadn't occurred in the November 1998 trial. This was the first sign that the lobsters were becoming moribund at a faster rate than in the November study, perhaps due to the higher temperature. This point will be examined further as we account for sampled verses control mortality rate. Note that in terms of initial mortality, lobsters stored in humid air in this trial were apparently performing better than those stored in sprays.
Acceptable or rejected lobsters entered the bled and unbled (ie. control) groups and were ultimately tanked in correct proportion to the original grade. This meant that average levels of hemolymph parameters in the bled lobsters (below) reflects that of lobsters from each treatment. Experience showed that there were adequate numbers of rejects so it was not necessary to amplify the number of rejected lobsters that were hemolymph sampled. Further, the previous practice of sampling all rejects (sections 7.4 and 7.5) was discontinued because it complicated interpretation of the treatment effects.

7.6.3.2 Hemolymph chemistry of accepted and rejected lobsters

Since treatment had such a dramatic effect on hemolymph parameters, it was once again necessary to consider hemolymph physiology of accepted and rejected lobsters on a treatment by treatment basis. The mean levels of at least one hemolymph parameter (eg. lactate) differed significantly between accepted lobsters and rejected lobsters, but there was no consistent pattern applying to all treatments (Table 36). As few lobsters in the flow submerged treatment were rejected, no statistics were performed on this treatment.
Lobsters rejected from the recirculating submerged treatment had a significantly different glucose concentration (Table 36), though the difference involved is so slight that the biological or commercial importance of this finding is doubtful. Even in the non-submerged treatments, there was no consistency in the differences between accepted and rejected lobsters. In humid air and the flow spray treatment, the lactate concentration, perhaps not surprisingly distinguished the rejected lobsters. The grades in the flow spray treatment also differed in chloride concentration but in the recirculating spray treatment rejects differed significantly from accepted lobsters in protein concentration! This latter finding is difficult to interpret. Only 10 lobsters were accepted in this treatment, and the lower sample size had the higher variance, probably making it difficult to demonstrate statistical significance in lactate concentration.

Table 36. Comparison of average hemolymph chemistry tests (± standard error) from western rock lobsters accepted or rejected by an experienced grader following each 6 h storage treatment. Data pooled for trials conducted on three consecutive days in March 1999.

	Flow subm		Recirc subm		Humid air		Flow spray		Recirc spray	
	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected
Lobsters sampled	60	4	58	9	24	40	16	40	10	50
Calcium	14.6±0.2	16.3±0.1	14.2±0.4	13.7±0.5	16.3±0.5	16.2±0.4	17.7±0.7	17.7±0.6	15.4±1.8	16.8±0.3
Chloride	542.4±12.4	594.7±52.5	539.0±9.0	561.0±21.3	558.3±14.1	542.4±12.8	561.5±14.4	516.7±8.0†	522.7±20.4	525.3±9.4
Glucose	0.6±0.1	0.5±0.1	0.6±0.1	0.4±0.1†	2.0±0.2	2.2±0.2	1.7±0.2	1.9±0.2	3.0±0.4	2.2±0.2
Potassium	10.2±0.2	11.7±0.6	10.2±0.2	9.3±0.4	12.3±0.4	12.2±0.4	11.6±0.4	11.7±0.4	12.1±0.5	11.8±0.2
Lactate	2.7±0.2	2.4±0.1	2.9±0.3	2.4±0.3	8.5±0.8	11.1± 0.8†	13.6±1.5	17.5± 0.8†	13.6±1.7	16.4±0.6
Magnesium	11.4±0.2	11.8±0.4	11.7±0.3	11.6±0.4	13.9±0.5	14.6±0.3	13.0±0.6	14.4±0.4	13.2±1.6	14.2±0.3
Sodium	591.8±7.6	623.7±22.8	607.3±10.9	565.3±16.4	610.0±14.7	605.2±11.4	614.7±21.9	585.4±14.2	592.8±33.8	576.3±7.5
Protein	85.4±3.1	102.1±16.5	92.1±3.7	85.0±11.2	83.5±3.8	83.6±2.8	85.0±3.6	88.0±3.5	117.0±11.7	90.0± 3.4†

Units are mmol L^{-1} except protein, which was g L^{-1}. † indicates that averages for accepted and rejected lobsters from this treatment are significantly different at 5%.

7.6.3.3 Survival of lobsters following tanking

Most of the rejected lobsters were dead within a day. This early mortality was unexpected based upon the previous trials. This trial was unusual in that that some lobsters were already dead when unloading the treatment cubicles (section 7.6.3.1). By comparing with the rate of mortality for the November 1998 data it is clear that the current group of lobsters were also dying in the tanks at a greater rate in the days immediately after treatment and sampling. (Figure 35). Indeed, many lobsters died that night (time 0 in the figure). Beyond the initial losses in the tanks, mortality following loadout and re-tanking of lobsters was only minor (Table 37).

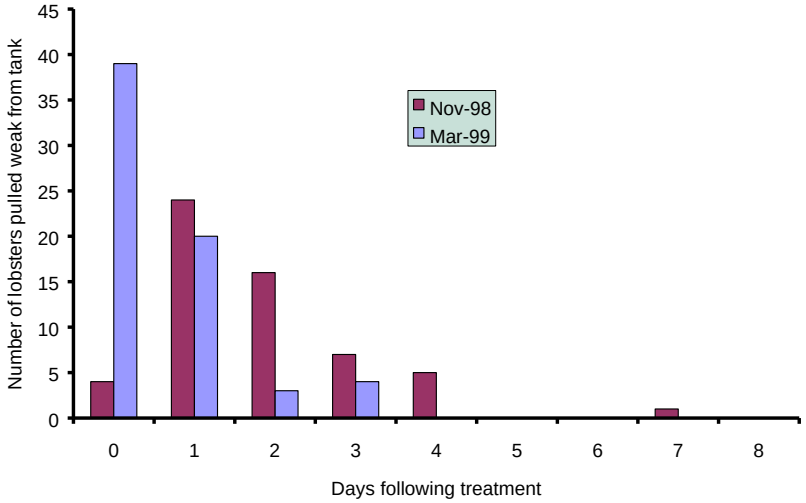


Figure 35. Numbers of tank rejects removed from the tanks on the night of the treatment (0) and on each day during the subsequent week for the November 1998 and March 1999 factory-based trials.

The lobsters from the submerged treatments showed little or no mortality when returned to the factory tanks, (Table 37). Even the lobsters previously stored in recirculating seawater showed negligible mortality over the subsequent week. This indicates that the water quality changes, under the conditions operating here, were not detrimental to the lobsters. Short term storage/transport of rock lobsters in recirculating tanks may be a feasible alternative when conditions warrant it.

Table 37. Losses of tagged lobsters from the tanks and during simulated transport and numbers of survivors for each treatment in the March 1999 factory based trial.

	Storage treatments				
	Flow subm.	Rec. subm.	Humid air	Flow spray	Rec. spray
Tank rejects	0	1	10	28	28
Dead in box	0	1	1	1	0
Re-tank rejects	4	2	3	0	1
Survivor	60	63	52	27	31
Totals	64	67	66	56	60

Lobsters originating from the non-submerged treatments showed poorer survival, though surprisingly, the worst result was in the spray treatments. The humid air result was surprisingly good, though it does back up the finding in the November study that sprays are not necessarily better for lobsters than simple humid air. Seawater sprays at very least should keep the conditions humid, so these results imply that spraying seawater on the lobsters is in some way detrimental to their survival. This finding needs to be investigated further.

The proportion of tagged lobsters that died after about a week in the tanks (tank rejects, Table 37) shows a similar profile for the treatments to that shown by the grader's figures (Table 35). The similarity of the grader's result for each treatment to the tank losses and total losses for these treatments after simulated packing is summarised in Figure 36.

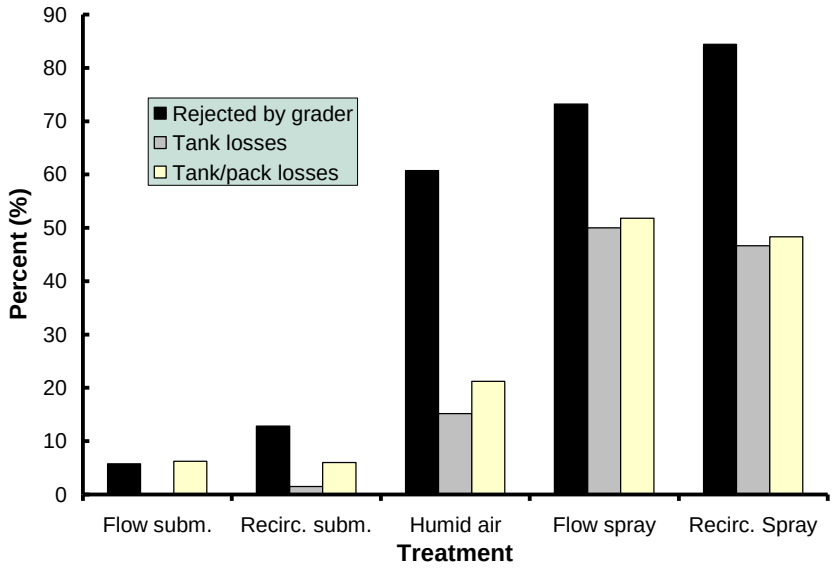


Figure 36. Comparing the level of rejection by the grader to the proportions lost in tanks and combined tank and packout losses in the March 1999 factory-base trial.

Overall, the outcome in the tanks was better than predicted by the grader, who was again systematically rejecting lobsters (Table 38).
About half of the lobsters that were originally rejected by the grader nevertheless survived in the tanks to be loaded out. However, as reported in previous experiments, the grader correctly rejected 92.5% of the lobsters that would later die in the tanks in the group of lobsters rejected. The flip-side of this grading 'bias' is that only 3% of lobsters were incorrectly graded as acceptable (i.e. 5 out of 169 lobsters). This is more clearly seen by grouping the lobsters as either tank rejects or 'not tank rejects' (Table 39).

Table 38. Comparing the grade of the lobster against its fate in the factory following the treatment in the March 1999 factory based trial.

	Rejected	Accepted
Tank rejects	62	5
Dead in box	2	1
Re-tank rejects	2	8
Survivor	78	155
Totals	144	169

Table 39. Examining the grading outcome versus the tank mortality in the March 1999 factory based trial by pooling lobsters that were 'not tank rejects' into a single category.

		Grade		Totals
Observed result	% correct	Rejected	Accepted	
Tank reject	92.5	62	5	67
Not tank rejects	66.7	82	164	246
Totals	72.2	144	169	313

7.6.4 Treatment effects

Lobsters from the submerged treatments, flowing or recirculating, were at no point significantly different in any of the parameters measured (Table 40).

Table 40 . Comparison of average hemolymph chemistry tests from western rock lobsters following each 6 h storage treatment. Data pooled for trials conducted on three consecutive days in March 1999.

