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Differential tolerance of body fluid dilution in two species of tropical hermit crabs: not due to osmotic/ionic regulation

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Abstract

The tropical intertidal hermit crabs *Clibanarius taeniatus* and *Clibanarius virescens* were examined for differences in survival and physiological responses in low salinity. We found that *C. taeniatus* survived better in dilute seawater than *C. virescens* and that these species did not differ in their abilities to regulate haemolymph osmolarity, ionic concentration of the haemolymph or body fluid volume. We also found no difference in oxygen consumption between the species when acutely exposed to a range of temperature and salinity combinations. It is concluded that the greater survival in dilute seawater by *C. taeniatus* compared to *C. virescens* is due to a greater tolerance of dilution of body fluids by *C. taeniatus*. Differences in tolerance to dilute seawater may influence the habitat preferences of these species within the same geographical area.

Keywords: Anomura; Hermit crabs; *Clibanarius*; Osmoregulation; Survival; Low salinity

1. Introduction

Intertidal organisms in tropical regions are frequently subjected to rapid changes in salinity and temperature (Moore, 1972; Brosnan, 1992; Coates, 1992). In Hong Kong, for example, tidepool temperatures range from 14 to 42 °C (Morton and Harper, 1995; Chan, 2000) and their inhabitants may also be exposed to sudden, heavy rainfall or extreme evaporation so that salinities may vary from 1.3 to 100‰ (Morritt and Williams, 2000a,b). In addition, salinity and temperature stress may increase with height on the shore (Newell, 1969, 1978; Brosnan, 1992; Metaxas and Scheibling, 1993). Thus, temperature and salinity stresses are important environmental influences affecting the survival of intertidal organisms (Gilles and Pequeux, 1983; Péqueux and Gilles, 1984; Hawkins, 1995; Stillman and Somero, 1996).

The hermit crabs *Clibanarius taeniatus* (Milne Edwards, 1848) and *Clibanarius virescens* (Krauss, 1843) are closely related, common inhabitants of rocky intertidal areas of tropical eastern Australia. Field observations (Dunbar, 2001; Dunbar et al., 2003) showed that the two species have very similar geographical distributions along the Queensland coast. However, within this broad area the two species prefer different habitats. Dunbar (2001) and Dunbar et al. (2003) found high numbers of *C. taeniatus* in areas that experience frequent episodes of lowered salinity, such as in estuaries or near river mouths. In contrast, *C. virescens* is abundant in areas that rarely, if ever, experience freshwater inundation, while *C. taen-*

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iatus is rare in these areas. An obvious question is how do the two species differ in their physiological responses to low salinity that might be correlated with a difference in tolerance of diluted seawater?

In Crustacea, the ability to osmoregulate appears to be correlated with tolerance of diluted seawater. Euryhaline crustaceans are generally better osmoregulators than stenohaline crustaceans, which tend to be osmoconformers (Kinne, 1964; Davenport, 1972; Gilles and Pequeux, 1983; Jury et al., 1994; Bromberg et al., 1995; McGaw et al., 1999; Castilho et al., 2001).

Although the osmoregulatory ability of crustaceans has received considerable attention (for reviews see Mantel and Farmer, 1983; Gilles and Pequeux, 1983; Péqueux, 1995), relatively few studies have been reported for the anomuran decapods (but see Thomas and Rice, 1992), with even fewer on the relationship between osmoregulatory ability and survival in hermit crabs. Existing studies of hermit crabs tend to support the hypothesis that osmoregulatory competence is correlated with the ability to tolerate decreased salinities. The hermit crab *Clibanarius vittatus* can be found in salinities of less than 20‰ and greater than 35‰ in the Gulf of Mexico (Sharp and Neff, 1980). In work by Sharp and Neff (1980), *C. vittatus* was shown to be an osmoregulator in salinities between 5 and 50‰ and Sweeny (in Sharp and Neff, 1980) reported a 75% survival rate in 11‰ at 25 °C for more than 14 days. Young (1979) and Sabourin and Stickle (1980) also reported that *C. vittatus* was able to regulate haemolymph osmolarity in media below 25‰. Furthermore, Young (1979) showed that *C. vittatus* survived low salinity conditions longer than the subtidal hermit crabs *Pagurus pollicaris* and *Pagurus longicarpus*, which appear to be osmoconformers (but see Sherman and Eichrodt, 1982). The hermit crab, *Diogenes bicristimanus*, which inhabits the intertidal regions of India (Tirmizi and Siddiqui, 1982; A. Asakura, personal communication) was shown to be a good osmoregulator and, when exposed to salinities of 15‰ or less, maintained haemolymph osmolarity hyperosmotic to the medium (Sarojini and Nagabhushanam, 1968). *Diogenes bicristimanus* was also tolerant of changes in salinity from 5 to 30‰ and able to survive prolonged exposure to salinities as low as 5‰ (Sarojini and Nagabhushanam, 1968).

