Predicting survival of western rock lobsters *Panulirus cygnus* using discriminant analysis of hemolymph parameters taken immediately following simulated handling treatments

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Abstract Instances of morbidity amongst rock lobsters (*Panulirus cygnus*) arriving at factories in Western Australia (WA) have been attributed to stress during post-harvest handling. This study used discriminant analysis to determine whether physiological correlates of stress following a period of simulated post-harvest handling had any validity as predictors of future rejection or morbidity of western rock lobsters. Groups of 230 western rock lobsters were stored for 6 h in five environments (submerged/flowing sea water, submerged/re-circulating sea water, humid air, flowing sea water spray, and re-circulated sea water spray). The experiment was conducted in late spring (ambient sea water 22°C), and repeated again in early autumn (ambient sea water 26°C). After 6 h treatment, each lobster was graded for acceptability for live export, numbered, and its hemolymph was sampled. The samples were analysed for a number of physiological and health status parameters. The lobsters were then stored for a week in tanks in the live lobster factory to record mortality. The mortality of lobsters in the factory was associated with earlier deviations in hemolymph parameters as they emerged from the storage treatments. Discriminant analysis (DA) of the hemolymph assays enabled the fate of 80-90% of the lobsters to be correctly categorised within each experiment. However, functions derived from one experiment were less accurate at predicting mortality when applied to the other experiments. One of the reasons for this was the higher mortality and the more severe patho-physiological changes observed in lobsters stored in humid air or sprays at the higher temperature. The analysis identified lactate accumulation during emersion and associated physiological and hemocyte-related effects as a major correlate of mortality. Reducing these deviations, for example by submerged transport, is expected to ensure high levels of survival. None of the indicators tested predicted mortality with total accuracy. The simplest and most accurate means of comparing emersed treatments was to count the mortality afterwards.

INTRODUCTION

Handling practices in the western rock lobster fishery (*Panulirus cygnus* George) are similar to those applied to other species of rock lobster. The lobsters are caught in pots and begin to tail-flap vigorously as they leave the water and during sizing and handling on the boat. Following storage on the boats, some lobsters are unloaded directly at the factory but others arrive after a journey by truck (stored cool in air or under a chilled seawater spray). Others are stored in floating pens and taken by carrier boats, again under seawater sprays, to the factory (Paterson & Spanoghe 1997). At the factory, the lobsters are sorted for size, colour, injury, and vigour. Lobsters deemed suitable for live export are placed in storage tanks where they are kept for several days before they are packed. Physiological recovery may occur relatively rapidly after similar stress in *Homarus gammarus* (Taylor & Whiteley 1989; Whiteley & Taylor 1992) unless the lobsters have suffered permanent injury and later weaken and die in captivity. Ideally, the handling practices used should be such that they minimise the numbers of lobsters that are brought into the live factory but are later found unsuitable for export.

Losses of lobsters during storage are often attributed to ”stress” during post-harvest handling. Accumulation of metabolic wastes and associated physiological changes may be important in this process (Whiteley & Taylor 1992; Hunter & Uglow 1993), but whether minimising these changes improves lobster survival is unclear. No attempt has been made to relate the physiological changes to commercial losses. Presently, the simplest way to find out if a change to handling practices improves the survival of lobsters is to make the change, store the lobsters, and count the number of lobsters that die. This is of course costly and wastes lobsters. Simple indices of future factory mortality could perhaps replace this method and, furthermore, help elucidate the mechanisms of morbidity in western rock lobsters. There are a large number of possible indicators of stress in lobster hemolymph that are relatively easy to

measure (Paterson & Spanoghe 1997). These include inorganic ions, various substrates and wastes of metabolism, and total protein concentration. Emersion and transportation are known to influence levels of inorganic ions, such as calcium (Ca) and magnesium (Mg), and concentrations of lactate and glucose (Johnson & Uglow 1985; Vermeer 1987; deFur et al. 1988; Taylor & Whiteley 1989; Whiteley & Taylor 1992; Hunter & Uglow 1993; Paterson et al. 1997a,b). In this study, we were particularly interested in ions that western rock lobsters are known to regulate in their hemolymph (Dall 1974). Hemocyte and other host-defence information was also collected (Jussila et al. 2001; Tsvetnenko et al. 2001).

A multi-variate approach such as discriminant analysis (DA) is appropriate for assessing multiple analytes. This method is essentially a predictive tool that attempts to assign a new observation to a category on the basis of the characteristics of known members of that category (Lachenbruch 1975). *A priori* prediction using DA has been exploited in a number of areas, for example to anticipate quality characteristics of potatoes, tea, and meat (Oliver et al. 1991; Taylor et al. 1992; Girard & Nakai 1994b; Orr et al. 1994; Downey & Beauchene 1997). It has apparently never been applied to predict the long-term viability of live seafood.

If future rejection can be predicted, then alternative handling practices could be developed and studied by minimising levels of indicators that have been linked to later lobster morbidity. A pilot study using a single spray treatment was therefore conducted to assess the feasibility of the approach. A test of different storage environments was conducted, to ensure a variety of responses, treating discreet batches of lobsters for 6 h in a range of environments and then sampling their hemolymph and tagging them. Lobster mortality was then monitored at the factory. A second storage environment trial was repeated at another time of year, to further test the repeatability and generality of any relationships observed. This study then used discriminant analysis to determine whether hemolymph tests following different transport/storage treatments had any

validity as general predictors of future mortality of western rock lobsters stored in a live lobster factory.

METHODS AND MATERIALS

Collection and storage of animals

This study used pink A-sized “jetty” lobsters (c. 445 g wet weight) held in the factory for 24 h. Jetty lobsters arrive at the factory straight from the boat and typically show no post-capture mortality. This period of acclimation in factory live tanks standardised the condition of the lobsters before their use. The night before starting the trial, the lobsters were fed with chopped fish (pilchards) to simulate feeding on baited pots, as would normally occur in freshly caught lobsters.

Storage environments

The experimental treatments were set up in five custom-insulated cubicles (c. 1.5 m^3) at the lobster factory. The basic approach was to stress lobsters for a given period, then sample hemolymph from each before tagging them and returning them to the factory for monitoring of subsequent morbidity and mortality. The cubicles were fitted out to provide the following five storage treatments: tank-flow, tank-recirculation, humid air, spray-flow, and spray-recirculation (Table 1). The garden-style spray-heads used were replaced after the first replicate trial (late spring) with a head that better distributed sea water over the lobsters.

Application of stress

On the morning of the experiment, 230 lobsters were distributed amongst the cubicles in pairs of plastic mesh baskets and treated for 6 h. 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. Spare lobsters were included to compensate for occasions were lobsters died in the treatment or were too damaged to sample.

The treatments were maintained at the ambient seawater temperature at the relevant time, this was 22°C for the first trial (late spring) and 26°C for the second trial (early autumn). Water quality (DO, pH) was checked at hourly intervals during the storage period, using hand-held meters and electrodes (TPS WP 81). At each sample time a water sample was also taken and refrigerated for later determination of ammonia using the Berthelot method (Varley 1967).

Grading, sampling, and storage after the treatments

Each cubicle was opened after 6 h and the animals inside were graded by an experienced factory grader into the following categories: dead, damaged, weak, and accepted. “Dead” lobsters that showed a heartbeat were transferred into the weak category for the purposes of hemolymph sampling.

In the first trial, half of the weak lobster group were sampled followed by sampling enough accepted lobsters to bring to 20 the number of lobsters sampled daily per treatment on each of the three treatment days. The unsampled weak lobsters and enough accepted lobsters to give a total of 20 were set aside each time as un-sampled controls for the sampling and tagging process. This practice of using as many rejected animals as possible, although suiting the discriminant analysis, was discontinued in the second trial, because it prevented interpretation of the treatments' effects (not discussed in this paper).

In the second trial, half of the rejected lobster group had hemolymph samples removed from them and half of all accepted lobsters were sampled. As before, the unsampled lobsters remained as controls.

The sampled lobsters were visually assessed for vigour (Spanoghe & Bourne 1997) size, sex, and damage. A pleopod tip was taken from each for moult staging. All sampled lobsters were then tagged to allow later identification using a coloured,

individually-numbered, livestock ear-tag anchored firmly to the distal segment of the antennal peduncle.