	Flow subm.	Recirc. subm.	Humid air	Flow spray	Recirc. spray
n	64	67	64	56	60
Calcium	14.7±0.2a	14.1±0.4a	16.3±0.3b	17.7±0.5c	16.6±0.4c
Chloride ^{ns}	545.1±12.0	541.9±8.3	548.3±9.6	530.5±7.6	524.9±8.5
Glucose	0.6±0.1 a	0.6±0.1 a	2.1±0.1 bc	1.8±0.2 b	2.3±0.2 c
Potassium	10.2±0.2 a	10.1±0.2 a	12.2±0.3 b	11.7±0.3 b	11.9±0.2 b
Lactate	2.7±0.2 a	2.8±0.2 a	10.1±0.6 b	16.4±0.8 c	16.0±0.6 c
Magnesium	11.4±0.2 a	11.7±0.3 a	14.3±0.3 b	14.0±0.4 b	14.0±0.3 b
Sodium ^{ns}	593.4±7.4	601.5±9.8	607.0±8.9	593.8±11.9	578.3±7.6
Protein ^{ns}	86.5±3.0	91.2±3.5	83.5±2.3	87.1±2.7	94.7±3.7

Units are mmol L^{-1} except protein, which was g L^{-1}. In each row, averages with similar postscripts are not significantly different at 5%.

In general terms, the non-submerged treatments differed significantly in many of the measured parameters. Only chloride, sodium, and protein level did not differ between treatments.

Calcium, glucose, potassium, lactate, and magnesium levels rose in the non-submerged groups, though some rose more significantly than others. In humid air, calcium, and lactate concentrations were significantly lower from one or both of the spray treatments. For glucose, flow spray was significantly lower from recirculating spray but not humid air.

7.6.5 Hemolymph chemistry following treatment versus later survival

Given that the submerged lobsters showed no or negligible tank mortality, attention was focused here upon those treatments that showed significant levels of tank mortality, namely the emersed treatments. There were relatively few lobsters (n=6) that failed the load-out challenge ('Box mortality' in the Discriminant analysis below) so these will be ignored for the present and t-tests will be conducted on tank rejects and survivors.

Future survivors had significantly higher protein and sodium concentration and significantly lower lactate level than future tank rejects (Table 41).

Table 41. Average hemolymph chemistry parameters (±standard error) in western rock lobsters leaving emersed treatments in the March 1999 factory trial grouped by subsequent outcome during factory storage

	Outcome			t-test
	Tank rejects	Box mortality	Survivor	
Protein	83.4±1.7 (63)	82.9±5.9 (6)	91.4±2.5 (108)	**
Chloride	529.5±7.9 (63)	561.3±14.8 (6)	537.4±7.0 (98)	ns
Sodium	572.6±8.9 (61)	607.0±27.0 (6)	605.6±7.2 (97)	**
Calcium	16.7±0.4 (63)	17.4±1.3 (6)	16.9±0.3 (100)	ns
Magnesium	14.2±0.3 (63)	15.7±0.6 (6)	14.0±0.2 (100)	ns
Potassium	11.9±0.2 (61)	12.6±0.8 (6)	12.0±0.2 (98)	ns
Glucose	2.0±0.2 (64)	2.7±0.6 (6)	2.1±0.1 (108)	ns
Lactate	16.3±0.6 (63)	14.1±1.8 (6)	12.6±0.6 (107)	***

ns= not significant; ** = P<0.01; *** = P<0.001. Only tank rejects and survivors were compared. Sample size in brackets, Units are mmol L^{-1} except protein, which was g L^{-1}

Something immediately apparent when comparing these results to those of the previous trial (Table 26) was the higher levels of lactate measured in these lobsters and the fact that magnesium level appears to be high in all lobsters sampled here. Unfortunately, LDH activity could not be measured this time because of technical problems after transporting the spectrophotometers to Geraldton.

7.6.5.1 Hemolymph chemistry of tank rejects on the first night

Some lobsters were so stressed that they died that very night. It might be expected that there would be something unusual about those lobsters. To examine this, we tallied all the lobster tag numbers according to when they were removed from the tank and using this did an ANOVA of 'died first night' lobsters versus lobsters that died later 'Died later' and those that were not tank rejects 'Did not die.' Again, lobsters from the submerged treatments were excluded from this analysis to stop the characteristics of unstressed submerged lobsters from distracting us from differences between lobsters in the emersed treatments.

The groups differed significantly in hemolymph lactate and sodium concentration (respectively, P<0.001 and P<0.05). However, the 'died first night' and 'died later' groups did not differ significantly in these parameters. But the unprecedented 'first night' losses appeared to be reinforcing the position of low hemolymph sodium level as a characteristic of the tank rejects in this trial (Table 41). The hemolymph sodium concentration of lobsters in the 'died first night' group was significantly different from that of lobsters that were not tank rejects; 'did not die' (Figure 37). While the evidence is slight at this stage, low hemolymph sodium level appears to be a symptom of severe distress in these lobsters.

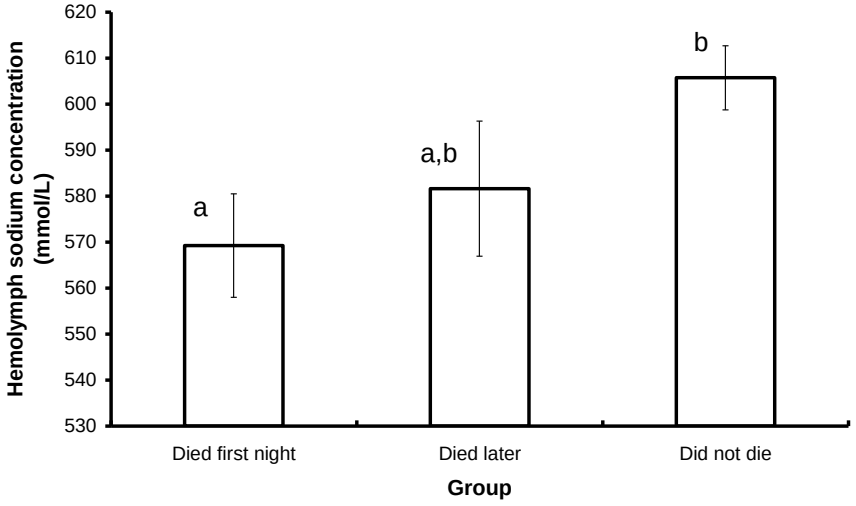


Figure 37. Hemolymph sodium concentration emerging from the treatments for lobsters that died on the night of the experiment, lobsters that died later and for survivors in the March 1999 factory-based trial.

Apart from differences in lactate and sodium discussed here, the tank rejects from the emersed treatments also differed significantly from survivors in hemolymph protein concentration (Table 41). However, the ANOVA showed no significant difference in protein level (P=0.09) between the groups 'died first night,' 'died later' and 'did not die.'

Generally, there were fewer physiological differences between tank rejects and survivors than seen in the previous study. The physiological parameters measured here may be ideal for measuring the responses of a lobster to emersion, but perhaps they are rapidly overtaken by pathological consequences that they do not adequately describe. The physiological parameters apparently deviate from baseline into a zone representing lethal 'stress' (that is, the lobster is likely to die) however after entering that band any further variation between lobsters in these parameters probably does not determine how soon individuals will die. Interestingly, the only parameter that differed significantly in these first night deaths was total hemocyte count. This is covered in more detail in the report of the accompanying project (FRDC 96/344).

7.6.6 Discriminant analysis

The tests above showed that when the lobsters were sampled emerging from the treatment cubicles, the group of future tank rejects and the survivors already differed significantly in a number of hemolymph parameters. The next step was to use discriminant analysis from this project, and the associated project 96/344, to see if lobsters could be classified into the three categories (tank rejects, box mortality and survivors) on the basis of their hemolymph test results. However, very few lobsters died as a result of being packed in the boxes. When a discriminant analysis was conducted using the three standard groups, the box mortality group did not separate well from the overall survivors in terms of the Mahalanobis Squared Distance. Since the lobsters that failed the packing challenge generally resembled the survivors, we repeated the analysis by pooling box mortality and survivors into the category of lobsters that survived until the pack out, the "not tank rejects." This was the same strategy adopted in the November 1998 study.

7.6.6.1 Stepwise discriminant analysis of 2 groups (tank rejects/ not tank rejects) using all treatments.

Forward selection using F-to-enter=2, arrived at a model requiring 5 variables, with a Wilks' Lambda of 0.655 (approx. F(5,173)=18.23***) (Table 42).

Table 42. Summary of stepwise discriminant function analysis (F to enter =2), for lobsters from all treatments sampled during the March 1999 factory trial and grouped according to 2 groups: tank rejects and not-tank-rejects.

Variable entered	Step	F to Enter	df	p-level	No. of vars.	Wilk's Lambda (Λ)	F value	df	p-level
√Lactate	1	47.73	1,177	0.000	1	0.788	47.73	1,177	0.000
Clot time	2	18.98	1,176	0.000	2	0.711	35.78	2,176	0.000
%granul.	3	5.79	1,175	0.017	3	0.688	26.43	3,175	0.000
Calcium	4	4.55	1,174	0.034	4	0.671	21.37	4,174	0.000
√BactRank	5	4.14	1,173	0.043	5	0.655	18.23	5,173	0.000

The variables with the largest individual contributions to discrimination were clotting time and lactate concentration (Table 43).

Table 43. Variables in the model following discriminant function analysis of lobsters from all treatments sampled during the March 1999 factory trial and grouped according to 2 groups: tank rejects, not tank rejects. N=179. Lobsters with missing data excluded.

	Wilks' Lambda if removed (Λ)	Partial Lambda of variable	F-to-remove (1,173)	p-level	Tolerance	1-Tolerance (R^2)
√Lactate	0.719	0.911	16.81	0.000	0.695	0.305
Clot time	0.724	0.905	18.12	0.000	0.899	0.101
%granul.	0.677	0.967	5.84	0.017	0.990	0.010
Ca	0.674	0.972	4.97	0.027	0.773	0.227
√BactRank	0.671	0.977	4.14	0.043	0.946	0.054

When the equations derived from this analysis were used to classify the same lobsters into 'not tank rejects' or tank rejects, the model got almost 90% of classifications correct (Table 44).

Table 44. Re-classification of lobsters using posterior probabilities calculated from the distances between cases and the group centroids, following discriminant function analysis of lobsters from all treatments sampled during the March 1999 factory trial and grouped according to RESULT (2 groups: tank rejects and not tank rejects).

		Predicted classifications	
Observed Group	%Correct	Tank reject	Not tank reject
Tank reject	65.6	21	11
Not Tank reject	94.1	10	160
Total	89.6	31	171

It was interesting that while there were classification errors in this table, there was no systematic bias toward identifying lobsters as 'not tank rejects' or 'tank rejects.' In the table, 32 lobsters were observed to be tank rejects, and 31 lobsters were classified as tank rejects. This contrasted to the outcome of the pilot study, which was biased toward survivors or 'not tank rejects.' The November 1998 study (Table 20), if anything, showed a slight bias toward classifying lobsters as tank rejects. The current classification functions are shown in Table 45.

Table 45. The classification functions obtained following discriminant function analysis of lobsters from all treatments sampled during the March 1999 factory trial and grouped according to 2 groups: tank rejects and not tank rejects.

	Classification	
Variable	Tank reject	Not tank reject
√Lactate	-1.2762	-2.4086
Clot. time	0.5999	0.485
%granul.	0.6827	0.8653
Calcium	3.0963	3.3839
√BactRank	1.2204	0.6487
Constant	-45.7084	-39.1719

Repeating the analysis using emersed treatments only (see section 7.6.6.2) retains a similar set of variables in the discriminant model, though there is no apparent compounding of treatment effects into the analysis.