Although the relationship remains unclear (Shumway, 1978; Péqueux and Gilles, 1984; Péqueux, 1995), osmotic regulation must result in some, even if indirect, metabolic cost (Kinne, 1971; Taylor et al., 1977; Mantel and Farmer, 1983; Péqueux and Gilles, 1984; Jury et al., 1994; Péqueux, 1995). The energy cost of osmoregulation could, in itself, constitute a barrier to tolerance of diluted seawater, especially in tropical environments where increased temperature may accompany osmotic stress.

Since it is integrally linked with osmotic regulation in crustaceans, ionic regulation might also be expected to play an important role in the tolerance of diluted seawater since the animal must maintain a haemolymph ionic composition which is different from that of the medium (Robertson, 1960). However, we know of no studies directly linking ionic regulatory ability with tolerance of diluted seawater.

In the present study, the survival of *C. taeniatus* and *C. virescens* was compared when exposed to prolonged low salinity conditions at different temperatures. The capabilities of the two species for haemolymph osmotic and ionic regulation, as well as volume regulation were also compared. As a means of assessing if there was any difference in the metabolic cost of osmoregulation between the two species, their oxygen consumption in diluted seawater was compared.

2. Materials and methods

For all experiments, intermolt adult *C. taeniatus* and *C. virescens* were collected from two sites on the Capricorn Coast region of Queensland, Australia (latitude: 22°17′–22°55′S; longitude: 150°15′E). Ambient salinity was measured throughout a 2-year period and found to be approximately 36‰, except during periods of river flooding. Crabs between 1 and 2.5 g total mass (including the shell) were acclimated in continuously aerated tanks with salinity of 36‰ at 25 ± 2 °C under a 12 h light:12 h dark regime for at least 7 days before being used in treatments. Algae were supplied by regularly adding algae-covered rocks to the aquaria and crabs were also fed frozen fish at the same time once a week. Animals were not sexed. Work on *Hemigrapsus nudus* and *Hemigrapsus oregonensis* by Todd and Dehnel (1960), *Carcinus maenus* by Siebers et al. (1972), *P. longicarpus* by Biggs and McDermott (1973) and *Callinectes sapidus* by Findley et al. (1978) have indicated no effect of gender on temperature tol-

erances, oxygen consumption and survival. For acclimation and for all experiments, seawater was collected from a tidal creek and diluted with distilled water to produce the required salinity.

2.1. Survival in low salinity

Fifteen *C. taeniatus* and 15 *C. virescens* were used for each treatment. Individuals were randomly placed in one of 30 numbered, 250-ml clear Perspex chambers containing 50 ml of 8‰ water and having a large surface area to volume ratio. Chambers were placed in a constant temperature water bath at 15, 25 or 35±2 °C without lids, for constant air saturation. Crabs were checked every 4–6 h for survival until all crabs of one species had died. Individuals were counted as 'Dead' if there was no antennal, antennule or maxilliped movement, even after they were prodded for 1 or 2 min. The experiment was done twice with each species. Thus, 30 individuals of each species were separately tested for survival in low salinity at each temperature. Controls were run in 36‰ at each temperature using 15 crabs of each species.