As these experiments were replicated on 3 consecutive days, the total number of lobsters sampled in each of the first and second trials was 300.

Monitoring during recovery

The sampled/tagged lobsters and the unsampled/untagged control lobsters were placed in an unoccupied seawater lobster storage tank in the live lobster factory. Different tanks were used for each of the 3 days of the first and second trials to keep the different batches of lobsters separate. After a delay of several hours to allow for initial recovery from handling, moribund or dead animals were removed and their tag numbers collated for identification purposes. Each tank was observed for at least 7 days.

Simulated load-out

All lobsters (tagged and untagged) remaining in the 3 tanks 7 days after the last stress treatment were then packed as if for commercial export and kept aside in an air-conditioned room for 36 h. The surviving animals were unpacked and placed in a single factory tank to recover for 24 h. Particulars of any animals that died during this period were recorded and a list of remaining tag numbers was obtained from the survivors.

Hemolymph sampling and initial measurements

Two hemolymph samples were drawn from the pericardial cavity of each lobster. For the first, a 2.5 ml sample was drawn for analysis of hemolymph constituents. 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,

using a Shibuya S-1 hand held refractometer (Paterson et al. 2000). Using an automatic pipette, a 250 µl sub-sample was drawn from each 1.5 ml vial of whole hemolymph and added to duplicate vials containing 500 µl of 1 mol $litre^{-1}$ perchloric acid (PCA), to precipitate protein. The pair of whole hemolymph vials and the pair of PCA-treated vials were then promptly capped and frozen in liquid N_2 for later analysis. The second sample (1 ml) was drawn using an ice-cold syringe, and stored on ice before analysis of the enzymatic activity of the hemolymph.

Analytical techniques

In the First trial using environments, sodium (Na), chloride (Cl), Ca, Mg, potassium (K), lactate, lactate dehydrogenase activity (LDH), glucose, protein content, hemocyanin absorbance, clotting time, total hemocyte count (THC), % granulocyte count, bacteria count, and antibacterial activity (ABA) were measured For the Second trial, LDH, THC, and ABA were dropped for technical/logistical reasons.

Lactate Dehydrogenase activity of serum/whole hemolymph was assayed using the Boehringer Mannheim MPR-1 kit. Levels of bacteremia and anti-bacterial activity were assayed as described elsewhere (Tsvetnenko et al. 2001). Hemocyte data were collected as total hemocyte counts, % granulocytes and clotting time, (Jussila et al. 1997; Jussila et al. 2001).

Serum was prepared from thawed whole hemolymph samples by allowing the samples to clot and then removing the clot with a needle. After centrifugation (at 5000 x *g*, 4ºC, 10 min) the serum was transferred to microcentrifuge tubes for storage at - 20ºC. The serum samples were analysed for Ca and Mg using Trace Arsenazo III and Calmagite kit methods respectively, with an Olympus model AU500 autoanalyser while Na and K 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 litre^{-1}. Cl concentration was determined using a Corning model 925 chloride analyser.

Acid precipitated samples were centrifuged at 5000 x *g* at 4ºC for 10 min. 500 μl of supernatant was removed and neutralised with 70 μl of 3 mol litre^{-1} KOH. Neutral perchlorate extracts were stored at -25ºC for subsequent analyses. Lactate and glucose were analysed using an Instrument Laboratories Multistat III analyser utilising respectively a Boehringer Mannheim kit (cat. no. 139 084) and a Roche Unimate 5 glucose HK kit (cat. no. 20736392122).

Statistics

Non-normal data were transformed before analysis. All general statistical tests such as t tests and analysis of variance were performed using Genstat 5, Release 4.1 (Lawes Agricultural Trust, Rothamsted Experimental Station). Multiple comparisons were performed using LSD tests. Discriminant analysis was conducted using Genstat 5, Release 4.1, and Statistica '99 version 5.5A (StatSoft, Inc. Tulsa, Oklahoma, United States).

RESULTS

First trial using storage environments

Water quality

Oxygen saturation remained at 85-100% saturation in both the submerged tanks and in the sumps for the spray treatments during the experiments. The pH was lower in the Recirc treatments than in the Flow treatments (Fig. 1). Water acidified gradually in the Spray recirc treatment, whereas Tank recirc lobsters reached a new

equilibrium immediately. Ammonia accumulated in both Recirc treatments at similar rates, reaching levels of 6-9 mg litre^{-1} in 6 h (Fig. 1).

Grading and survival after treatments

The grader rejected few lobsters from the Tank treatments, unlike lobsters stored in Humid air or Spray (Table 2, Fig. 2). The single grader used here correctly predicted the fate of 81.6% of the lobsters (Table 3), and c. 90% of lobsters accepted proved correctly to be “not tank rejects”. Subsequent losses occurred mainly during storage in the tanks or during recovery from the simulated box transport. The grader’s totals broadly resembled the number of lobsters surviving after a week in the factory tanks and after the simulated pack-out (Fig. 2). Note that the grades reflect the poor result in the Spray recirc treatment.

Second trial using storage environments

Water quality

Oxygen saturation remained at acceptable levels (>80% saturation) in the water in both the submerged tanks of lobsters and in the sumps of the spray treatments. The water pH and ammonia data (Fig. 3) showed a different pattern to that seen in the first trial. Acidification of the water was only seen in the Tank recirc treatment. The water in the Spray recirc system retained a similar pH to that of the seawater supply. That treatment also showed less ammonia accumulation than in the Tank recirc treatment though the later reached levels at 6 h similar to that observed in the First trial.

Grading and survival after treatments

The rate of mortality differed between the first and second trial (Fig. 4). In the second trial, the grader accepted most of the lobsters in the Tank treatments though a small

proportion of these lobsters was rejected as weak (Table 4). The lobsters from the Tank treatments showed little or no mortality when returned to the factory tanks, (Fig. 5). The result was poorer for the non-submerged treatments, particularly the Spray treatments, with a higher proportion of dead and weak lobsters (Table 4). Most of the surviving rejected lobsters were dead within a day, indeed, many lobsters died that night (time 0 in Fig. 4). Beyond the initial losses in the tanks, mortality following packaging in boxes and re-tanking of lobsters was only minor (Table 6). Overall, the outcome in the tanks was better than predicted by the grader, but only 3% of accepted lobsters were incorrectly graded (Table 5). The proportion of tagged lobsters that died after about a week in the tanks (tank rejects, Table 4) shows a similar profile for the treatments to that shown by the grader's figures (Table 4).

Step-wise discriminant analysis

Forward selection (F-to-enter=2) using the two groups (tank rejects and "not tank rejects") produced models with significant discrimination in each case but a varying suite of parameters in the model (First $\Lambda = 0.643$, c. $F_{3,137} = 32.07$ $p < 0.001$; First/Second $\Lambda = 0.625$, c. $F_{7,228} = 19.52$ $p < 0.001$; Second $\Lambda = 0.655$, c. $F_{5,173} = 18.23$ $p < 0.001$). The step-wise process for each model is summarised in Table 7. The relative discrimination provided by each variable in the model is provided by the F-to-remove statistic in Table 8.

Reclassification using "internal" discriminant functions and reciprocal predictions between data sets

These internal reclassifications were 85-90% correct (Table 9). The reciprocal predictions were 75 and 83% correct respectively (Table 10).

Predicting outcomes of different treatments

The various discriminant analyses were also used to predict the losses within each of the 5 treatments of the first and second trials. For the sake of brevity, only the predicted number of “tank rejects” is considered. The resulting predicted mortality profiles are compared to the known or observed mortality and to the grader’s initial rejection rate for those treatments (Fig. 6 and 7).

Interestingly, all models consistently and correctly predicted zero or negligible mortality in Tank treatments in all data sets— where mortality was of course expected to be and known to be minimal. Regarding the non-submerged treatments, in the first study, only the internal reclassification of those lobsters using the first model mirrored the treatment-by-treatment result from the mortality recorded, (Fig. 6). In the second study, the grader and several discriminant analyses correctly predicted that of all the non-submerged treatments, Humid air would give the lowest mortality. However, curiously, the various models detected a difference between the two Spray treatments that was not reflected in the recorded losses.

Classification functions

Group membership was described above in terms of posterior probability data calculated in the course of conducting the discriminant analyses. The classification functions recorded here (Table 11) are also suitable for predicting group membership of other lobsters for which these particular hemolymph test results are known.