This March study had a higher treatment temperature and showed a heightened level of stress and initial mortality. For example, the lactate levels were much higher in the current study- to the extent that 'not-tank rejects' in this data resemble the tank-rejects of the earlier work. This point will be canvassed when we examine the predictability of the discriminant equations across different trials in section 7.7.

The list of parameters in the equations obtained here differs from that found in the November study. The lobsters response to the treatment conditions this time around had apparently deteriorated to the point where a shift occurred in the parameters that best described the different groups of lobsters. Why did this happen? Sampling occurred after a defined 6 hours of treatment. The rate of change in each parameter had a bearing upon the profile of hemolymph parameters shown at the time of

sampling. The scope for some parameters to rise may have been limited, allowing the profile in lobsters that were less stressed to catch up with the others. This could explain why certain physiological parameters previously found to be useful stress indicators (eg. magnesium) were more or less uniformly high regardless of outcome in these lobsters (Table 41). It would also account for why the survivors from one trial accumulated enough lactate in one experiment (Table 41) that they resembled lobsters that died in a previous experiment (Table 26).
As shown, when sampled this time, the variation explaining impending mortality was now expressed amongst a gamut of immunological parameters. Judging by the mortality rate on the first evening, it could be argued that we were now describing the characteristics of morbid lobsters rather than predicting their future mortality. Perhaps this accounts for the greater accuracy of prediction in Table 44.

7.6.6.2 Stepwise discriminant analysis using 2 groups (tank rejects/ not tank rejects) of emersed lobsters

This analysis is included simply to show that the characteristics of the submerged lobsters are not a major issue when it comes to establishing the list of parameters.
Forward selection using F-to-enter=2, arrived at a model requiring a group of 5 variables largely corresponding to that obtained above with the larger data set, with a Wilks' Lambda of 0.692 (approx. F(5,93)=8.28***) (Table 46).

Table 46. Summary of stepwise discriminant analysis (F to enter >=2) of the emersed march 1999 lobsters grouped into 2 groups, tank rejects and pooled not tank rejects

Variable Entered	Step	F to enter	df	p-level	No. of vars.	Wilk's Lambda (Λ)	F-value	df	p-level
Clot time	1	19.42	1,97	0.000	1	0.833	19.42	1,97	0.000
%granul.	2	6.62	1,96	0.012	2	0.779	13.58	2,96	0.000
√Lactate	3	4.86	1,95	0.030	3	0.741	11.04	3,95	0.000
√BactRank	4	3.49	1,94	0.065	4	0.715	9.37	4,94	0.000
Protein	5	3.08	1,93	0.083	5	0.692	8.28	5,93	0.000

Clotting time provided the largest individual contribution to discrimination, followed by lactate concentration and %granulocytes (Table 47).

Table 47. Variables in the model following discriminant function analysis of lobsters from EMERSED TREATMENTS ONLY sampled during the March 1999 factory trial and grouped according to RESULT (2 groups: tank rejects, not tank rejects). N=99. Lobsters with missing data excluded.

	Wilks' Lambda if removed (Λ)	Partial Lambda of variable	F-to-remove 1,93	p-level	Tolerance	1-Toler. (R^2)
Clot time	0.787	0.879	12.78	0.001	0.976	0.024
%granul.	0.723	0.958	4.12	0.045	0.977	0.023
√Lactate	0.735	0.941	5.79	0.018	0.861	0.139
√BactRank	0.719	0.962	3.69	0.058	0.978	0.022
Protein	0.715	0.968	3.08	0.083	0.869	0.131

This model correctly classified 82.6% of lobsters. (Table 48). It is interesting that while there are classification errors in this table, there is no systematic bias toward identifying lobsters as 'not tank rejects' or tank rejects. That is, the observed (row) and predicted (column) totals for the Tank reject group are similar.

Table 48. Re-classification of lobsters using posterior probabilities calculated from the distances between cases and the group centroids, following discriminant function analysis of lobsters from EMERSED TREATMENTS ONLY sampled during the March 1999 factory trial and grouped according to 2 groups: tank rejects and not tank rejects.

		Predicted classifications	
Observed Group	%Correct	Tank reject	Not tank reject
Tank reject	64.5	20	11
Not tank reject	89.3	9	75
Total	82.6	29	86

The classification functions are presented in Table 49.

Table 49. The classification functions obtained following discriminant function analysis of lobsters from EMERSED TREATMENTS ONLY sampled during the March 1999 factory trial and grouped according to 2 groups: tank rejects and not tank rejects.

	Classification	
Variable	Tank reject	Not tank rejects
Clot time	0.6243	0.5294
%granul.	0.5084	0.6688
√Lactate	3.8556	2.9766
√BactRank	1.7969	1.2658
Protein	0.2656	0.2994
Constant	-41.6739	-35.2193

7.7 Using the key stress indicators to predict tank losses for particular storage treatments

7.7.1 Summary

Stress can be demonstrated during post-harvest handling and the more extreme changes in these parameters have been linked in trials here to lobster deaths. However, in terms of dealing with this on the factory floor, graders may still be the simplest and most effective judge or indicator of the condition of consignments of lobsters arriving at a factory or of the relative merits of particular handling methods.

The prediction accuracy was around 80% when predictions were made between the different data sets. To help avoid confusion with dates, the data sets can be identified as the first, second and third sets (Table 50). The equations derived from the Second data set (November 1998, Table 34) systematically over-estimated the level of tank losses in the Third (March 1999) data. Similarly, equations raised on the Third set of data (Table 45 and 49) systematically under-estimated the number of tank losses in the Second (November 1998) data set. What was perhaps unexpected was that given the different sets of parameters selected in this and previous trials the predictions were even as good as they were, (Table 50). Clearly, there was a large degree of predictability associated with mortality and the broad changes occurring in lobster hemolymph following stress. In other words, stress that kills lobsters leaves its mark in their hemolymph before they die.

Table 50. The key stress indicators. Hemolymph parameters associated with lobster mortality in the factory-based trials. Parameters occurring more than once given in bold type.

Trial	Parameters used in the discriminant function
FIRST March 1998	**Magnesium** > Potassium > **Calcium** > **√Lactate**
SECOND Nov. 1998	**√Lactate** > log LDH activity > **Magnesium**
THIRD March 1999	Clotting time > **√Lactate** > % granulocytes > **Calcium** > √Ranked Bacteria Count

The various discriminant equations derived at points during the study (Tables 21, 34, 45 and 49) were also used here to predict the mortality rate in each of the 5 treatments of the Second and Third factory trials (to maintain the notation adopted above). Rejection by the grader closely matched the pattern of mortality seen later in the tanks. Of the discriminant equations applied to the Second data set, only the predictions from those equations derived from the Second data set itself also mirrored the result from the observed mortality. Trial specific factors seem to lead to errors when using the First (March 1998) and Third equations to predict treatment mortality in the Second group of lobsters. Similar mismatches occurred when used the First and Second equations to predict for the Third data set. An interesting aside was that the discriminant equations accurately predicted that lobsters from the submerged treatments would survive, while the grader sometimes rejected up to 10% of them. The characteristics of unstressed lobsters are apparently more definitive than those of stressed lobsters.

The key physiological stress indicators, the variables contributing most often to the discriminant equations, were lactate and magnesium. These parameters indicate that the lobsters, particularly in the seawater spray treatments are not getting enough oxygen. It is questionable that the spray achieves anything under these circumstances. Calcium was selected by two trials but the case for this parameter being a key stress indicator was not strong. Perhaps other parameters could be found to give greater discrimination and while enzyme activity level showed promise it was technically difficult to do in the field.

The most accurate approach to comparing storage treatments of course is to simply observe the mortality following storage in different systems. This won't show why the lobsters died or why the

treatments failed, but it will indicate what isn't working. The key stress indicators provide a direction to head in and specifications to meet on the way to improving handling methods.

7.7.2 The situation

The premise of this study was that stress during post-harvest handling was responsible for losses of rock lobsters during storage in factory tanks. The objective was thus to find indicators of that stress so that they could be either used by some factories to grade live product for export or used in studies of alternative handling practices so that fewer lobsters were stressed.

The findings and steps of experimental work of this project, detailed in previous sections, can be summarised as follows:

1. Several physiological parameters changed from baseline levels (section 7.1) during the post-harvest handling of rock lobsters (section 7.2) and these changes parallelled the respiratory problems that developed when rock lobsters are kept out of water (Section 7.3),
2. Using these findings as a basis, a series of factory-based experiments, some using alternative ambient temperature storage methods, were used to establish which of these physiological changes could be linked to later mortality in stressed lobsters (sections 7.4 to 7.6).
3. Immediately after an imposed stress, the lobsters that eventually died over the following week were significantly different from future survivors with respect to some hemolymph parameters (particularly lactate and magnesium concentration).
4. Using discriminant analysis of several hemolymph test results, it was possible to correctly classify the fate of 80-90% of lobsters, however with each replicate of the study, a different set of hemolymph tests were required. Though there was some consistency from time to time, particularly regarding lactate's important in the analysis.
5. The highest level of lactate concentration and overall mortality were seen when lobsters were stored in air at ambient temperature, and at best spraying seawater on them had no beneficial effect. If anything the spray appeared to increase mortality in some replicates above that seen in humid air alone.
6. However, concerning the search for a "simple stress indicator", the grader assisting the study was also largely observing the same stress being described by these physiological tests. The spray treatments with the highest level of mortality also showed the highest level of initial rejection by the grader.
7.

In short, stress can be demonstrated during post-harvest handling, and the more extreme changes in these parameters have been linked in trials here to lobster deaths. However, in terms of dealing with this on the factory floor, graders may still be the simplest and most effective judge or indicator of the condition of consignments of lobsters arriving at a factory or of the relative merits of particular handling methods.

So, where does this leave physiological stress indicators? All we've done so far is generate the discriminant equations from particular groups of lobsters, and used them to classify lobsters in a broad sense. We haven't considered how well the equations classify lobsters overall, or how well they classify lobsters from different storage treatments.

This section will consider

- how well a discriminant equation raised on one group of lobsters can predict the fate of another group of lobsters,
- compare how the factory grader and the various equations based on the hemolymph tests predicted the performance of the different storage treatments in terms of the actual mortality seen in those treatments.
- discuss what these key indicators of lobster stress actually tell us about rock lobster handling.

7.7.3 Predicting mortality

So far, the discriminant equations raised in sections 7.4 to 7.6 have only been used to classify internally, that is, classifying the very lobsters used to derive the equations. To avoid congesting the previous analyses unnecessarily, the issue of actual external predictions applied to other data was put off until this point, where it could be considered alone. To reduce confusion about the dates and identity of the different trials and equations, the different data sets will be called here: First, Second and Third, corresponding to the pilot study (March 1998, section 7.4), and the November 1998 (section 7.5) and March 1999 (section 7.6) factory based trials. Recall again that because missing data removes a lobster from the analysis, the total number of lobsters classified (eg. the Total figure in the lower right corner of Table 51) can vary according to the completeness of the data sets for the various parameters.

The discriminant analyses used as the basis for these cross-predictions are essentially those presented in the previous sections with one important exception. LDH data was not obtained in the Third trial, so in order to use a discriminant analysis from the Second trial to predict the outcome in the Third trial, it was necessary to carry out another discriminant analysis of the Second trial data using only those variables measured in both the Second and Third trials. This analysis, (summarised below) will be denoted as "Second trial (Third variables only)."