2.2. Osmoregulation in low salinity

Osmoregulation of each species was tested in fifteen combinations of temperature (15, 25 and 35 °C) and salinity (4, 8, 15, 25 or 36‰). For each repetition of the experiment at a given temperature, 24 individuals of each species were randomly chosen from the acclimation tank. No effort was made to standardise size, since preliminary tests showed no affect of size on haemolymph osmolarity. Shells were not removed. Four specimens of each species were individually placed in separate 250-ml Perspex chambers containing 50 ml of one of the treatment salinities (4, 8, 15, 25 or 36‰) while four individuals had haemolymph withdrawn by thoracic puncture with 29-gauge syringe needles and measured upon removal from the acclimation tank to give an initial haemolymph osmolarity. Since the average exposure time of the intertidal zone in the Central Queensland region is approximately 6–8 h, all chambers were randomly placed in a large circulating water bath at the given treatment temperature for 7 h. Additionally, preliminary experiments on both species showed that haemolymph concentration reached a steady state after approximately 3–5 h. Haemolymph and an equivalent sample of the medium were withdrawn at the same time and measured by a Wescor VAPRO 5520 vapour pressure osmometer which had been calibrated using OPTI-MOLE™ 100, 290 and 1000 mmol/kg sodium chloride standard solutions. Each experiment was repeated three times for both species. Measurements of haemolymph osmolarity at each of the three temperatures tested (not including repetition 2 of 25 °C, which was discarded due to acclimation tank abnormalities, resulting in a sample size of eight individuals of each species for each treatment) were analysed by Model III, two-way ANOVA for 'Species' and 'Repetition'. Haemolymph osmolarities for both species at each temperature were compared to treatment water osmolarities by two-tailed *t*-tests.

2.3. Volume changes in low salinity

Adult, intermoult hermit crabs were gently shaken to remove water trapped within the shell, then weighed. Groups of four similar sized crabs of the same species were exposed to one of nine combinations of temperature (15, 25 and 35 °C) and salinity (8, 20 and 36‰) for 6 h in a flow-through, recirculating system. At the completion of exposure, all crabs were gently shaken again and reweighed to detect any changes in wet weight as an indirect measure of volume regulation. Experiments were repeated four times ($n=4$).

2.4. Ionic regulation in low salinity

During survival experiments in 8‰ at 15 and 35 °C, haemolymph samples of more than 50 μl were withdrawn from 15 individuals each of *C. taeniatus* and *C. virescens* by thoracic puncture with 29-gauge syringe needles. Both dying hermit crabs and individuals that survived until the end of the experiments were sampled with the preliminary assumption that the ionic concentration of the haemolymph for crabs at the point of dying would be the same as for surviving hermit crabs. Since Davenport (1972) and Sherman and Eichrodt (1982) used lack of movement and lack of resistance to being pulled from the shell to indicate dying, or death of hermit crabs exposed to treatment, we identified dying crabs as those demonstrating little resistance to being removed from the shell and had a delayed physical response to abdominal prodding. The experiment was done once with animals maintained at 15 °C and twice

with animals at 35 °C. Samples were taken at 4-h intervals in both 15 and 35 °C experiments. However, in some intervals in the 15 °C experiment, the number of dying crabs was so low that intervals were pooled into groups and are here reported as 12-h intervals. For figures in which *C. taeniatus* haemolymph ion concentration is graphed, the final data point represents the ionic concentration in haemolymph from surviving crabs (Fig. 4A, Fig. 5A, Fig. 6A and Fig. 7A).

Fifty microlitres of haemolymph from each specimen were pipetted into 10 ml vials and diluted to 5.0 ml by the addition of 4.95 ml of 2% nitric acid. As a control, 50 μl haemolymph samples were taken from four individuals of each species in the acclimation aquaria (36‰ at 25 °C) and analysed in the same way. These are presented as 'Initial' haemolymph ion concentrations. Five, 5.0 ml control blanks of 2% nitric acid and 8‰ seawater were also prepared. All samples were kept refrigerated at 4.0 °C until analysed.

Samples were analysed by a Perkin Elmer Optima 3000DV inductively coupled plasma emission spectroscope (ICPES) for the concentrations of sodium (Na^+), potassium (K^+), calcium (Ca^{++}) and magnesium (Mg^{++}). Standard solutions were used to calibrate readings for each of these ions. For initial and final measurements, haemolymph concentrations of each ion were compared with the concentrations of the medium by one-way ANOVAs.