Data summaries

Since it is difficult to relate the classification functions to actual concentrations of parameters in the hemolymph, and to enable comparisons with other studies, the average hemolymph test results for lobsters in each study that proved to be survivors and tank rejects are also summarised (Tables 12 and 13).

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DISCUSSION

Discriminant analysis was able to successfully classify the fate of lobsters using hemolymph measurements. Previous studies of lobsters and other decapods during commercial handling have shown that they are stressed and that levels of various parameters change, for example in response to emersion (Vermeer 1987; deFur et al. 1988; Taylor & Whiteley 1989; Whiteley & Taylor 1992). However, the present study has demonstrated that some of these changes are not simple deviations from baseline values but indicate something about the future wellbeing of the lobsters. Whether the indicators are causative or merely symptomatic remains to be seen. Although this finding confirmed the premise of this study, that lobsters can become moribund during live storage because they have been stressed beforehand, no single overriding indicator of stress, or pattern of indicators, emerged from this study. The grader who assisted the study was apparently just as accurate at predicting relative future mortality levels in the experiments as were the discriminant functions based on the hemolymph tests. Where it is practical to do so, the most accurate method of assessing mortality, particularly across different handling treatments, was to directly measure the outcome. The benefit provided by the hemolymph indicators is that they highlight how the lobsters respond to the treatments.

Several aspects of the results need to be discussed further. First, the classification process was not completely accurate. It indicated that the hemolymph tests were no more accurate than an individual grader at assessing lobsters— and they were certainly more complex and time consuming. Second, each repeat of the work discriminated the tank rejects and survivors (“not tank rejects”) using different sets of parameters, possibly driven by the difference in water temperature. Third, the discriminant equations raised from other data sets could not predict the relative mortality in the different non-submerged treatments but they could predict with close

to 100% accuracy that submerged lobsters from any data set would survive when stored in the factory. The discriminant functions raised were only 83-90% accurate at internal classification (i.e., within each replicate of the experiment) of the lobsters sampled, and this accuracy fell when equations derived from one sample set were applied to other data sets (Tables 9 and 10). It is likely that the hemolymph parameters chosen did not cover all possible causes of mortality. The grader who assisted the study was just as accurate as the discriminant models (Tables 3 and 5). Interestingly, the internal reclassification process (Table 9) and to a lesser extent the cross-data set predictions (Table 10) were, like the grader used, better at isolating probable survivors than picking tank rejects (though presumably for different reasons). Although the first and second experiments returned different equations (Table 11), some parameters are worthy of closer scrutiny, particularly since they were also associated with emersion of lobsters. In some instances the differences arose because of problems redeploying particular methods (e.g., ABA and LDH) in the field during the second study. In other cases, a previously useful parameter was displaced by new parameters that provided better discrimination (note the effect that exclusion of LDH had upon the number of parameters required to categorise lobsters in the first study).

The inclusion of lactate in the model was surprising because it was clear that the absolute level of lactate in the hemolymph was not the issue. Lactate acid is a product of anaerobic glycolysis and accumulates in crustacean muscle and hemolymph when oxygen consumption exceeds rate of uptake at the gills (e.g., exercise, aquatic hypoxia and emersion). Emersion is the primary factor involved here, though occasional tail-flaps during handling will contribute to the lactate level. Higher hemolymph lactate levels are expected in emersed lobsters at higher temperatures (Whiteley & Taylor 1990). What was not expected was that the survivors in the second trial showed hemolymph lactate levels (Table 13) similar to that of tank-rejects in the first trial (Table 12), and this difference in magnitude was a

major contributor to the difficulties in applying one discriminant equation across the two data sets. However, the implication is that the relative accumulation of lactate, rather than the concentration reached at any given time was associated with whatever problem contributed to the deaths of many of these lobsters.

Magnesium level in the hemolymph of the western rock lobster is normally kept at a concentration considerably lower than that of the surrounding sea water (Dall 1974). Inside the cells, Mg interacts with and helps to shape enzymes and other important proteins (Doumen & Ellington 1992). Perhaps the acidity associated with emersion (Taylor & Whiteley 1989), compromises the integrity of the lobster's cells and allows this Mg to leak into the hemolymph. Mg did not accumulate in the hemolymph continuously in the same manner that lactate did, but appeared to plateau at c. 14 mmol $litre^{-1}$ (Tables 12 and 13). In the second trial, with its elevated lactate levels, and prominence of health-related parameters in the discriminant equation (Table 11), the hypermagnesemia in the group of survivors or "not tank-rejects" (12.7 ± 2.4 mmol $litre^{-1}$), appears to have approached the plateau in the tank-reject group (Table 13) and, as a consequence, the parameter lost its usefulness.

Calcium appeared in two of the equations but never as a parameter contributing much discrimination to the analysis. The literature often links elevated Ca level with emersion-induced acidosis (deFur et al. 1980; Burnett 1988). Naturally, this distinction is little help when discriminating emersed survivors.

Enzyme activity measurements showed some promise in the first trial but these methods were labour-intensive and difficult to work with because of coagulation and problems with transporting the spectrophotometers. The rationale for using them was similar to that for including K, namely that enzymes like LDH are constituents of cells, and their presence in the hemolymph is an indicator of cellular damage. The lactate in the hemolymph also originates in cells, so the damage may be directly linked to effects of relatively higher rates of lactate accumulation (e.g., acidosis). Perhaps these and other cellular or molecular markers could supply more discrimination in

studies where lobster stress needs to be categorised (Dillon & Fisher 1983; Chang et al. 1999).

The “classical” stress indicator, hemolymph glucose concentration, gave mixed results in trials conducted here. Some lobsters that were severely stressed nevertheless had “baseline” glucose levels. This finding is consistent with other recent studies (Hall & van Ham 1998) that question the orthodoxy that stressed crustaceans are necessarily hyperglycaemic. Perhaps the elevated glucose level in the hemolymph cannot be sustained indefinitely and falls back to baseline levels if the stress is prolonged.

The second trial also returned a number of hemocyte-related parameters as predictors of future mortality, the significance of which have been discussed elsewhere (Fotedar et al. 2001; Jussila et al. 2001; Tsvetnenko et al. 2001) rather than the simple physiological or biochemical variables observed in the first analysis. The outcome of the second trial, conducted at a higher sea water temperature, was more severe than that of the first trial; some lobsters in the second study died in the treatment chamber (Table 4) whereas others died during the first hours of storage (Fig. 4). The discriminant equations from the second trial were probably describing the secondary effects of the stress and morbidity on the lobsters rather than the primary effects of the storage environments themselves, hence the within-data prediction accuracy was near 90% (Table 9).

Clearly the dynamics of the lobster’s stress response are important to explain why particular discriminant equations could not be applied accurately across data sets. The stress studies were conducted for a definite 6-h period, during which various parameters rose, fell, or responded in more complex ways. Introducing temperature as a variable (i.e., by repeating the study at a different time of year), meant that the “stress” or deterioration within the lobsters progressed at different rates, so sampling at a discreet point in time inevitably profiled the lobster’s hemolymph at a different stage in their stress response. Aside from problems of applying the discriminant

equations to an entire data set, the equations raised from other data sets could not predict the relative mortality in each of the different treatments with complete accuracy— with one fundamental exception (Fig. 6 and 7). Nearly all of the equations predicted correctly that submerged lobsters from any data set would survive when stored in the factory. To put this into context, the grader's "prediction" is similar to the relative treatment mortality, save for some rejection of submerged lobsters and some differences in rejection between treatments. Understandably, submerged storage of lobsters gave a very favourable outcome, even when the same sea water was recirculated through the tank for the duration of the trial. Submerged transport of large quantities of lobsters by road is impractical and this method is more suited to transport at sea (e.g., carrier boats). The stress indicators show that under the conditions of these experiments, the use of sprays seemed no better than the humid air treatment. Judging by the accumulation of lactate observed, the sprays do not support aerobic metabolism in emersed lobsters at these temperatures. Of course, sprays may still have their uses when transporting lobsters by boat under the more hostile conditions of an open or semi-enclosed deck. In the field, water sprays are a good means of preventing dehydration as well as cooling the lobsters from the effect of the sun (e.g., evaporative cooling). Further work is needed to examine the treatment effects— and particularly to understand the impact of low temperature on the lobster's responses.