7.7.3.1 Stepwise discriminant analysis using 2 groups (tank rejects, non-tank rejects) of all treatments from the November 1998 factory based trial using parameters common with the March 1999 factory based trial

Forward selection using F-to-enter=2, arrived at a model requiring a group of 7 variables largely corresponding to that obtained above with the larger data set, with a Wilks' Lambda of 0.625 (approx. F(7,228)=19.52***) (Table 51).

Table 51. Summary of stepwise discriminant function analysis, for lobsters from all treatments sampled during the November 1998 factory trial (using variables used in both November 1998 and March 1999 studies) and grouped according to 2 groups: tank rejects and not-tank-rejects. Lobsters with missing data excluded.

Variable Entered	Step	F to enter	df	p-level	No. of vars.	Wilk's Lambda (Λ)	F-value	df	p-level
√Lactate	1	100.38	1,234	0.000	1	0.700	100.38	1,234	0.000
Mg	2	7.80	1,233	0.006	2	0.677	55.55	2,233	0.000
Protein	3	5.86	1,232	0.016	3	0.660	39.76	3,232	0.000
Clot time	4	4.08	1,231	0.044	4	0.649	31.24	4,231	0.000
Na	5	2.85	1,230	0.093	5	0.641	25.76	5,230	0.000
Ca	6	2.99	1,229	0.085	6	0.633	22.15	6,229	0.000
√Glucose	7	2.72	1,228	0.101	7	0.625	19.52	7,228	0.000

Lactate concentration provided the largest individual contribution to discrimination, followed by magnesium concentration and clotting time (Table 47). This was a similar situation to that observed in the original analysis (section 7.5.6.2). The major difference this time is that without data for LDH activity, a large number of variables were required to classify the lobsters.

Table 52. Variables in the model following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (using variables used in both November 1998 and March 1999 studies) and grouped according to 2 groups: tank rejects and not-tank-rejects. N=236. Lobsters with missing data excluded.

	Wilks' Lambda if removed (Λ)	Partial Lambda of variable	F-to-remove 1,228	p-level	Tolerance	1-Toler. (R^2)
√Lactate	0.726	0.861	36.71	0.000	0.484	0.516
Mg	0.662	0.944	13.50	0.000	0.519	0.481
Protein	0.640	0.977	5.39	0.021	0.675	0.325
Clot time	0.642	0.974	6.18	0.014	0.928	0.072
Na	0.635	0.985	3.48	0.064	0.738	0.262
Ca	0.634	0.987	3.12	0.079	0.482	0.518
√Glucose	0.633	0.988	2.72	0.101	0.626	0.374

This model correctly classified about 86% of lobsters. (Table 48). It is interesting that while there are classification errors in this table, there is no systematic bias toward identifying lobsters as ‘not tank rejects’ or tank rejects. That is, in the data examined, there were 208 "not tank rejects" for example, while the analysis classified 213 lobsters into this category.

Table 53. Re-classification of lobsters using posterior probabilities calculated from the distances between cases and the group centroids, following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (using variables used in both November 1998 and March 1999 studies) and grouped according to 2 groups: tank rejects and not tank rejects.

		Predicted classifications	
Observed Group	%Correct	Tank reject	Not tank reject
Tank reject	58.0	29	21
Not tank reject	92.3	16	192
Total	85.7	45	213

The classification functions are presented in Table 49.

Table 54. The classification functions obtained following discriminant function analysis of lobsters from all treatments sampled during the November 1998 factory trial (using variables used in both November 1998 and March 1999 studies) and grouped according to 2 groups: tank rejects and not tank rejects.

	Classification	
Variable	Tank reject	Not tank rejects
√Lactate	1.283	-0.968
Mg	-1.313	-1.834
Protein	0.571	0.602
Clot time	-0.003	-0.043
Na	0.663	0.677
Ca	3.222	3.491
√Glucose	3.179	4.002
Constant	-222.418	-222.39

7.7.3.2 Predicting the fate of lobsters sampled in the Second trial

The discriminant functions derived from the pilot study, the First trial, included parameters used in the subsequent two factory-based trials (Table 50). These can therefore be applied to the Second data set to predict which lobsters resembled 'not tank rejects' or "tank rejects" on the basis of the characteristics established under that previous, single spray treatment, regime. Rather than calculating from scratch using the relevant classification functions, we used the capacity of STATISTICA to select new sets of data for classification after completing each discriminant analysis.
Interestingly, the First equations were just as accurate when applied to predict the fate of the Second group of lobsters as they were when used to re-classify the First data set. The predictions made by the First equations on the Second group of lobsters were 84.5% correct (Table 55), whereas the re-classification of the First data (used to generate the First discriminant functions) was 83.3% correct (Table 20). Indeed, the First equations predicted the fate of the Second group of lobsters almost as well as the Second equations did (87.4%, Table 33). However, it should be noted that 31 tank rejects were predicted in Table 55, but 56 rejects were observed amongst the lobsters analysed.

Table 55. Prediction for the Second group of lobsters (November 1998) using posterior probabilities calculated from the distances between cases and the group centroids established during the First factory trial. 2 groups (tank rejects and not tank rejects).

		Prediction		
Observed	Correct	Tank reject	Not Tank reject	Totals
Tank reject	39.3%	22	34	56
Not Tank reject	96.0%	9	213	223
Total	84.5%	31	247	279

The Third discriminant equations, applied to the Second group of lobsters, consistently overestimated the number of 'not tank rejects' in the sample (227 lobsters against the 194 'not tank rejects,' Table 56). Some of the Second group mortalities resemble the survivors of the Third group, to the point that the analysis is convinced that nearly all the lobsters survived (227 of the 240 lobsters classified). There is a systematic "error" in the predictions. To find this bias in the classification tables given here (eg. Table 56), note whether the row and column totals for observed and predicted 'not tank rejects' (for example) are unequal or not.

Table 56. Prediction for the Second group of lobsters (November 1998) using posterior probabilities calculated from the distances between cases and the group centroids established during the Third factory trial. (2 groups: tankrejects and not tank rejects)

		Predicted		
Observed	%Correct	Tank reject	Not Tank reject	Totals
Tank reject	19.6	9	37	46
Not Tank reject	97.9	4	190	194
Total	82.9	13	227	240

7.7.3.3 Predicting the fate of lobsters sampled in the Third trial

The different magnitude of stress and mortality rate observed in the Third trial had consequences when using equations raised on previous data sets to predict survival of those lobsters. This worked each way, both when using the equations raised on the Third group of lobsters to predict the fate of lobsters sampled in previous trials (above) and using earlier equations to predict the outcome for the Third data set.

Interestingly, the First group analysis was not affected to the same degree as the Second (Third variables) analysis; predicting more Third group lobsters would survive than actually did (Table 57), which is a systematic bias often seen in the discriminant predictions in this project. Of the lobsters predicted to be 'not tank rejects,' about 90% of those predictions were correct, but less than half of the 'tank reject' predictions were correct.

Table 57. Prediction for the Third group of lobsters (March 1999) using posterior probabilities calculated from the distances between cases and the group centroids established during the First factory trial. (2 groups: tankrejects and not tank rejects).

		Predicted		
Observed	%Correct	Tank reject	Not Tank reject	Totals
Tank reject	35.0	21	39	60
Not Tank reject	91.6	18	197	215
Total	79.3	39	236	275

The predictions based upon the new Second group (Third variables) analysis (given in section 7.7.3.1) showed the expected bias toward incorrectly rejecting lobsters that follows on from the observed differences between the results (Table 58). Fewer lobsters were predicted to be 'not tank rejects' than

was actually the case, an unusual outcome given the tendency for such predictions to be optimistic. In the process, the predictions for the two categories both turn out to be about 80% correct- which again, is unusual. In other cases, there was usually a marked disparity seen in the percentages derived from each category.

Table 58. Prediction for the Third group of lobsters (March 1999) using posterior probabilities calculated from the distances between cases and the group centroids established during the Second factory trial (using variables in common with the Third study). (2 groups: tankrejects and not tank rejects)

Observed	%Correct	Predicted Tank reject	Not Tank reject	Totals
Tank reject	89.2	33	4	37
Not Tank reject	72.0	54	139	193
Total	74.8	87	143	230

7.7.3.4 How well do the equations predict?

The prediction accuracy was around 79-85% when the First and Second (Third variables) equations were used to predict for the Third group of lobsters, and when the Third equations were used to classify the Second data set. However, the equations raised on the Second data set systematically over-estimated the level of tank losses in the Third (March 1999) data. Similarly, equations raised on the Third set of data systematically under-estimated the number of tank losses in the Second (November 1998) data set. Many 'survivors' in the Third data set, resembled tank rejects seen in the previous trials. This was consistent with the physiological changes in the Third trial having advanced further than seen before (perhaps because of the warmer temperature) within the 6 h timeframe of the experiment (page 98, 105).

Table 59. How successfully could predictions made using discriminant analyses of the results of the various trials be used to predict the outcome in new data (bold)

Data set	Predicted by which analysis	% correct
Second	**First**	**84.5**
	Second	87.4
	Second (Third)	85.7
	Third	**82.9**
Third	**First**	**79.3**
	Second	n.a.
	Second (Third)	**74.8**
	Third	89.6

What is perhaps unexpected is that given the different sets of parameters selected in this and previous trials (Table 50), the predictions are even as good as they are. Clearly, there is a large degree of predictability associated with mortality and the broad changes occurring in lobster hemolymph following a stress. In other words, the stress that kills lobsters leaves its mark in their hemolymph before they die.

So, in terms of broad predictability, the various equations perform surprisingly well within the context. But of course, they still make mistakes. What we haven't considered yet is whether the equations make more mistakes with some storage treatments than with others?

7.7.4 Predicting mortality in the treatments

The most accurate approach to comparing storage treatments of course is to simply observe the mortality following storage in different systems. This won't tell you why the lobsters died or why the treatments failed, but it will tell you what isn't working. Of course keeping large numbers of lobsters aside in a factory to watch some of them die is unlikely to be practical in all circumstances. Fortunately, as will become plain here, at least grading appears to be just as acceptable, at least when differences between treatments are large, (in the context of these experiments), for rating the storage regimes as complex mathematical assessments of hemolymph tests, though with some interesting quirks.
The various discriminant equations derived at points during the study were used here to predict the mortality rate in each of the 5 treatments of the Second and Third factory trials (to maintain the notation adopted above). For the sake of brevity, the particulars of the statistical analysis is passed over here and only the predicted number of 'tank rejects' is considered. The resulting predicted mortality profiles are then compared to the observed mortality and the grader's rejection rate for those treatments (Figure 38 and Figure 39).

In the figures,

- 'Mortality' is the observed number of tank rejects
- 'Grader' is the number of lobsters originally rejected immediately after treatment
- 'First' refers to predictions based upon the First (March 1998) study equations.
- 'Second' refers to predictions using equations based upon all treatments/all parameters in the Second (November 1998) data set,
- 'Second/3rd' equations using only parameters from the Second study that were also measured in the Third study,
- 'Third' refers to predictions based upon all treatments the Third data set.