2.5. Oxygen consumption

A flow-through recirculating water system was used to measure oxygen consumption. The apparatus was made of three 250-ml Perspex chambers connected in series by inflow and outflow rubber tubing. The chambers on the incurrent and excurrent sides were fitted with oxygen electrode probes connected to two TPS 90D DO_2 meters and the differences in percent saturation of oxygen were measured every 300 s.

Groups of four similar sized hermit crabs of the same species were acutely exposed to one of nine combinations of temperature (15, 25 and 35 °C) and salinity (8, 20 and 36‰) for 6 h. All treatment and control experiments were replicated four times ($n=4$). Before the group was placed in the animal chamber of the oxygen consumption apparatus each individual was gently shaken to remove trapped water from within the shell and then weighed. Hermit crabs were then kept together in their experimental group in a Perspex container for some time (up to ~20 min) between being shaken to rid them of shell reservoir water and being placed within the experimental chamber prior to the start of the experiment. Animal groups were acclimated in the experimental set-up for approximately 1 h, in order to acclimate to it before measurements for oxygen consumption began. Although the crabs moved about the chamber initially, they soon adopted a relaxed position with the antennae and antennules slightly protruding out from beneath the shell, as described by Sabourin and Stickle (1980). Data points used to calculate oxygen consumption were taken from recordings some 3–5.5 h into the experimental regime—a point at which oxygen consumption under experimental conditions had levelled off to a relatively steady state. Controls for water borne microbes and algae were performed with seawater only. Groups of four empty shells from which hermit crabs had been removed were also tested over the same treatment time in all combinations of temperature and salinity. These shells were not scrubbed clean of epibionts, since the aim of this test was to see if shell epibionts had a significant effect on the rates of oxygen consumption measured in crabs within their shells.

Upon completion of treatments crabs were removed from the shell, dabbed dry and weighed again. Weight-specific oxygen consumption was calculated from percent oxygen saturation readings using the formula:

$$\left[(S_i - S_e)(C'_{ts})(F)\right]/W \quad (1)$$

where S_i and S_e are the measured percent saturation of oxygen in the incurrent and excurrent treatment water, respectively; C'_{ts} is the value for oxygen solubility at a given temperature and salinity in ml O_2/l seawater (SW); F is the average flow rate per hour; W is the total wet weight of tissue for samples in the treatment.

The appropriate (negligible) values of shell-only oxygen consumption were subtracted from oxygen consumption values of live, shelled hermit crabs to give a corrected value of oxygen consumption. Correction for microbial oxygen consumption was unnecessary because results of tests showed no oxygen consumption by seawater only. After correction, oxygen consumption was ana-

lysed by Model I, three-way ANOVA for 'Species', 'Salinity' and 'Temperature' followed by post hoc Tukey pairwise analyses.

3. Results

3.1. Survival in low salinity

Fig. 1A shows the comparison of percent survival between *C. taeniatus* and *C. virescens* when exposed to 8‰ at a temperature of 15 °C. For *C. virescens*, the time of 50% survival (i.e. t_{50}) occurred at approximately 38.5 h, while that for *C. taeniatus* did not occur until approximately 65 h. In control experiments where both species were held at 15 °C in 36‰, all *C. taeniatus* and *C. virescens* survived 83 h of exposure.

In Fig. 1B, the percent survival of the two species exposed to 8‰ at 25 °C is shown. Fifty percent of *C. virescens* survived approximately 41.5 h of exposure. However, *C. taeniatus* maintained greater than 50% survival for the experimental duration of 76.5 h. In contrast, all *C. taeniatus* ($n=15$) and all *C. virescens* ($n=15$) survived at 25 °C in 36‰ for the 73 h control experiment.

The survival of the two species was also different when exposed to 8‰ at 35 °C (Fig. 1C). After only approximately 5.5 h, *C. virescens* survivorship was reduced to 50%, while 93% of *C. taeniatus* remained alive. Within 13 h, only 3% of *C. virescens* remained, while 70% of *C. taeniatus* were still alive. The t_{50} for *C. taeniatus* occurred at 16.5 h.