The analysis correctly classified the survivability of lobsters that had been submerged before sampling. This also occurred when the discriminant equations were derived solely from the results of emersed lobsters (data not presented here). The reason for the difference in accuracy between submerged and non-submerged treatments may be because submerged lobsters show characteristics that are more consistent and closer to baseline than the individuals from the emersed treatments. Variation in type or magnitude of the deviations from baseline in lobsters in the emersed treatments would be expected to increase the chance of classification errors.

The parameters used here as predictors of mortality are not equations that identify submerged or emersed lobsters, because the latter happen to die more often. The process of deriving the functions selects parameters that provide greatest discrimination between the chosen categories, and since all emersed lobsters did not die, parameters that only define emersed lobsters are passed over by the analysis. Although this study highlighted the broad changes occurring in lobsters under stress and focused on key indicators associated with mortality, there are some drawbacks to the multi-parametric approach. While some physical tests, such as protein concentration or clotting time, are performed immediately, laboratory analysis of metabolites or ions takes considerably longer than an experienced human grader would take to judge a lobster. The high number of hemolymph tests included also increases the likelihood that data is lost as a result of sampling errors or methodological problems. If a lobster missed data from one variable it was excluded from the discriminate analysis. Ideally, this sort of work is better suited to a single instrument returning a number of values for each example or individual, for example, veterinary blood panels, HPLC, gas chromatography, or reflectance spectroscopy (Girard & Nakai 1994a; Chen et al. 1995, 2003; Downey & Beauchene 1997).

To conclude, discriminant analysis was able to successfully predict the future survival of lobsters, though with only 80-90% accuracy, and any one model did not suit all data sets. Still, the parameters in these models can legitimately be called key stress indicators, with the added knowledge that they are at the least correlated with, if not the cause of, mortality. The contribution of lactate to the discriminant models is understandable because of the role that oxygen deprivation plays in the physiology of emersed crustaceans and it provides a clear means to study the responses of lobsters to alternative handling methods. However, no particular level of hemolymph lactate concentration was associated with lobsters that would later die. Many of the lobsters that died were apparently disadvantaged by having generally high rates of

lactate accumulation which in turn may have had follow-on effects on their physiology.

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Table 1 Summary of seawater supply and relevant flow rates and water volumes applying to the storage environment treatments used in this study. (N/A, not applicable).

	Treatment				
	Tank-flow	Tank-recirc.	Humid air	Spray-flow	Spray-recirc.
Seawater supply	Flow-through	Recirculated	None	Flow-through	Recirculated
Flow rate litre min^{-1}	10	10	N/A	10	10
Volume (litre)	N/A	79	N/A	N/A	79
Quantity of lobsters (kg) (in 2 baskets)	21	21	21	21	21

Table 2 Number of rejected and accepted lobsters sampled, and tagged during the first storage environment trial.

	Grading result by treatment				
	Tank flow	Tank recirc.	Humid air	Spray flow	Spray recirc.
Rejected	1	5	14	12	33
Accepted	59	54	44	44	27
Total	60	59	58	56	60

Table 3 Relationship between the grader's figures and the losses following tanking for lobsters, packing in boxes, losses following re-tanking the lobsters, and final survival during the first storage environment trial**.**

Outcome No. of lobsters	Rejected	Accepted	Total
Tank reject	34	23	57
Dead in box	2	4	6
Re-tank reject	4	22	26
Survived	25	179	204
Total	65	228	293

Table 4 Grading result when assessing the lobsters leaving the treatments during the second storage environment trial.

	Storage treatments				
	Tank flow	Tank recirc.	Humid air	Spray flow	Spray recirc.
Dead	0	0	1	20	19
Weak	8	18	81	81	100
Leg-loss	9	6	3	4	4
Accepted	122	116	50	33	18
Totals	139	140	135	138	141

Table 5 Relationship between the grader's figures and the losses following tanking for lobsters, packing in boxes, losses following re-tanking the lobsters, and final survival during the second storage environment trial**.**

	Rejected	Accepted	Totals
Tank rejects	62	5	67
Dead in box	2	1	3
Re-tank rejects	2	8	10
Survived	78	155	233
Totals	144	169	313

Table 6 Losses of tagged lobsters from the tanks and during simulated transport and numbers of survivors for each treatment during the second storage environment trial.

	Storage treatments				
	Tank flow	Tank recirc.	Humid air	Spray flow	Spray recirc.
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

Table 7 Summary of stepwise discriminant function analysis of hemolymph test results for lobsters that later became either tank rejects or not tank rejects for all treatments in the first and second trials. First/second refers to a reanalysis of the first study data set using variables common with the second study, required to make predictions in Table 10.

	Variable to Enter	Step	*F* to enter	d.f.	*p* level	No. of vars. in model	Wilks' Lambda (Λ)	*F* value	df	*p* level
First	√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
First/ Second	√Lactate	1	100.38	1, 234	0	1	0.7	100.38	1, 234	0.000
	Mg	2	7.8	1, 233	0.006	2	0.677	55.55	2, 233	0.000
	Protein	3	5.86	1, 232	0.016	3	0.66	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
Second	√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
	Ca	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

Table 8. Summary of variables in three models based upon step-wise discriminant function analysis of the data sets according to the pooled groups (tank rejects and not tank rejects). First/second refers to a reanalysis of the first study data set using variables common with the second study, required to make predictions in Table 10.

		Wilks' Lambda (Λ)	Partial λ of variable	F-to-remove	p-level	Tolerance
				d.f.=1, 173		
First	√Lactate	0.72	0.90	19.5	0.00	0.72
N = 177	LogLDH	0.69	0.93	12.8	0.00	0.94
	Mg	0.66	0.97	5.0	0.03	0.75
				d.f.=1, 228		
First/Second	√Lactate	0.73	0.86	36.7	0.00	0.48
N = 236	Mg	0.66	0.94	13.5	0.00	0.52
	Protein	0.64	0.98	5.4	0.02	0.68
	Clot time	0.64	0.97	6.2	0.01	0.93
	Na	0.64	0.99	3.5	0.06	0.74
	Ca	0.63	0.99	3.1	0.08	0.48
	√Glucose	0.63	0.99	2.7	0.10	0.63
				d.f.=1, 173		
Second	√Lactate	0.72	0.91	16.8	0.00	0.70
N = 179	Clot time	0.72	0.91	18.1	0.00	0.90
	%granul.	0.68	0.97	5.8	0.02	0.99
	Ca	0.67	0.97	5.0	0.03	0.77
	√BactRank	0.67	0.98	4.1	0.04	0.95

Table 9 Observed classification, reclassification using posterior probabilities, and percentage of reclassifications that were correct following Discriminant Function Analysis of samples of tagged western rock lobsters (*Panulirus cygnus*) that were stressed and their mortality tracked for a week (tank rejects or not-tank rejects) while stored in a live lobster factory. First/second refers to a reanalysis of the first study data set using variables common with the second study, required to make predictions in Table 10. (TR, tank rejects; N-TR, not tank reject.)

	First			First/ Second			Second		
	TR	N-TR	% correct	TR	N-TR	% correct	TR	N-TR	% correct
Observed									
TR	29	19	60.4	29	21	58	21	11	65.6
N-TR	11	180	94.2	16	192	92.3	10	160	94.1
Total	40	199	87.4	45	213	85.7	31	171	89.6

Table 10 Observed classification, predictions, and percentage of predictions correct for the first and second data sets analysed using posterior probabilities calculated from the distances between examples and the group centroids of the other data sets. (TR, tank rejects; N-TR, not tank reject.)

Data set	First			Second		
Predictions	Second			First/Second		
	TR	N-TR	% correct	TR	N-TR	% correct
Observed						
TR	9	37	19.6	33	4	89.2
N-TR	4	190	97.9	54	139	72
Total	13	227	82.9	87	143	74.8

Table 11 Classification functions obtained following discriminant function analysis of all treatments of the first and second studies. First/second refers to a reanalysis of the first study data set using variables common with the second study, required to make predictions in Table 10. (TR, tank rejects; N-TR, not tank reject.)