7.7.4.1 Predictions for treatments in the Second (November 1998) trial

The grader's 'prediction' reflects the relative treatment mortality almost exactly (Figure 38) even if we know that numerous mistakes are made on the basis of individual lobsters. This was mentioned in earlier sections but should be emphasised again. A grader could easily reject a single lobster in error, but it appears that if the grader rejects large numbers of lobsters then there is something wrong with that treatment. Caution is of course needed, since only a single grader was used here, but it still puts the relative efficacy of complex hemolymph tests into perspective. Apart from identifying physical injury, the grader assesses factors such as the posture, muscle tone and liveliness of each lobster, and while selections by individuals may differ slightly, there would probably be good agreement between the relative grade awarded by different individuals to a range of treatments.

Of the discriminant equations available, only the predictions from those derived from the Second data set itself (Second & Second/3rd) also mirror the result from the actual mortality (Figure 38). Trial specific factors seem to lead to errors when using the First and Third equations to predict treatment mortality in the Second group of lobsters. The Third equations seriously underestimate mortality in the Second set of data. This difference in the magnitude of apparent stress between the Third and Second studies was noted earlier (page 98, 105).

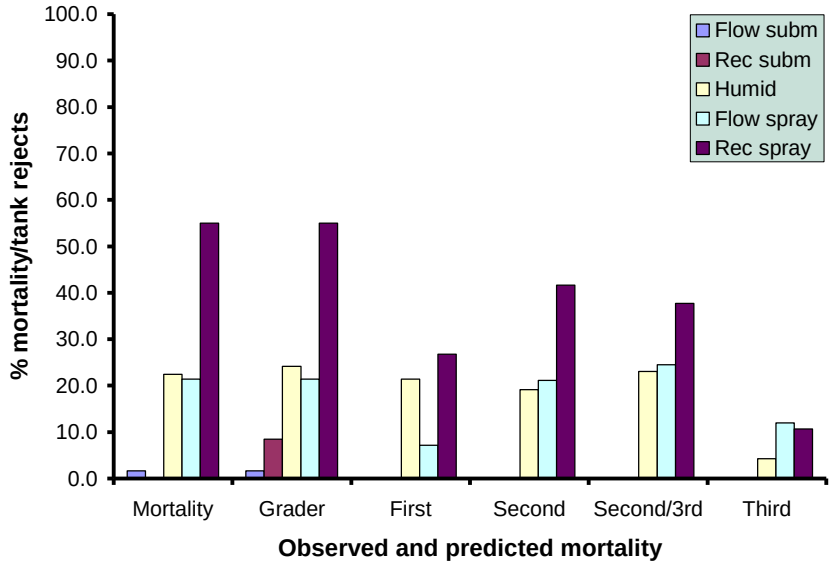


Figure 38. Observed mortality for each treatment during the Second (November 1998) trial versus the grader's predicted mortality (rejects) and mortality predicted using different discriminant analyses from the First, Second and Third trials profiles.

7.7.4.2 Predictions for treatments in the Third (March 1999) trial

The grader and several discriminant tests all predicted that of the emersed treatments, humid air would result in the lowest mortality, but none seemed to predict the relative mortality in both spray treatments.

In March, the grader substantially over-estimated mortality all-round, even predicting 10-20% mortality in the submerged treatment (this also happened in one submerged treatment in the November trial, Figure 38). These false negatives are perhaps an inevitable result of strict grading to minimise numbers of tank rejects in the tanks.

In contrast, the discriminant equations consistently predicted that lobsters from submerged treatments survived. Interestingly, this outcome even holds for the Second/3rd trial equations when the statistics could be expected to be classifying ‘not tank rejects’ incorrectly as tank rejects.

Of course, it is no real surprise that the best way to store or transport lobsters is in water, so its hard to see this finding as a vindication for complex multi-parametric statistics. Yet, the consistency with which the equations were able to identify minimally stressed lobsters in this context does give pause for thought. Perhaps they function best as “non-stress” indicators? That is, normal unstressed lobsters probably represent a more uniformly defined group than do stressed lobsters, which can be stressed in different ways and to different degrees.

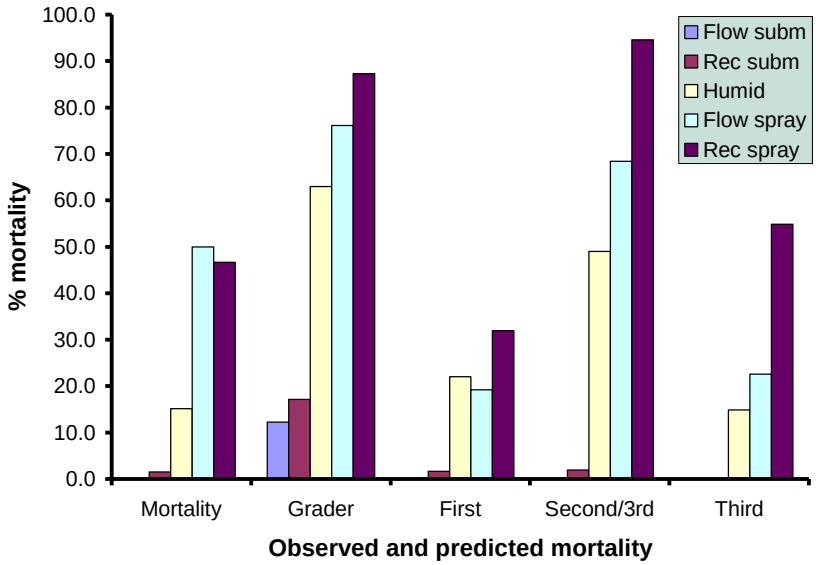


Figure 39. Observed mortality for each treatment during the Third (March 1999) trial versus the grader's predicted mortality (rejects) and mortality predicted using different discriminant analyses from the First, Second and Third trials.

7.7.5 The key stress indicators

The physiological roles of the key indicators have been touched upon at points elsewhere in this report but it is worth re-iterating their characteristics once more.

7.7.5.1 Lactate

Lactate or more correctly, lactic acid, is well known as a product of anaerobic metabolism in crustaceans and many other animals. When tissues are starved of oxygen, they resort to an alternative metabolic pathway (anaerobiosis= life without oxygen) to sustain cellular process, and in crustaceans this leads to the accumulation of lactic acid. Keeping a lobster in air impairs its gill function and the oxygen level in its hemolymph falls dramatically. Of course, a similar problem applies to a lobster submerged in poorly oxygenated water. Then it is a case of oxygen uptake failing. On the other hand, the lobster can go anaerobic if it consumes oxygen faster than it can usually obtain from its surroundings. If the lobster exercises violently by tail flipping it cannot supply oxygen quickly enough to its tail muscle to support that activity. Lactic acid then accumulates. It may be not so much the lactate molecule as the acid (H^+) released at the same time which is the major problem to the lobster. This is further reinforced by the dramatic differences in lactate levels between the November 1998 and March 1999 factory studies. It is likely that the absolute value of lactate concentration observed is not as important as the rate at which that lactate accumulates.

While elevated levels of lactate are associated with mortality in many lobsters, that does not mean that this single parameter explains all lobster deaths. This point has been elaborated upon elsewhere (Paterson et al in press, see Appendix 2).

7.7.5.2 Magnesium

Magnesium level in the hemolymph of the western rock lobster is normally kept at a concentration considerably lower than that of the surrounding seawater. This metal ion is abundant in seawater, but

if allowed to equilibrate into the hemolymph is expected to interfere with nerve and muscle function. The fact that magnesium level can rise in lobsters kept out of water suggests that the source is intrinsic to the animal- even if it may turn out to be entering from the gut fluid. Inside the cells themselves, magnesium has an important contribution to metabolism because it interacts with and helps to shape enzymes and other important proteins. Perhaps the acidity associated with emersion compromises the integrity of the lobsters cells and allows magnesium to leak into the hemolymph. The magnesium ions did not continually accumulate in the manner that lactate did. In the final factory-based trial, which showed high levels of mortality in the treatment itself and shortly afterwards, the ion was also elevated in future survivors as well as future mortalities, suggesting that the most severely stress lobsters had stopped accumulating magnesium and that the survivors had caught up.

7.7.5.3 Calcium

The support for calcium as a key stress indicator is not strong. It did appear in two of the equations but never as a parameter contributing much discrimination to the analysis. It appears to play a minor role, though the literature would link elevated calcium level with emersion induced acidosis. Survivors did not differ significantly from tank rejects in calcium concentration in any of the factory-based trials conducted in this study, and consistent significant changes were not seen in calcium concentration during post-harvest handling or laboratory emersion. However, in both the November 1998 and March 1999 factory based trials, there was a significant difference in hemolymph calcium concentration between submerged and non-submerged lobsters.

7.7.5.4 Other assays

Potassium appeared in the equation derived in the First trial. The concentration of this ion is regulated strongly by cell membranes since it plays a crucial role in muscle contraction and nerve function. It normally occurs in large amounts inside cells, and hence any significant elevation in the hemolymph is evidence either that potassium has left the cells through transport or diffusion or that many cells have been damaged and released their contents into circulation. The levels of this ion in lobsters also showed a number of interesting baseline ‘shifts’ during various experiments in this study, but this may simply be due to homeostatic regulation and may have no commercial significance.

Enzyme activity measurements showed some promise in the Second trial but these methods were labour intensive and difficult to work with due to coagulation and problems with transporting sensitive equipment. The rationale for using them is similar to that for including potassium, namely that enzymes like lactate dehydrogenase are constituents of cells, and their presence in the hemolymph is an indicator of cellular damage. Perhaps these and other cellular or molecular markers could supply more discrimination in studies where lobster stress needs to be categorised, but a good case would have to be put to support further work in this area.

7.7.5.5 What didn’t get up

Perhaps just as surprising is that a ‘classical’ stress indicator like hemolymph glucose concentration did not figure in the list of key stress indicators, despite the fact that it clearly changed during post-harvest handling and responded unambiguously to emersion in controlled laboratory trials. However, the glucose concentration gave mixed signals in trials conducted with storage environments in the factory-based trials. Some lobsters that were without doubt severely stressed were nevertheless returning ‘baseline’ glucose levels upon analysis. This finding is consistent with other recent studies (Hall and van Ham 1998) that question the orthodoxy that stressed crustaceans are necessarily hyperglycaemic- it may be that the elevated glucose level in the hemolymph cannot be sustained indefinitely and falls back to baseline levels if the stress is prolonged.

7.7.6 What the physiological indicators do tell us

We are not saying that these key indicators are causing the deaths of lobsters several days later. At best we can say that the appearance of these materials in the hemolymph is associated, or correlated, with the processes that killed the lobsters. A working hypothesis would be that the severe acidosis accompanying emersion in some lobsters is triggering pathological damage severe enough to rapidly kill them. At the same time, parallel disturbances in the numbers and types of hemocytes may leave other lobsters, already compromised by the physiological stress, unable to manage established or developing bacterial infections, leading to losses up to 4 days after the treatment.

The stress indicators not only provide some direction with regard to improving handling methods they also provide a direction to head in and specifications to meet on the way to improving handling methods. In the context of this study, the work shows that spraying seawater on rock lobsters stored at ambient temperature in air does not ameliorate the effects of emersion. Lobsters under seawater sprays still accumulate large amounts of lactic acid. Perhaps the spray is not available to the lobsters for respiration, running over the outside of the animal rather than entering the gill chamber. Lobsters piled into a basket will be in no particular orientation to the water flow and some positions may prevent any gill irrigation altogether. This applies mainly to ambient temperature carrier boat transport, where the benefit gained in the switch to submerged storage is pretty clear. The current ranking of treatments may not hold for truck transport of lobsters. Lower temperatures may change the responses of the lobsters and indeed, a cold seawater spray may be particularly efficient at cooling lobsters down.