The control in 36‰ at 35 °C (data not shown) suggested that *C. virescens* was less tolerant of the higher treatment temperature than *C. taeniatus*. The number of live individuals of both species decreased slightly in the first 6 h, but there was no statistical difference in survival between species for up to 29 h of exposure ($\chi^2_1=1.88$, $P>0.05$).

3.2. Osmoregulation in low salinity

Fig. 2A–C, demonstrate the establishment and maintenance of haemolymph osmolarity hyperosmotic to the medium over the range of treatment salinities (4–36‰) at 15, 25 and 35 °C, respectively. As salinity decreases, the trendlines become increasingly hyperosmotic. While at 15 and 25 °C there was no overall significant differences in haemolymph osmolarity between *C. taeniatus* and *C. virescens* over the range of salinities tested (two-way ANOVA; 15 °C; $F_{1,117}=0.781$, $P>0.05$; 25 °C; $F_{1,76}=99.830$, $P>0.05$), at 35 °C there was a slight difference, the mean haemolymph osmolarity of *C. taeniatus* being higher (two-way ANOVA, $F_{1,113}=21.439$, $P=0.044$).

3.3. Volume changes in low salinity

At all of the experimental temperatures both *C. taeniatus* and *C. virescens* showed significant differences in the change of tissue wet weight when exposed to different salinities (Fig. 3, Model I, three-way ANOVA, $F_{3,116}=114.3$, $P<0.05$). However, there was no overall differences between species. Volume changes were highest for both *C. taeniatus* and *C. virescens* in the most dilute medium (8‰) at all three temperatures, while volume changed least when crabs were tested at the acclimation salinity (36‰).

3.4. Ionic regulation in low salinity

The haemolymph concentrations of Na^+, K^+, Ca^{++} and Mg^{++}, analysed by ICPES, in dying and surviving crabs followed similar trends in both species over time at 15 and 35 °C. In addition, we found that haemolymph samples taken from *C. taeniatus* demonstrated little or no difference in ionic concentration between dying and surviving crabs (Fig. 4A, Fig. 5A, Fig. 6A and Fig. 7A).

In both species exposure to 8‰ at both 15 and 35 °C resulted in a sharp reduction in haemolymph Na^+ concentration to near, or below, ambient. At 15 °C in both species and at 35 °C for *C. taeniatus*, the concentration of haemolymph Na^+ remained virtually the same as ambient until the end of the experiment (Fig. 4A and B). At 15 °C the concentration of this ion in haemolymph from *C. taeniatus* that were still alive at the end of the experiment was significantly lower than ambient Na^+ concentration (one-way ANOVA, $F_{1,4}=16.915$, $P<0.05$) (Fig. 4A). By the end of 48 h in 15 °C, all *C. virescens* in the experiment were dying and the haemolymph Na^+ concentration in these individuals remained below that of the water, although not significantly so (one-way ANOVA, $F_{1,7}=2.594$, $P>0.05$) (Fig. 4B).

Fig. 5A and B show the changes in K^+ concentration for *C. taeniatus* and *C. virescens*, respectively, over 72 h when subjected to 8‰ at 15 °C and over 36 h at 35 °C. There is a clear tendency