First			First/ Second			Second		
Variable	TR	N-TR	Variable	TR	N-TR	Variable	TR	N-TR
√Lactate	-1.6643	-3.2878	√Lactate	1.283	-0.968	√Lactate	-1.2762	-2.4086
LogLDH	13.4545	11.322	Mg	-1.313	-1.834	Clot. time	0.5999	0.485
Mg	4.0699	3.7443	Protein	0.571	0.602	% granul.	0.6827	0.8653
			Clot time	-0.003	-0.043	Ca	3.0963	3.3839
			Na	0.663	0.677	√BactRank	1.2204	0.6487
			Ca	3.222	3.491			
			√Glucose	3.179	4.002			
Constant	-41.682	-28.5933	Constant	-222.418	-222.39	Constant	-45.7084	-39.1719

Table 12 Summary of hemolymph test results (mean±SD, number of measurements in brackets) for tank rejects and survivors (not-tank rejects) taken during the first study. (ns, not significantly different.) Unless stated otherwise, units are mmol litre^{-1}.

	All treatments				
	Survivors		Tank rejects		*p* <
ABF	0.28± 0.26	(216)	0.42± 0.30	(44)	0.01
Ranked bacterial counts	4.2± 4.0	(237)	6.1± 4.9	(57)	0.001
Ca	14.1± 2.0	(223)	15.6± 1.9	(56)	0.001
Cl	471.4± 32.7	(223)	465.3± 30.3	(56)	ns
Clotting time (s)	46.4± 13.0	(222)	55.2± 15.2	(51)	0.001
Glucose	1.2± 1.2	(235)	2.3± 1.9	(56)	0.001
Granulocytes (%)	10.2± 4.1	(218)	8.7± 4.3	(51)	0.05
Hcy (Abs. 340 nm)	0.3± 0.1	(225)	0.3± 0.1	(55)	ns
K	11.9± 1.2	(223)	13.1± 1.1	(56)	0.001
Lactate	2.8± 2.9	(235)	8.1± 4.7	(56)	0.001
LDH	60.6± 83.6	(222)	232.1± 306.1	(51)	0.001
Mg	11.6± 1.9	(223)	14.2± 2.6	(56)	0.001
Na	541.0± 33.1	(223)	536.6± 30.5	(56)	ns
Protein (g litre^{-1})	83.6± 22.0	(234)	82.9± 15.7	(55)	ns
Total cell count (millions)	6.4± 4.1	(236)	5.8± 3.4	(57)	ns

Table 13 Summary of hemolymph test results (mean±SD, number of measurements in brackets) for tank rejects and survivors (not-tank rejects) taken during the second study. (ns, not significantly different.) Unless stated otherwise, units are mmol $litre^{-1}$.

	All treatments				
	Survivors		Tank rejects		p<
Ranked bacterial counts	2.2± 3.0	(244)	4.8± 4.5	(64)	0.001
Ca	15.6± 2.8	(226)	16.7± 3.1	(65)	0.01
Cl	541.2± 73.7	(221)	529.7± 61.7	(65)	ns
Clotting time (s)	47.8± 10.4	(226)	60.4± 10.7	(42)	0.001
Glucose	1.3± 1.2	(244)	2.0± 1.3	(66)	0.001
Granulocytes (%)	8.1± 3.4	(204)	5.3± 3.3	(54)	0.001
K	11.0± 1.9	(223)	11.9± 1.8	(63)	0.001
Lactate	7.4± 6.4	(243)	16.1± 5.3	(65)	0.001
Mg	12.7± 2.4	(226)	14.2± 2.3	(65)	0.001
Na	600.9± 68.2	(220)	576.2± 71.3	(63)	0.05
Protein (g $litre^{-1}$)	89.9± 26.1	(242)	83.2± 13.7	(64)	0.01
Total cell count (millions)	7.5± 3.2	(246)	6.1± 3.0	(67)	0.001

List of figures

Fig. 1 A, Water pH and B, ammonia measured during the Tank and Spray treatments during the first storage environment trial.

Fig. 2 Comparison of rejection by the grader for each storage treatment, percentage of western rock lobsters (*Panulirus cygnus*) removed from the tanks, and total losses (from the tanks and following simulated pack-out and storage) during the first storage environment trial.

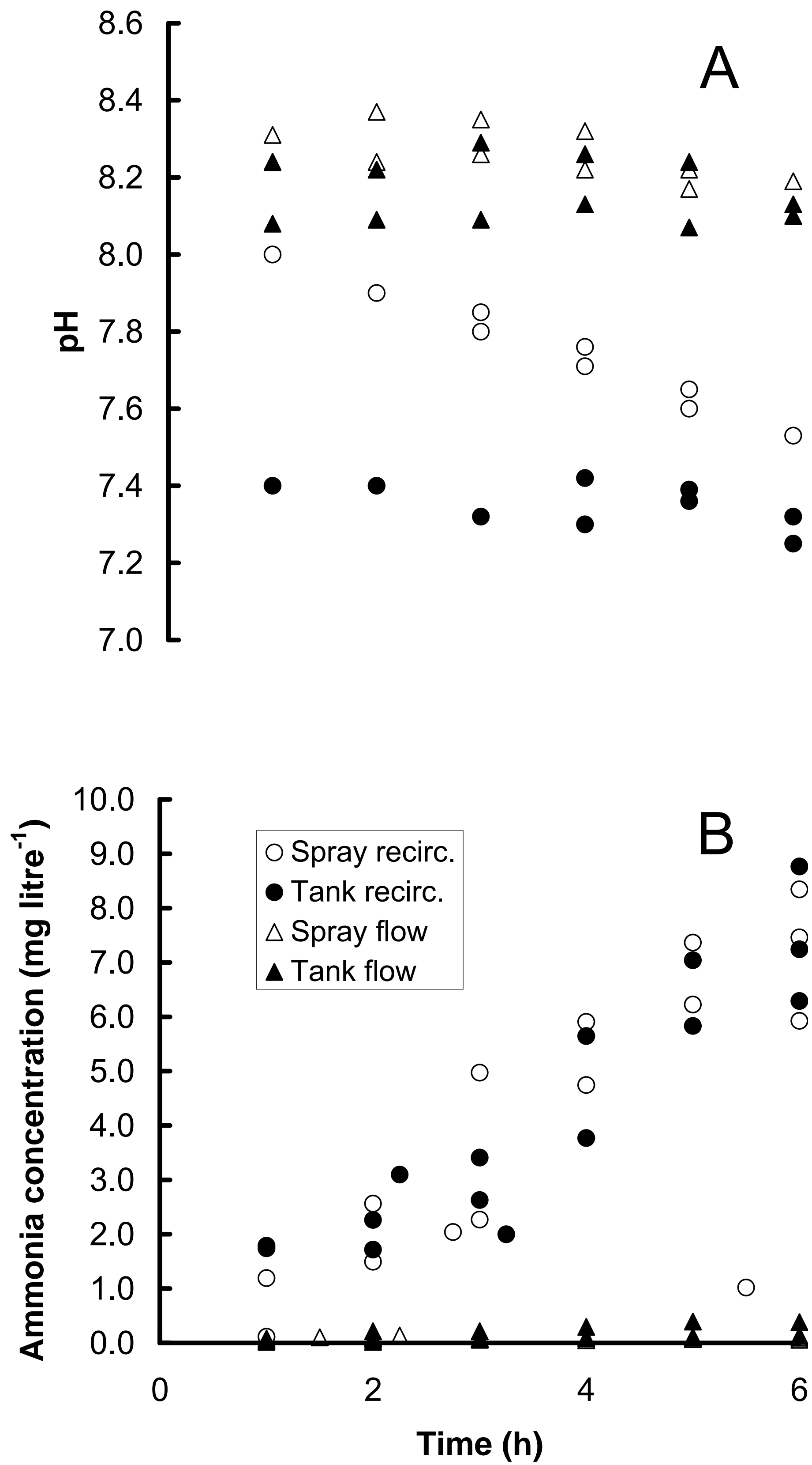
Fig. 3 A, Water pH and B, ammonia measured during the Tank and Spray treatments during the second storage environment trial.