8 BENEFITS

The benefits provided by this study are

- a greater understanding of the impact of stress on rock lobsters
- key stress indicators that allow us to monitor the performance of lobsters in alternative handling regimes

This program of research has provided a greater understanding of the impact of stress on rock lobsters and emphasised the fact that not all stress is necessarily a commercial problem. Lobsters are going to be stressed during post-harvest handling but they can still recover if the effects are not severe.

Secondly and specifically, this research has shown that lobsters that have been stressed to the point where they die soon afterwards during tank storage in the factory can be identified with a moderate degree of certainty using a small number of hemolymph tests. However, the actual tests required to bring this about did change slightly from time to time.

While this study was not a comprehensive examination of commercial grading practices, the results available indicated that grading lobsters for vigour remains the simplest and most practical means of selecting lobsters that will not weaken during tank storage. Furthermore, in the trials conducted here, grading appeared to be a valid means of assessing the performance of lobsters in alternative storage/transport systems. In any case, counting actual mortality arising from storage/transport treatments was preferable because it gave unambiguous comparisons of mortality in different treatments.

While a physiological stress indicator simple enough to rival grading in effectiveness was not found, nevertheless, the key stress indicators identified were still appropriate for examining the responses of lobsters to alternative storage regimes. Counting mortality tells how many lobsters died, but the stress indicators help to explain why.

This project enables future studies of lobster handling to use indicators of commercially significant stress. This allows the performance of lobsters in handling regimes to be studied more effectively, with information gained on the extent of improvements or changes. This would particularly apply to questions about why seawater sprays fail to improve survival in air under ambient conditions.

The results of the storage environment trial help to promote the idea that lobster storage/transport methods can be simple. It is recommend that the best way to transport lobsters for short periods is submerged. While this may not be surprising, there is a need to explore submerged methods as much as in practical.

Water quality deterioration did not appear to be a problem in the context of the experiments conducted here. This has implications for arguments about whether biological filtration of transport/storage water is necessary for short term haulage of submerged product. These observations are confined to ambient temperature and hence are strictly applicable to bulk transport of lobsters on carrier boats. Cooling the lobsters introduces another variable, requiring studies at lower temperature more in keeping with truck transport (see further development below).

The objective of improving handling practices is not necessarily to simply export more live lobsters, but instead to improve overall fitness for live export, so that as many lobsters as possible arrive at a factory in a state fit for live export. This widens the options for marketing of lobsters. Processors who concentrate upon this live product may well seek to move more live lobsters, however a lobster fit for live export is fit for anything that the processors requires.

9 FURTHER DEVELOPMENT

The key stress indicators found here could be used in further studies to refine truck transport practices to aid in the interpretation of mortality figures.
The discriminant statistical approach used here could be used in examining the responses of crustaceans for key indicators of problems in other cases where physiological responses are intrinsically linked to production, such as aquaculture of rock lobsters or production of soft-shelled crabs.

10 CONCLUSIONS

Stress indicators exist, but no 'simple' indicator, and short of using mortality, human graders shouldn't be discounted. The advantage of stress indicators is that they show why lobsters are dying and suggest changes to regimes to improve outcomes in terms of fit for live.

If minimising deviations of these indicators are used as the proviso for choosing storage/transport environments, then this criterion would continue to emphasise the need for submerged storage/transport of lobsters. The storage environment trials also showed that the prognosis was excellent for lobsters stored in recirculated seawater for 6 hours.

Changes to the code of practice would seem warranted. In recommending submerged transport as the best method for short term movement of lobsters, this work suggests that as long as aeration is maintained that water quality deterioration is not a priority issue, and provision of biological filtration of the water does not seem to be a priority.

Submerged transport will not always be possible or practical. This research shows that contrary to what you might first expect, that spraying seawater onto lobsters in air serves no apparent benefit in terms of lobster condition over and above that provided by simple humid air. Further work may be required to establish exactly why sprays fail to benefit lobsters in air. Oxygen uptake by the gills appears to be severely curtailled in air under ambient temperature. Perhaps wetting the lobsters using sprays tends to 'drown' them, by keeping the gills water-logged when they should be drying in order to access oxygen available in the air.

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12 APPENDICES

APPENDIX I

STAFF

Name	Position	Qualifications	% time
Glen W. Davidson	Seafood Physiologist	PhD	100
Brian D Paterson (Principle Investigator)	Crustacean Physiologist	PhD	40
Patrick T. Spanoghe	Crustacean Physiologist	PhD	100

APPENDIX II

RELATED MATERIAL (ATTACHED)

Paterson B, Davidson G, Spanoghe P. (1999) Identifying stress when western rock lobsters are stored out of water: the average and individual blood lactate concentrations. In 'Proceedings of the International Symposium on Lobster Health Management,' (Eds LH Evans and JB Jones) pp. 35-41 (Aquatic Science Research Unit, Curtin University of Technology).

Paterson B, Davidson G, Spanoghe P. (1999) Measuring total protein concentration in blood of the western rock lobster *(Panulirus cygnus* George) by refractometry. In 'Proceedings of the International Symposium on Lobster Health Management,' (Eds LH Evans and JB Jones) pp. 110-115 (Aquatic Science Research Unit, Curtin University of Technology).

Identifying stress when western rock lobsters are stored out of water: the average and individual blood lactate concentrations

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ABSTRACT

Currently western rock lobsters, *Panulirus cygnus*, are graded a number of times during post-harvest handling, to remove injured or damaged lobsters, as well as to remove individuals, on the basis of posture and responsiveness, that experience shows may die during factory storage. Trials have been undertaken, using blood tests, to try to identify lobsters that survive a handling treatment but which die subsequently. We have subjected large numbers of lobsters to a controlled period of stress, then sampled their blood immediately to measure a number of physiological parameters. The lobsters were tagged and stored in the factory and their fate recorded. In this paper we examine the lactate results in detail to see how well results for individual lobsters compare with average findings. The group of lobsters that later survived had significantly lower blood lactate concentration leaving the treatment than those lobsters that did not. Clearly, reducing the stress that leads to lactate accumulation will improve the outcome. However, examining the frequency distributions of this parameter shows that the average lactate concentration in lobsters that did not survive following treatment does not convey well the diverse responses of individual lobsters and shows how difficult it is to use one parameter to predict death. Lobsters may die for more than one reason and using several parameters in combination may explain more of the mortality. Counting mortality is undeniably the simplest way to measure lethal stress, but stress indicators help explain why the lobsters died, and knowing that may suggest ways to alter practices to reduce mortality.

Keywords: Mortality, stress, lactic acid, live handling

I. INTRODUCTION

Western rock lobsters (*Panulirus cygnus* George) are graded a number of times during post-harvest handling to remove injured or damaged lobsters as well as to remove individuals that are considered to be “weak” on the basis of their posture and responsiveness. These stressed lobsters are singled out because it is thought that they are unlikely to survive for long in the factory. While the number of lobsters involved might only be a small fraction of the average annual catch of 10,500 tonnes, reducing this stress and ensuring that the bulk of the catch arrives fit for live export remains a priority. To reduce this stress we need to be able to measure it first.

Lobsters are stressed when a factor, sometimes called a stressor, causes their internal physiology to deviate from normal. Many of the parameters that can be

measured in crustacean blood are stress indicators of some kind (Paterson and Spanoghe, 1997). Of course, simply knowing that these lobsters are stressed after harvest may not be of much practical use. We want to identify lobsters that survive a handling treatment, but that die subsequently. Having early indicators for this kind of stress or delayed mortality would allow us to objectively compare handling methods using key tests, ones associated with the processes that kill lobsters. The results of these tests will also help to tell us why lobsters die or weaken following handling.

To find these indicators we have undertaken two major trials where we subjected large numbers of lobsters to a controlled period of stress, then sampled their blood immediately to measure a number of physiological parameters. The lobsters were then tagged and stored in the factory and their fate recorded. A full exposition of the data collected in these trials is beyond the scope of this article. Here we report data for one parameter, blood lactate concentration, in greater detail than would perhaps normally be the case. As this symposium is aimed at a broad audience, and the paper examines the implications of statistics when applied to individual lobsters, some readers may find more than usual attention given to explaining basic statistics.

Lactate, or lactic acid, was not chosen at random. When living cells are short of oxygen, they produce lactic acid. This is the same chemical produced when human muscles are deprived of oxygen and contributes to the fatigue experienced during strenuous exercise. Lactate is present in very low amounts in tissues and blood of resting unstressed lobsters and it accumulates when lobsters are stored and transported out of water (Spanoghe and Bourne, this volume). Indeed, a number of studies would have it that this progressive rise in lactate concentration, and the associated acidification of the blood and tissues, is a major cause of mortality when lobsters are transported (Vermeer, 1987; Whiteley and Taylor, 1990).

But does lactate concentration allow us to predict mortality, thus establishing the supposed cause and effect relationship proposed? This paper focuses upon the extent to which the test results of individual lobsters, and not so much the average for a group of lobsters, may or may not allow us to predict whether they will live or die following an imposed stress.

II. MATERIALS & METHODS

Two replicated studies of lobsters in different transport/storage environments were completed in Geraldton, Western Australia in November 1998 and March 1999. ‘A’ sized lobsters (approx. 450g) with pink shell colour, that had been in factory tanks for at least 24 h, and which originated from boats delivering directly to the factory were used in the experiments. On the evening before use, the lobsters were fed with chopped fish at approximately 3% of lobster weight, to simulate feeding on bait.

Each time, the basic design required that about 200 lobsters were distributed equally across five different treatments (submerged in flow-through seawater, submerged in recirculated seawater, humid air, sprayed with flow through seawater, sprayed with recirculating seawater) and stored under these conditions for six hours at ambient temperature. Following treatment, all lobsters from each of the 5

treatments were graded (acceptable or rejected), and half of the accepted and the rejected lobsters (about 100 lobsters in total) were tagged and their blood sampled before placing them in a factory storage tank. The remaining lobsters were returned to the factory tanks without being sampled to act as controls for sampling mortality. The trials were repeated on three consecutive days, using three different tanks of lobsters so that a total of about 300 lobsters were treated, tagged, sampled and survival noted for each of the November and March replicates of the study.

After the treatments, lobster mortality (tank rejects) was monitored for up to a week following treatment. At the end of this observation period, the remaining lobsters were packed as if for export and stored for 36 hours in an air-conditioned room, a period similar to that required for live export. The lobsters were unpacked and placed back in the factory tanks and monitored for a further 24 h. Mortality while in the box or immediately upon re-tanking was monitored (box mortality). Lobsters that remained alive at the end of the process were designated as 'survivors.'

The blood samples were analysed for a number of the physiological and hematological parameters, some of which are mentioned elsewhere at this symposium; for example protein, lactate, glucose, calcium, magnesium, potassium, total hemocyte count, percentage granulocytes, and anti-bacterial factor (Evans *et al.*, this volume). The results were then considered in terms of individual tests, to compare the average responses of lobsters that survived and those that did not. Groups of blood tests were also considered together using a statistical technique known as discriminant analysis to try to develop a short list of parameters that explain most, if not all, of the mortality. Rather than attempting to give the complete account of these studies (Paterson *et al.*, in preparation), this paper discusses the results of lactate assays conducted during the November trial, to explore the shortcomings of using one parameter alone to predict mortality. Only the data for lobsters stored out of water (in moist air or under sprays) is presented, as mortality was low for lobsters following submerged storage.