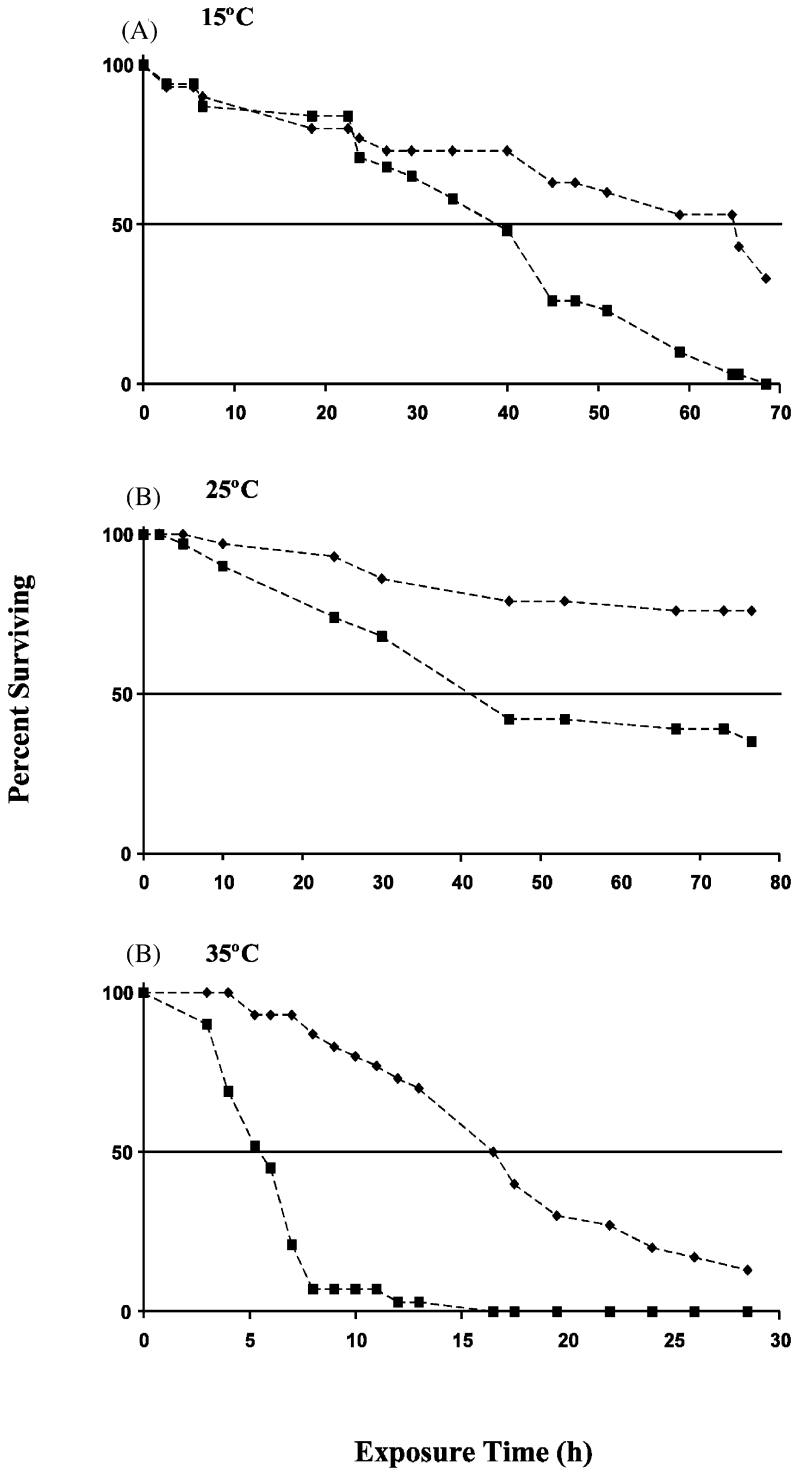

Fig. 1. Comparison of survival between *C. taeniatus* (♦) ($n=30$) and *C. virescens* (■) ($n=30$) exposed to 8‰ medium A: at 15 °C over 68.5 h, B: at 25 °C over 76.5 h and C: at 35 °C over 28.5 h.