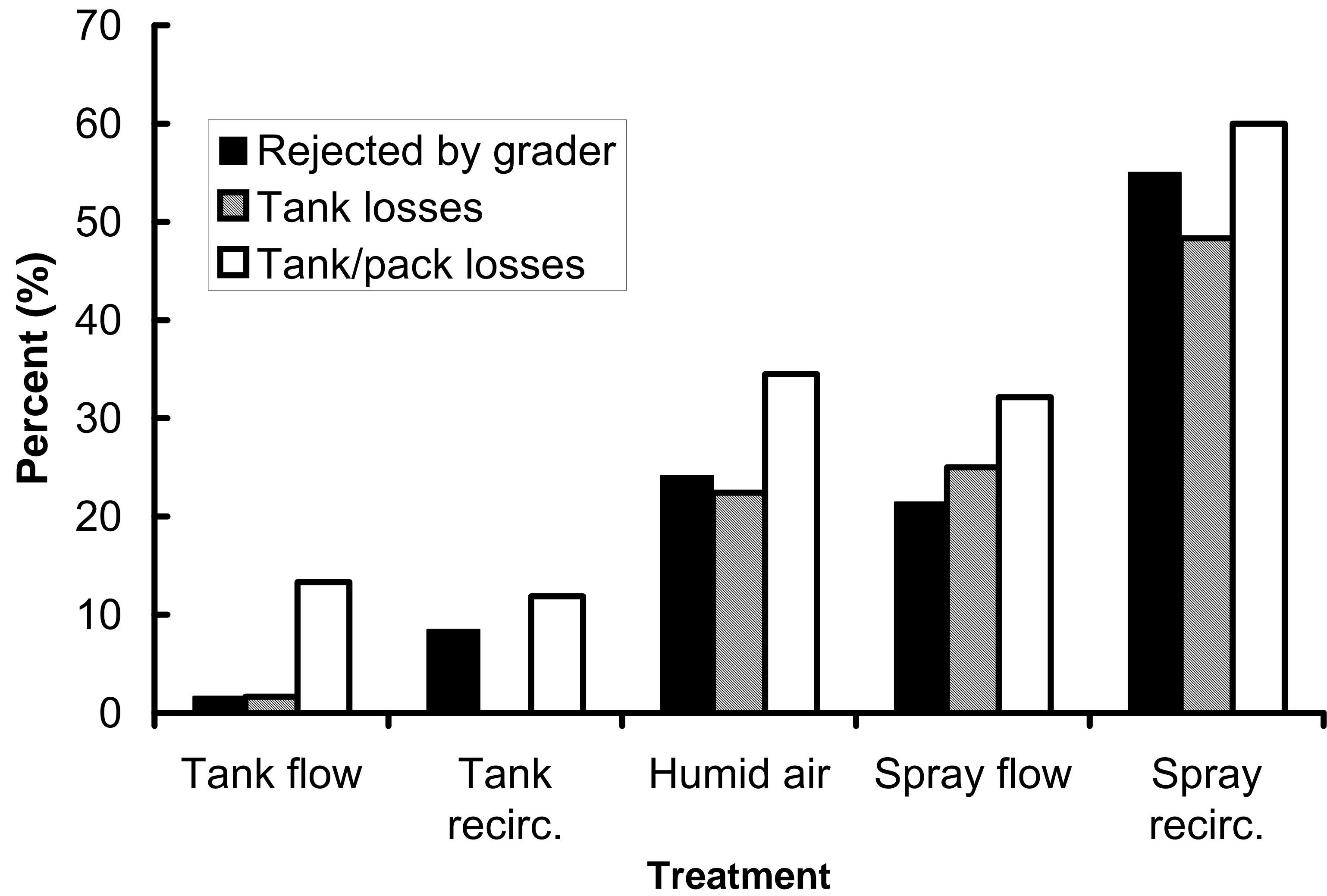
Fig. 4 Numbers of tank rejects removed from the tanks on the night of the treatment (0) and on each day during the subsequent week for the first and second storage environment trial.

Fig. 5 Comparing the level of rejection by the grader to the proportions of western rock lobster (*Panulirus cygnus*) lost in tanks and the combined losses following tanking and pack-out in the second storage environment trial.

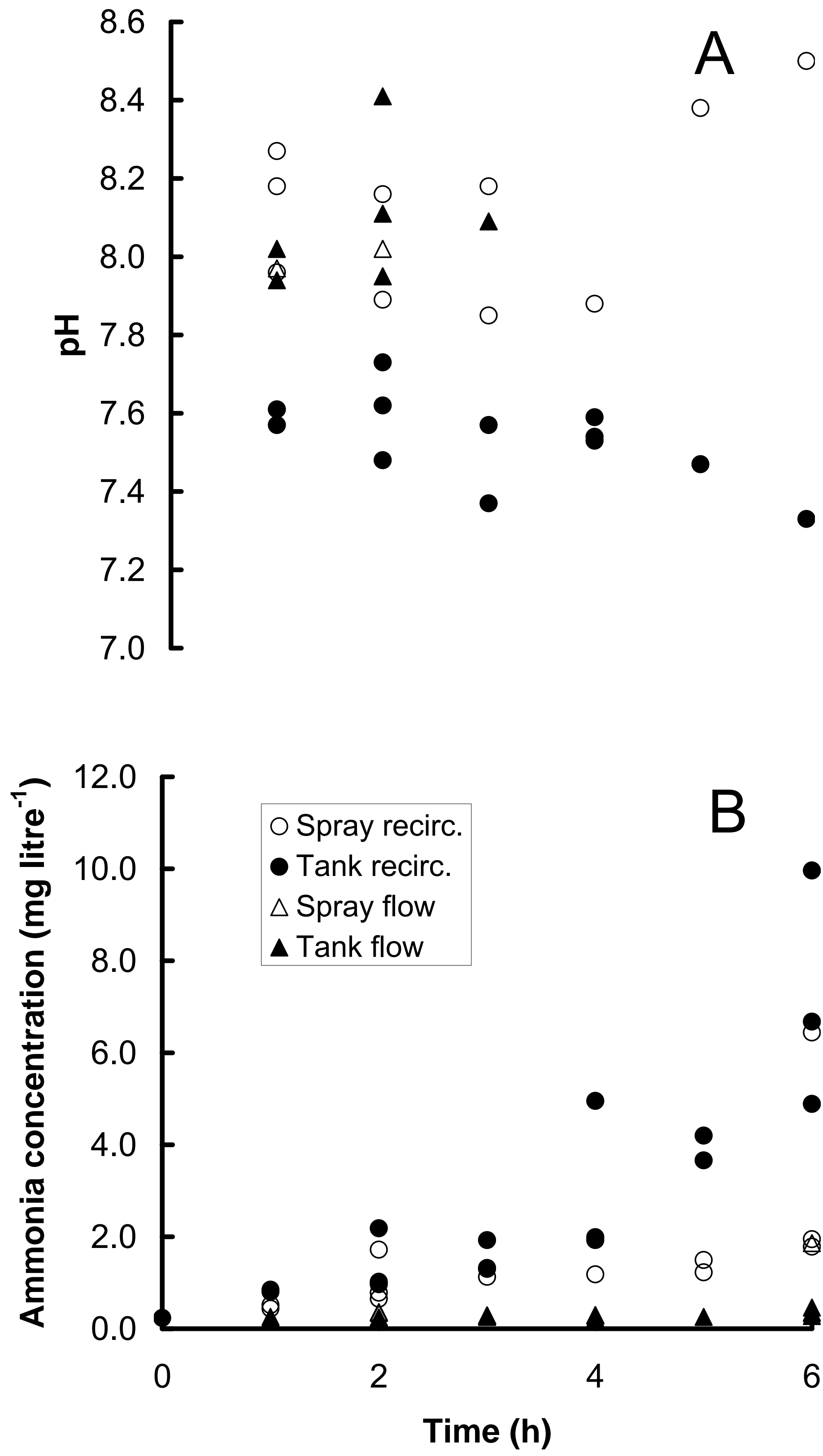
Fig. 6 Observed lobster mortality for each treatment during the first trial versus the grader's predicted mortality (rejects) and the mortality predicted using different discriminant analyses from the first, and second trials.