Lactate was measured using a standard enzymatic test kit method (Boehringer Mannheim catalogue number 139 084). Differences between the averages of the different groups were tested using Analysis of Variance (Statistix For Windows, Analytical Software, Tallahassie, Florida, USA). The results were transformed by taking the square root of the blood lactate concentration prior to Analysis of Variance. This transformation of the data was necessary prior to Analysis of Variance because the distribution of results was not symmetrical about the average.

III. RESULTS

On average, lobsters that died in the days following the treatment emerged from the treatment with a blood lactate concentration higher than that of lobsters that later survived (compare tank rejects and survivors in Fig. 1). This difference was statistically significant. This means that if two groups of lobsters were picked at random from a larger population of lobsters we'd expect both groups to have a similar average lactate concentration. When they were sampled leaving the treatments, chance alone is unlikely to give the two groups such widely divergent average lactate concentrations, and we conclude that significantly elevated lactate concentration immediately after treatment is associated with lobsters that later die.

At the risk of labouring the point, this bare, statistical conclusion refers only to the differences between the averages. It makes no claims at all about the biological significance of the averages themselves.

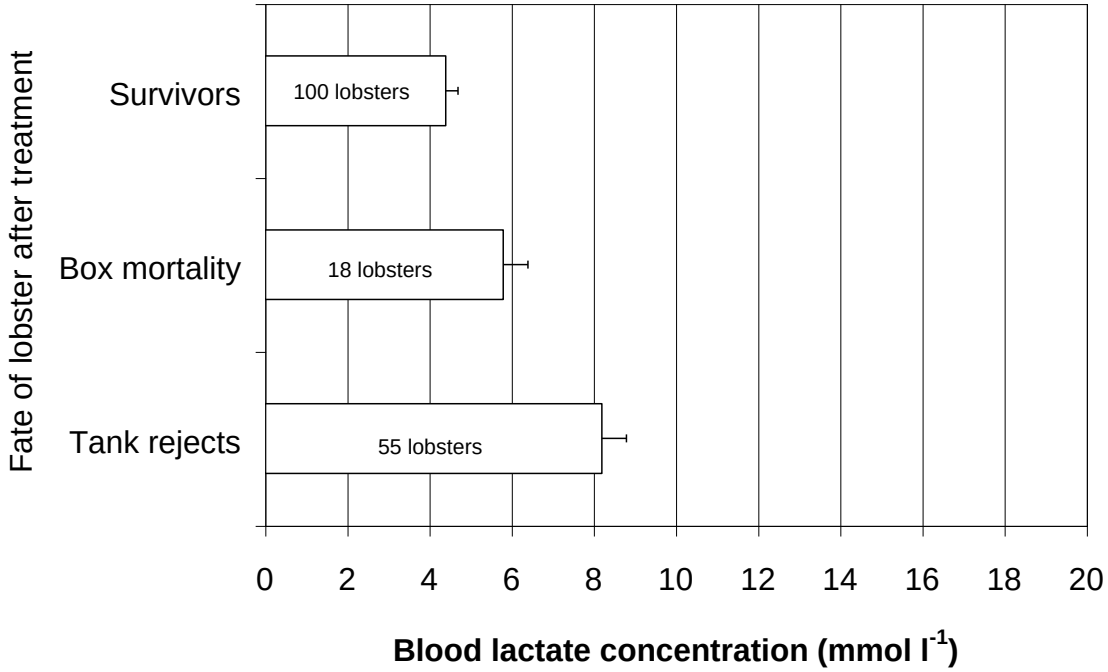

Figure 1 Average blood lactate concentration of lobsters sampled immediately after the stress treatment, and grouped according to their fate during later storage in factory tanks.

Nevertheless, the case for a lactate level of around 8 mmol l^{-1} predicting future mortality (the average for 'tank rejects' taken from Fig. 1) seems pretty watertight at this stage. But consider now the frequency distribution of the same data set (Fig. 2). This figure shows the number of lobsters sampled (the height of the bars) whose blood lactate concentration lies within the 1 mmol l^{-1} concentration interval chosen. This distribution is plotted for the three categories used to group the lobsters.

Notice that the lobsters that eventually died ('tank rejects') have a distribution that is much flatter, and its upper 'tail' extends to higher concentrations (up to 20 mmol l^{-1}) than the other distributions. This is a common response when animals deviate from normal resting levels for a parameter, they tend to show no typical degree of deviation but instead get spread across a considerable range of concentrations. In this case, lobsters with the highest lactate concentration were the most stressed. In contrast, the distribution of 'survivors' is weighted toward lower, more 'normal' concentrations. A large number of lobsters that were survivors had, immediately after treatment, a blood lactate concentration that was at or below 4 mmol l^{-1}. In some 'survivors' however, the blood lactate level had risen to levels of up to 11 mmol l^{-1} by the end of the 6 hour treatment, similar in fact to many of the lobsters that later died.

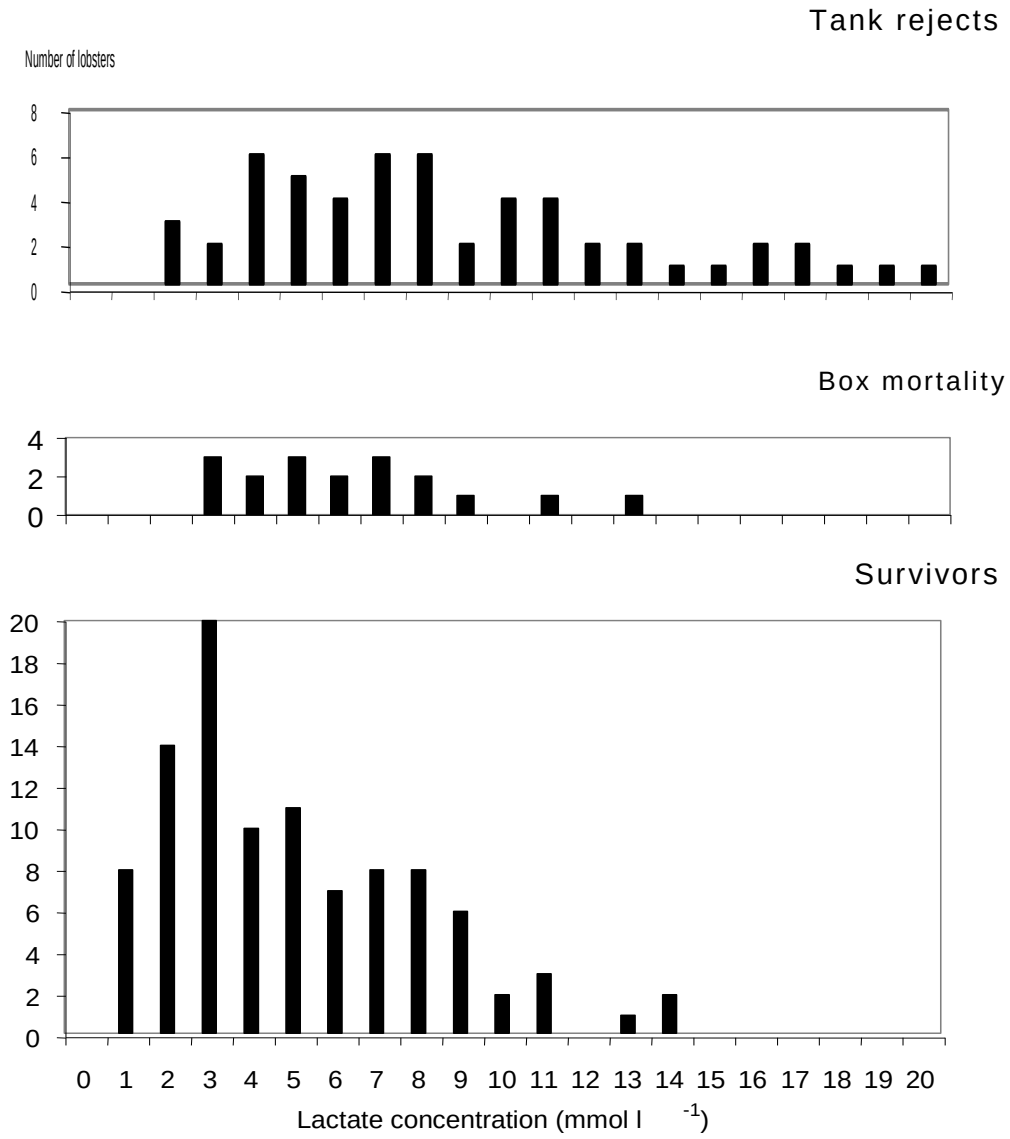

Figure 2 Frequency distributions of blood lactate concentrations of lobsters sampled immediately after the stress treatment, but grouped according to their fate during later storage in factory tanks

No lobster survived in this trial if its blood lactate concentration was greater than 14 mmol l^{-1} after the stress treatment. Comparing the group averages from Fig. 1 to the distributions in Fig. 2 shows the extent of overlap between the groups. The average lactate level in the lobsters that later died (Fig. 1), which could be concluded to be 'undesirable' is apparently physiologically acceptable, judging by the number of survivors which also had around 8 mmol l^{-1} lactate when sampled immediately after the treatment (Fig. 2). Indeed, a lobster with a blood lactate concentration of 10 mmol l^{-1} following treatment might live or might die, and some lobsters with low lactate concentrations in their blood, nevertheless died afterwards.

IV. DISCUSSION

We should approach with considerable caution any statement that lobsters die because *this* or *that* parameter changes. This is the key message to draw from Fig. 2. A similar story emerges if we look at other parameters measured in these studies, but these aren't explored here simply for the sake of brevity. So what does it mean if lobsters that die following stress earlier had a significantly higher average level of a certain parameter? Certainly we can conclude that high stress leads to mortality, and that deviation of parameters such as lactate can be linked to increased mortality in lobsters. The statistics are still valid when applied to groups of lobsters. We compare averages in order to draw objective conclusions about samples of lobsters. But we cannot use this approach to make judgements about individuals. It is arguable that it is not the 'average' individuals that we should be concerned about in this case, but rather the individuals with extremely above average levels of lactate following treatment.

Incidently, the average levels of lactate found here in all treatments were similar to those reported in emersed Florida lobster *Panulirus argus* (Latreille) and European lobster *Homarus gammarus* (L.) (Vermeer 1987; Whiteley and Taylor 1990). To put this in context, relatively high levels of lactate (an average of 16.5 mmol l^{-1}) have been reported in tropical rock lobsters, *P. ornatus* (Fabricius) kept briefly in air on small dinghy following capture, (Paterson, *et al.* 1997). Lactate accumulation, or the processes underlying it, may only be killing some of the lobsters. There could be some very simple reasons why no one parameter explains all of the lobster mortality. The average lactate concentration for tank rejects in Fig. 1 includes levels for all lobsters that died, many of which may have been killed by something unrelated to lactate. The added stress associated with handling and blood sampling the lobsters is clearly one possible source of mortality. The mortality rate of the sampled lobsters in the trial from which this data was drawn was about 10% higher than that of the unsampled ones. Sampling mortality might account for why some lobsters die without any pointers in the blood sample for their eventual fate. But is it that simple? A case could be put that the highly stressed lobsters, which we already expect to die, are even more likely to die because of the added manipulation involved in sampling? If sampling mortality alone was a major problem, we wouldn't have seen the low mortality that occurred following the submerged storage treatments (Paterson *et al.*, unpublished data). Those lobsters were also sampled.