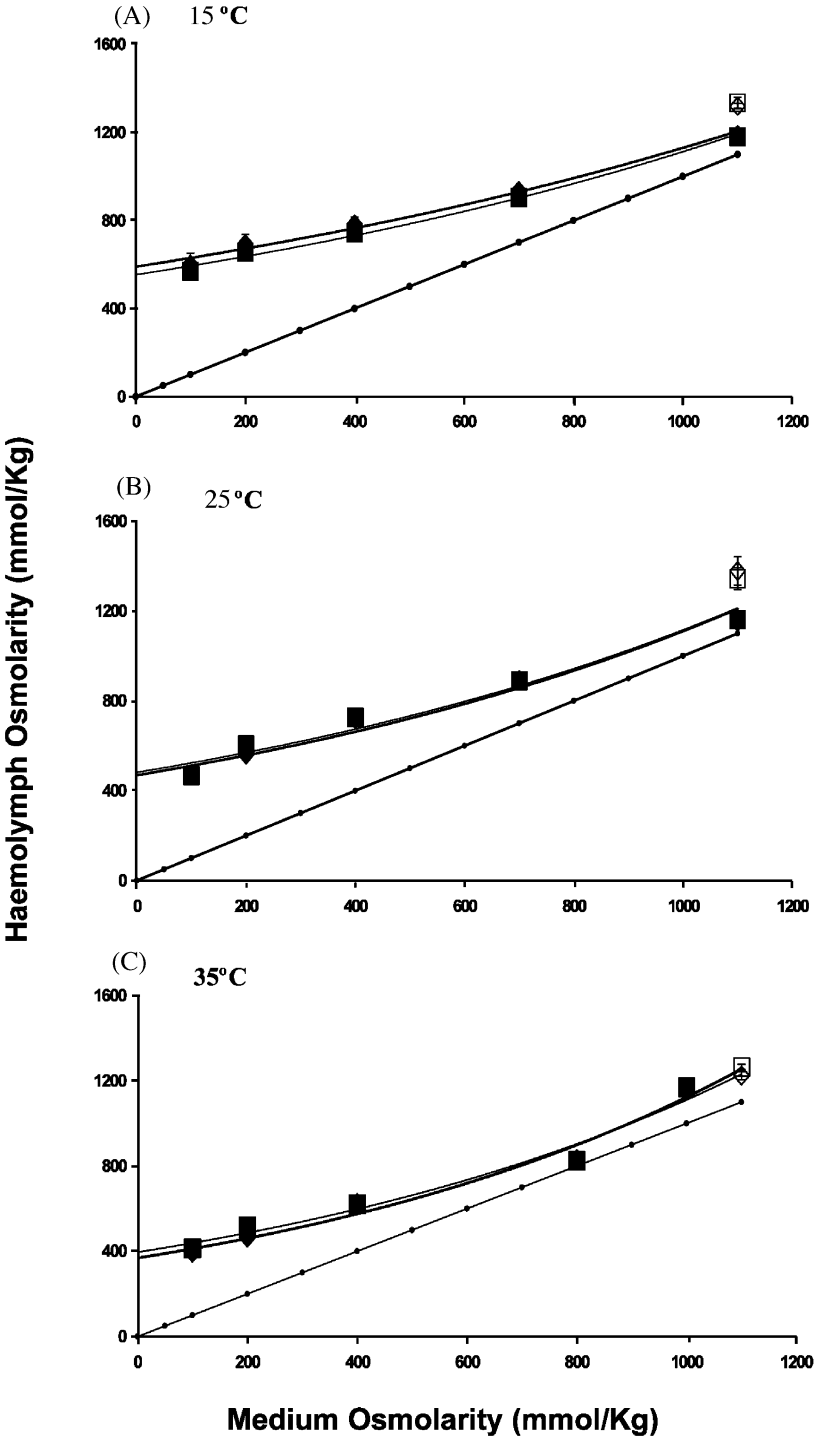

Fig. 2. Comparison of *C. taeniatus* (♦, dark line) and *C. virescens* (■, light line) haemolymph osmolarity in salinities from 4 to 36‰ at A: 15 °C, B: 25 °C and C: 35 °C in relation to an isosmotic line. Values for initial *C. taeniatus* (◇) and *C. virescens* (□) haemolymph osmolarity are presented.