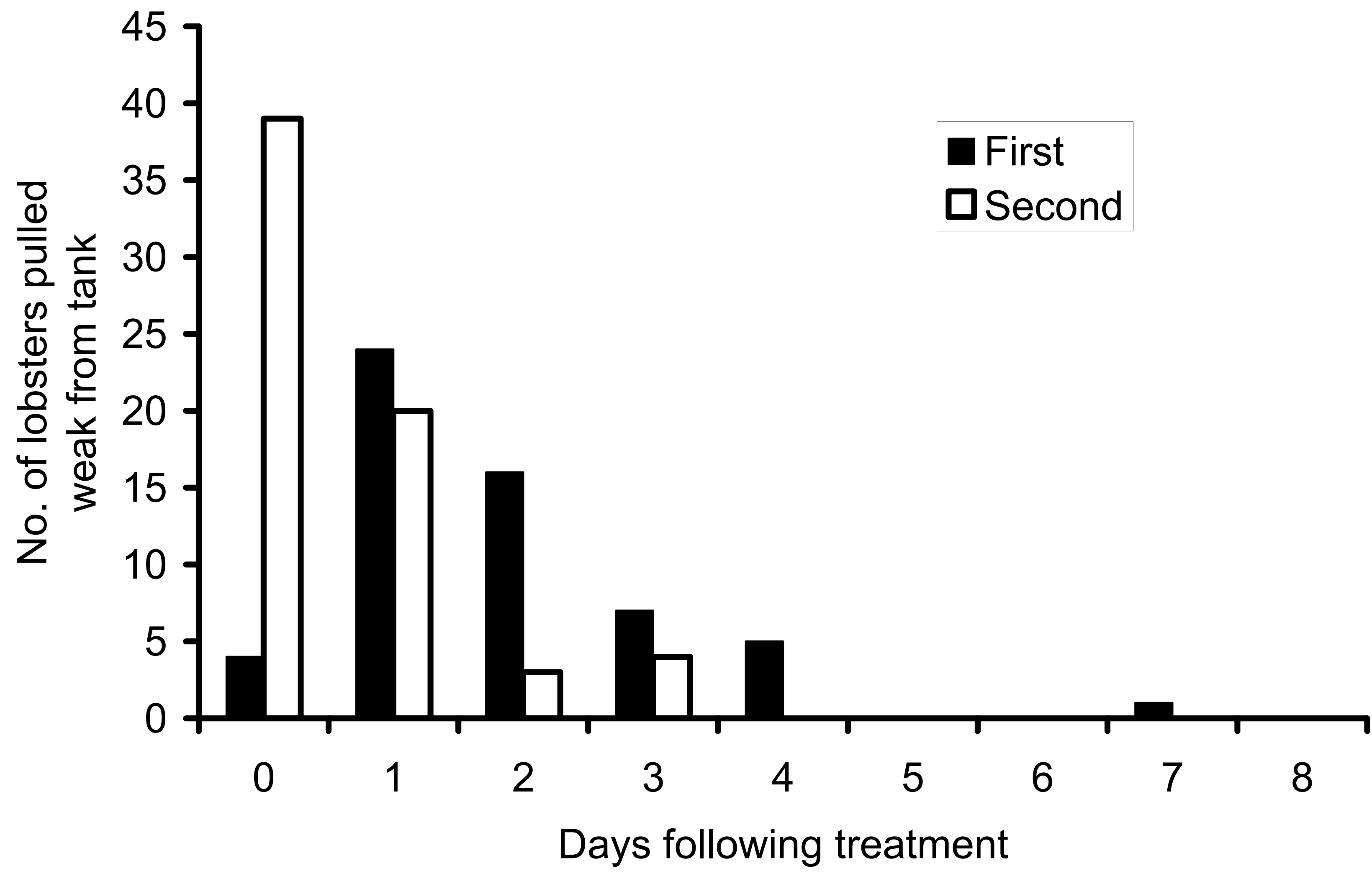
Fig. 7 Observed mortality for each treatment during the second trial versus the grader's predicted mortality (rejects) and mortality predicted using different discriminant analyses from the first and second trials.

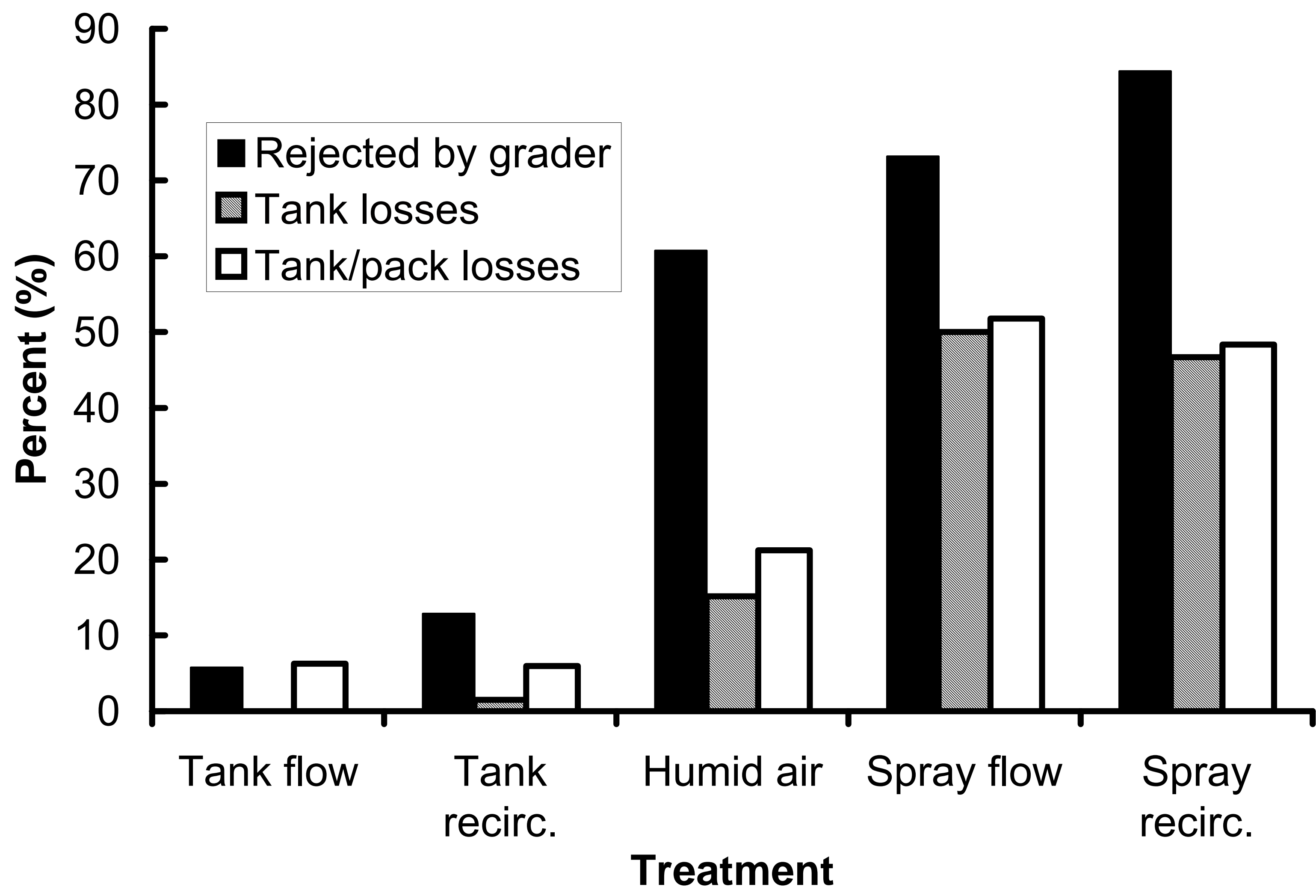

A
pH
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
B
Ammonia concentration (mg litre-1)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Spray recirc.
Tank recirc.
Spray flow
Tank flow
0
2
4
6
Time (h)