Other factors besides blood sampling may also be killing some of the lobsters. This would account for why lobsters which apparently weren't stressed, that is, had low lactate levels, still died following the trial. The lobsters might have been injured during the treatment, or perhaps became 'sick' following the trial. Using several indicators may help to account for more of these deaths.

Of course, the more parameters that have to be measured, the more complex the task becomes and even after that effort it is still possible that some mortality will remain unexplained. A point is very soon reached at which it is simpler to use mortality itself as the measure of lethal stress. However, stress indicators help us to understand why the lobsters died and knowing that may suggest ways to alter practices to reduce mortality.

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Measuring total protein concentration in blood of the western rock lobster (*Panulirus cygnus* George) by refractometry

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ABSTRACT

Research on western rock lobsters *Panulirus cygnus* has shown that refractometry is a simple non-destructive field technique for assessing the blood protein concentration and hence the tissue mass or "condition" of rock lobsters. However, a conversion from refractive index (RI) to protein concentration has not been published for this species. In order to convert RI values to protein concentrations, the refractive index of a number of blood samples were measured at ambient temperature using a Shibuya S-1 salinometer calibrated at ambient temperature with distilled water. Total protein concentrations were determined colourimetrically by the biuret method. Comparing the data gave the following regression equation:

$$\text{Total protein (mg ml}^{-1}) = 5402.398 \times \text{RI} - 7214.877,\ r^2 = 0.947,\ n = 28$$

This analytical method represents a simple and useful way to obtain important information about the condition of rock lobsters entering commercial handling and transport. The measurement involves a straightforward physical phenomenon and it is probably not surprising that the conversion equation obtained in this study is similar to that derived from a study of the American lobster *Homarus americanus*. These conversions may be a satisfactory method of estimating blood protein concentration in other large marine crustaceans. Though if large numbers of measurements are to be made for other species using this method, it is relatively easy to establish a calibration to ensure the accuracy of the technique.

Keywords: condition, hemolymph, tail meat, portable instrument

I. INTRODUCTION

Blood protein concentration in lobsters changes when the blood volume changes in response to moulting or starvation. Research on western rock lobsters *Panulirus cygnus* George has shown that refractometry is a simple non-destructive field technique for assessing the blood protein concentration and hence parameters such as blood volume, tissue mass or "condition" of rock lobsters (Dall, 1974, 1975).

The use of refractometry to determine serum or blood protein concentration is an established clinical technique and has a number of benefits over more complex methods. 1) The refractometers themselves are relatively cheap, most cost less than $500. 2) The method provides immediate results, and 3) the data is at least as reliable as those of commonly employed colourimetric methods that require

samples to be stored and later analysed (Dall, 1975; Alexander and Ingram, 1980). 4) Clotting of blood is not an issue as protein determinations can be performed rapidly using whole blood within the time it takes for the blood to clot. Of course, when using the instrument in the field, it is important to note that unless a temperature compensated refractometer is used, the temperature of the device should be kept as constant as possible and the calibration of the instrument must be checked periodically with distilled water during measurements.

II. MATERIALS & METHODS

Blood samples were obtained from the pericardial sinus (beside the heart) of lobsters via the thin arthrodial membrane between the posterior margin of the carapace and the abdomen using ice-cold disposable hypodermic syringes. The refractive index (RI) of each sample was measured immediately at ambient temperature (~25°C) using a Shibuya S-1 salinometer calibrated at ambient temperature with distilled water.

After drawing the sample, 2 or 3 drops of blood were immediately placed on the prism of the refractometer and the RI was recorded before the blood clotted. If clotting did occur, the reading did not appear to alter, but the demarcation line on the prism became indistinct and difficult to read. Between readings the prism was rinsed with distilled water and wiped with a tissue. Calibration checks demonstrated that in terms of the RI recorded, it did not matter if the temperature of the few drops of sample (eg. 17°C) was initially different from the operating temperature of the refractometer at ambient temperature. Therefore, realistic differentials in ambient air and seawater temperature were not a significant source of measurement error.

In order to convert RI values to protein concentrations, the refractive index of a number of fresh blood samples was measured and total protein concentrations were determined immediately by the biuret method (Varley, 1967) using a Shimadzu UV 1201 spectrophotometer. Serial dilution of a solution of bovine serum albumin (150 mg ml^{-1}) provided standards. A calibration relationship for the two parameters was then calculated by regressing RI on total protein concentration using least squares linear regression techniques.

III. RESULTS & DISCUSSION

Several studies have shown that the protein scale on clinical serum refractometers, while correlated to protein concentration, sometimes overestimates the values returned by colorimetric assays of protein in fish serum, (Alexander and Ingram, 1980; Hunn and Greer, 1990; Ikeda and Ozaki, 1982; Wells and Pankhurst, 1999). Further calibration of the device is required in these cases.

The instrument used here was chosen because it has a slightly wider refractive index scale than typical serum protein refractometers, allowing RI values to be measured above 1.360. In the approximately 3000 lobsters used in our studies, values of RI varied between 1.3430 - 1.3695.

The calibration relationship for predicting blood total protein from RI was determined to be:

(1) Total protein (mg ml^{-1}) = (RI - 1.3364379)/ 0.0001757, r^2 = 0.947, n = 28

A similar relationship has been calculated for *Homarus americanus* Milne-Edwards by Leavitt and Bayer (1977) but these authors regressed total protein concentration on RI yielding the following equation:

(2) Total Protein (mg ml^{-1}) = 5449.417 x RI - 7295.321

If, for comparison, we express our data the same way we arrive at the following equation:

(3) Total protein (mg ml^{-1}) = 5402.398 x RI - 7214.877, r^2 = 0.947, n = 28

For comparison, the data and equation obtained from this study are plotted alongside the regression for *Homarus americanus* in Fig. 1. Note that the slopes are quite similar but that the intercepts on the RI axis (the projected RI of a sample lacking any protein) differ slightly, implying a difference between the two species in the contribution to refractive index of solids other than protein. Dall (1975) also calculated this relationship in the western rock lobster but did not report the whole equation. In addition, Dall (1975) used a different method for calibrating the refractometer, setting RI to 1.3400 with a 3% salt solution. The reason for doing so is unclear, given that the RI of a 3% salt solution is 1.3380 on a refractometer calibrated with distilled water. Regardless of which calibration is used the slope of the line remains unaffected.

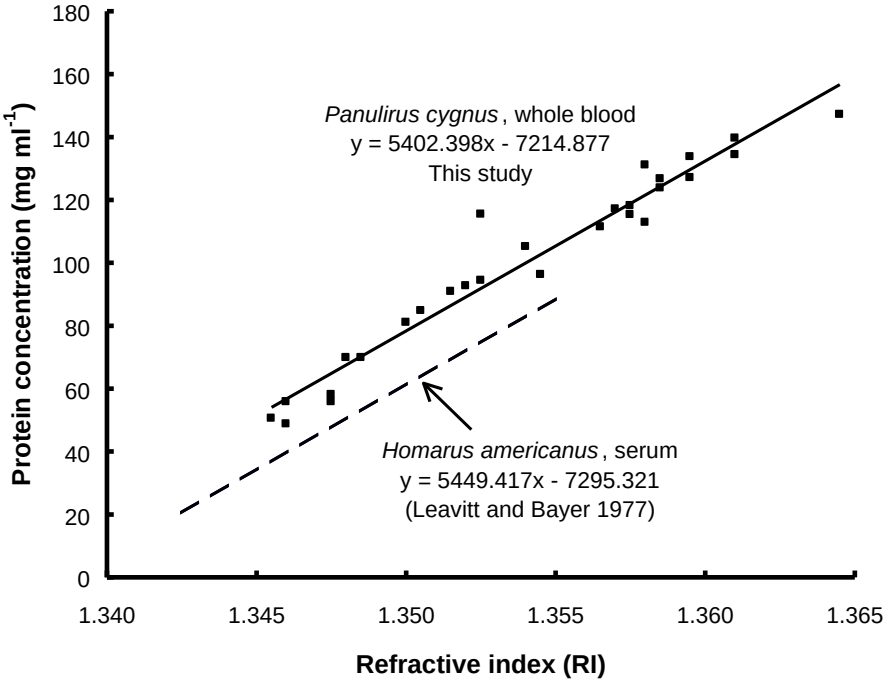

Figure 1 Comparing the relationship between protein concentration and blood refractive index (RI) for western rock lobster *Panulirus cygnus* and the published calibration for serum of American lobster *Homarus americanus*.

The biuret reagent reacts with peptide bonds to form a reddish-purple complex, the absorbance of which can be measured at 540 nm. The intensity of colour is proportional to the number of peptide bonds and hence the protein concentration (mg ml^{-1}). Whereas biuret reacts only with protein, RI is affected by all solutes in the sample. The use of refractometry for protein determinations relies upon the overwhelming contribution of protein to the total solute concentration and the relative constancy of the concentrations of other solutes. However, in human clinical studies there are a number of conditions that may interfere with the protein vs. RI relationship. For example RI is affected by, amongst other things, alterations in the albumin/globulin ratio and hyperglycaemia (Tietz, 1926). The first of these is probably of little concern in crustacea since by far the greatest proportion (75 - 95%) of blood protein is attributable to hemocyanin, the protein that transports oxygen in the blood. Hyperglycaemia (elevated glucose levels) in crustaceans is also likely to be less of a concern than in mammalian studies since hyperglycaemic values in mammals are much higher than in decapods. In addition, the concentration of salts in the blood of marine decapods is much greater than in mammals, therefore the relative effect on RI of an increase in glucose will be much less in decapods.

The method is already recommended for grading the condition of live *H. americanus* arriving at factories, allowing industry operators to make decisions about the marketing of individual lobsters based on information from a single drop of blood (Leavitt and Bayer, 1977). It has also been used for studies of live lobsters, crabs, penaeid shrimp and fish (Vezina, 1978; Smith and Dall, 1982; Alexander and Ingram, 1980, Moore *et al*., 2000). One other example of where protein refractometry has been used applied to commercial problems relates to monitoring the spoilage of fish (Vyncke, 1995).

This analytical method represents a simple and useful way to obtain important information about the condition of rock lobsters entering commercial handling and transport. The measurement involves a straightforward physical phenomenon and it is probably not surprising that the conversion equation obtained in this study (equation 2) is similar to that derived from a study of the American lobster (Leavitt and Bayer, 1977).

It is probably safe to assume therefore, that for some purposes, the equations reported here, or others published (e.g. equation 2), may be a satisfactory estimation of blood protein concentration in other large marine crustaceans, especially if knowing relative changes is sufficient. Though if large numbers of measurements are to be made for another species using this method, it is relatively easy to calculate a calibration relationship to ensure the accuracy of the technique.

Aside from being an index of condition (eg. blood volume and tissue mass), protein concentration in the blood of crustaceans also varies with the moult cycle, being lowest during post-moult and highest pre-moult. This needs to be taken into account when interpreting why a given lobster has low or high protein level (Dall, 1974). Preliminary data suggest that migratory sub-adult *P. cygnus*, or “whites”, have the characteristically low blood protein concentration expected of post-moult lobsters.

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