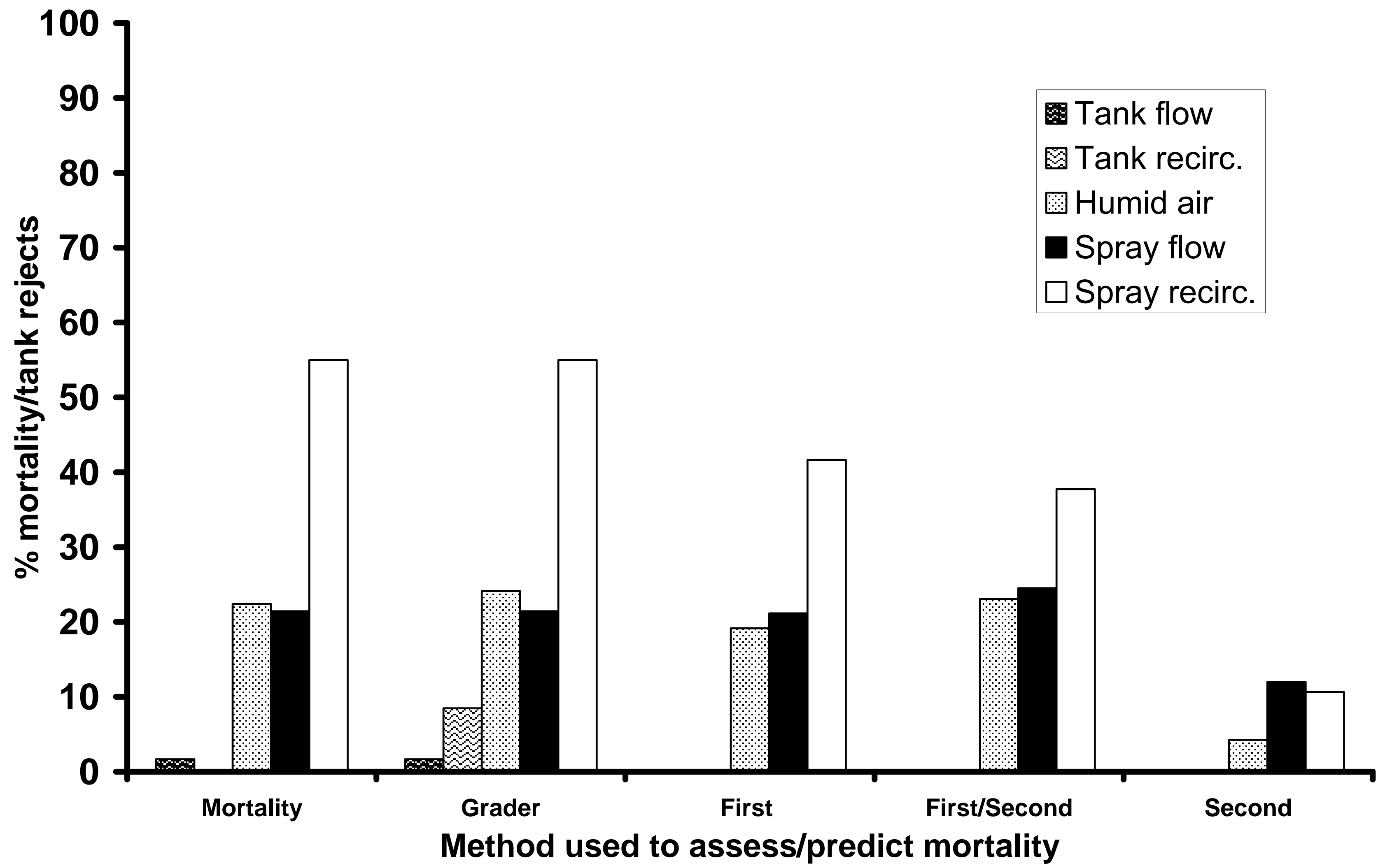


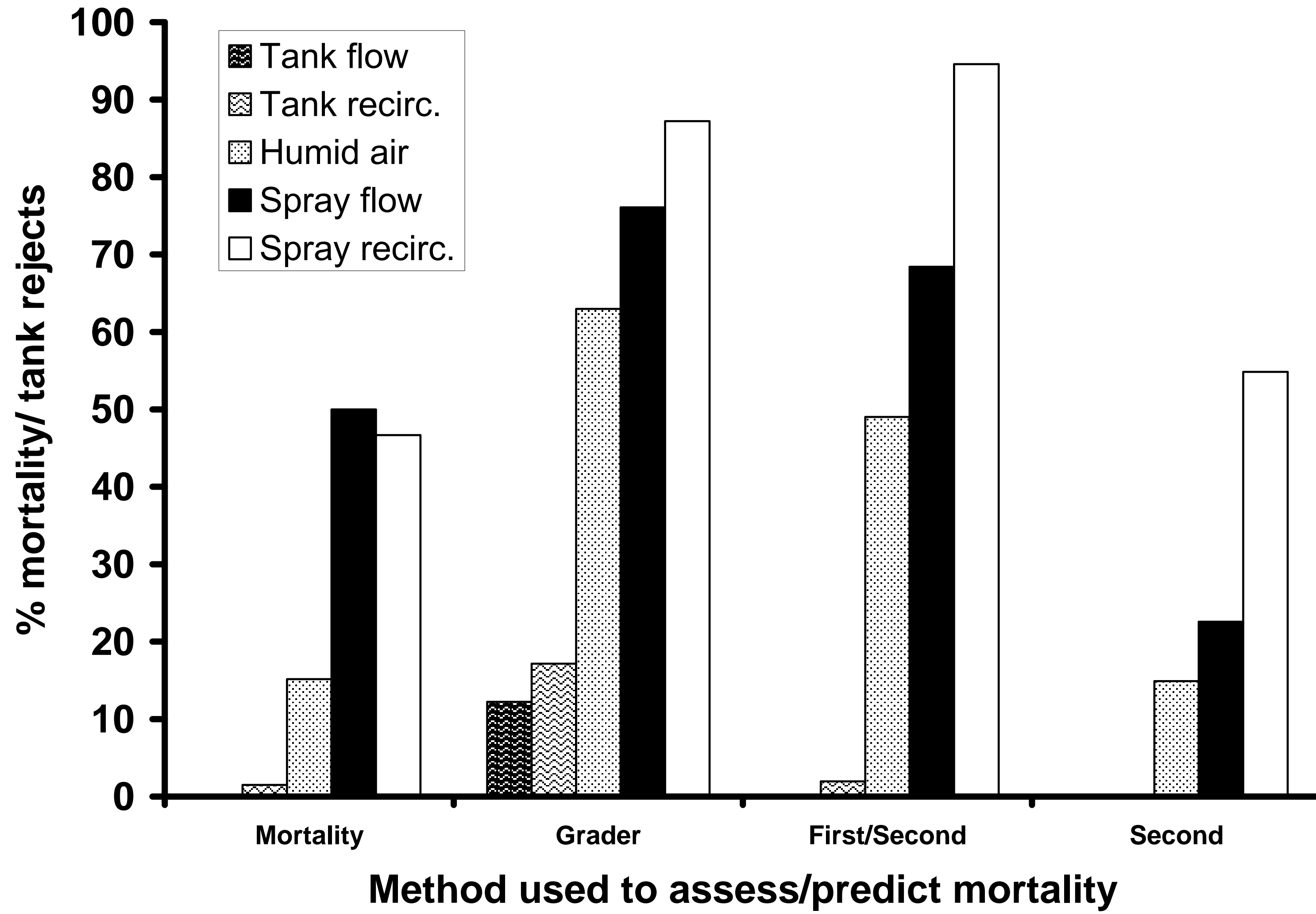
70
60
50
40
30
20
10
0
Percent (%)
Rejected by grader
Tank losses
Tank/pack losses
Tank flow
Tank recirc.
Humid air
Spray flow
Spray recirc.
Treatment


8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
pH
A
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Ammonia concentration (mg litre-1)
B
Spray recirc.
Tank recirc.
Spray flow
Tank flow
0
2
4
6
Time (h)


45
40
35
30
25
20
15
10
5
0
No. of lobsters pulled weak from tank
First
Second
0
1
2
3
4
5
6
7
8
Days following treatment


90
80
70
60
50
40
30
20
10
0
Percent (%)
Rejected by grader
Tank losses
Tank/pack losses
Tank flow
Tank recirc.
Humid air
Spray flow
Spray recirc.
Treatment


100
90
80
70
60
50
40
30
20
10
0
% mortality/tank rejects
Tank flow
Tank recirc.
Humid air
Spray flow
Spray recirc.
Mortality
Grader
First
First/Second
Second
Method used to assess/predict mortality


100
90
80
70
60
50
40
30
20
10
0
% mortality/ tank rejects
Tank flow
Tank recirc.
Humid air
Spray flow
Spray recirc.
Mortality
Grader
First/Second
Second
Method used to assess/predict mortality