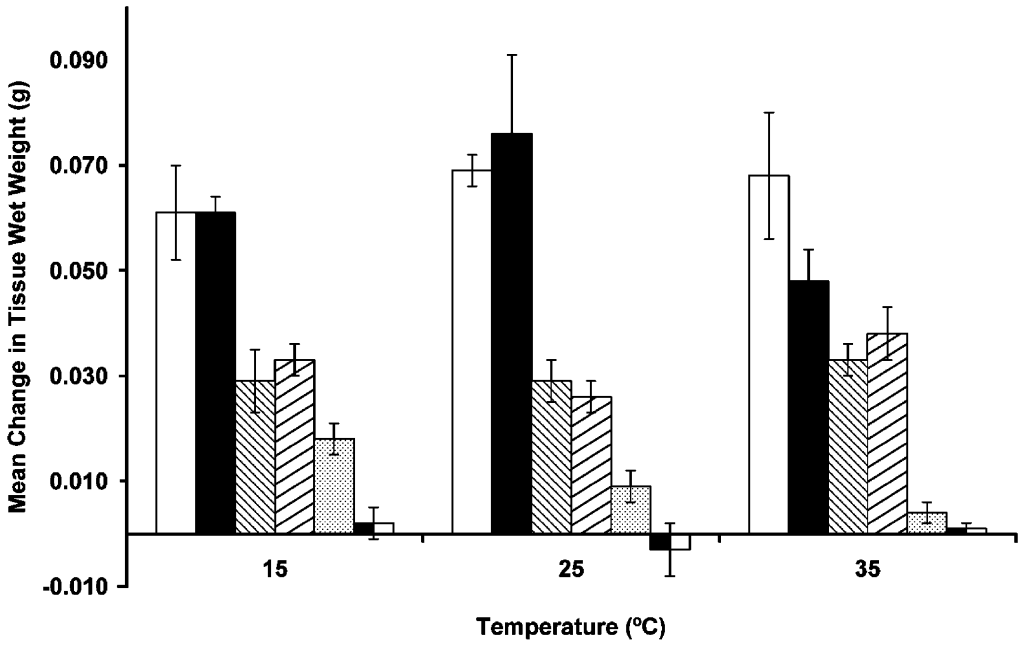

Fig. 3. Comparison of mean changes in tissue wet weight between *C. taeniatus* (light fill) and *C. virescens* (dark fill) in combinations of salinity and temperature. □■ = 8‰ SW; ▨ ▨ = 20‰ SW; ▨ ◧ = 36‰ SW. Bars represent 1 standard error.

for both species to maintain haemolymph K^+ concentration above ambient at both temperatures.

Fig. 6A and B show the concentration of Ca^{++} in the haemolymph of *C. taeniatus* and *C. virescens* in 8‰ at 15 and 35 °C. In both species the haemolymph concentrations of Ca^{++} fell at first in both temperatures, but at 15 and 35 °C in *C. taeniatus* and at 15 °C in *C. virescens* haemolymph Ca^{++} concentrations were maintained above ambient until the end of the experiment.

When the haemolymph concentrations of Mg^{++} were compared between species it was seen that the concentration in the haemolymph of both species dropped sharply at both temperatures. At 15 and 35 °C in *C. taeniatus* and at 15 °C in *C. virescens*, haemolymph concentration of Mg^{++} then remained essentially no different from ambient (Fig. 7A and B).

Two unexpected results occurred at 35 °C. In both *C. taeniatus* and *C. virescens*, the initial concentrations of Na^+ and Mg^{++} were much lower at 35 °C than at 15 °C, although throughout the remainder of the exposure at 35 °C the concentrations of these ions followed the same general trend as at 15 °C (Figs. 4 and 7). We are unaware of any discrepancies in our methods and are unsure why initial concentrations of these ions were low. A second exception was that in *C. virescens* at 35 °C, the haemolymph concentration of K^+ steadily increased from initial measurements equivalent to that in the acclimation medium, to a significantly higher concentration (one-way ANOVA, $F_{1,14} = 14.131$, $P < 0.01$) after 8 h (Fig. 5B). We cannot be certain of the accuracy of the final point for haemolymph concentration of K^+ for *C. virescens* at 35 °C, since we were able to obtain measurements from only a single sample.

3.5. Oxygen consumption

The results of shell-only controls showed negligible oxygen consumption which, when subtracted from the oxygen consumption of shelled hermit crabs, had no effect on the trends reported. Mean mass-specific oxygen consumption rates for *C. virescens* and *C. taeniatus* at different temperatures and salinities are presented as $\mu l\ O_2/h/g$ in Table

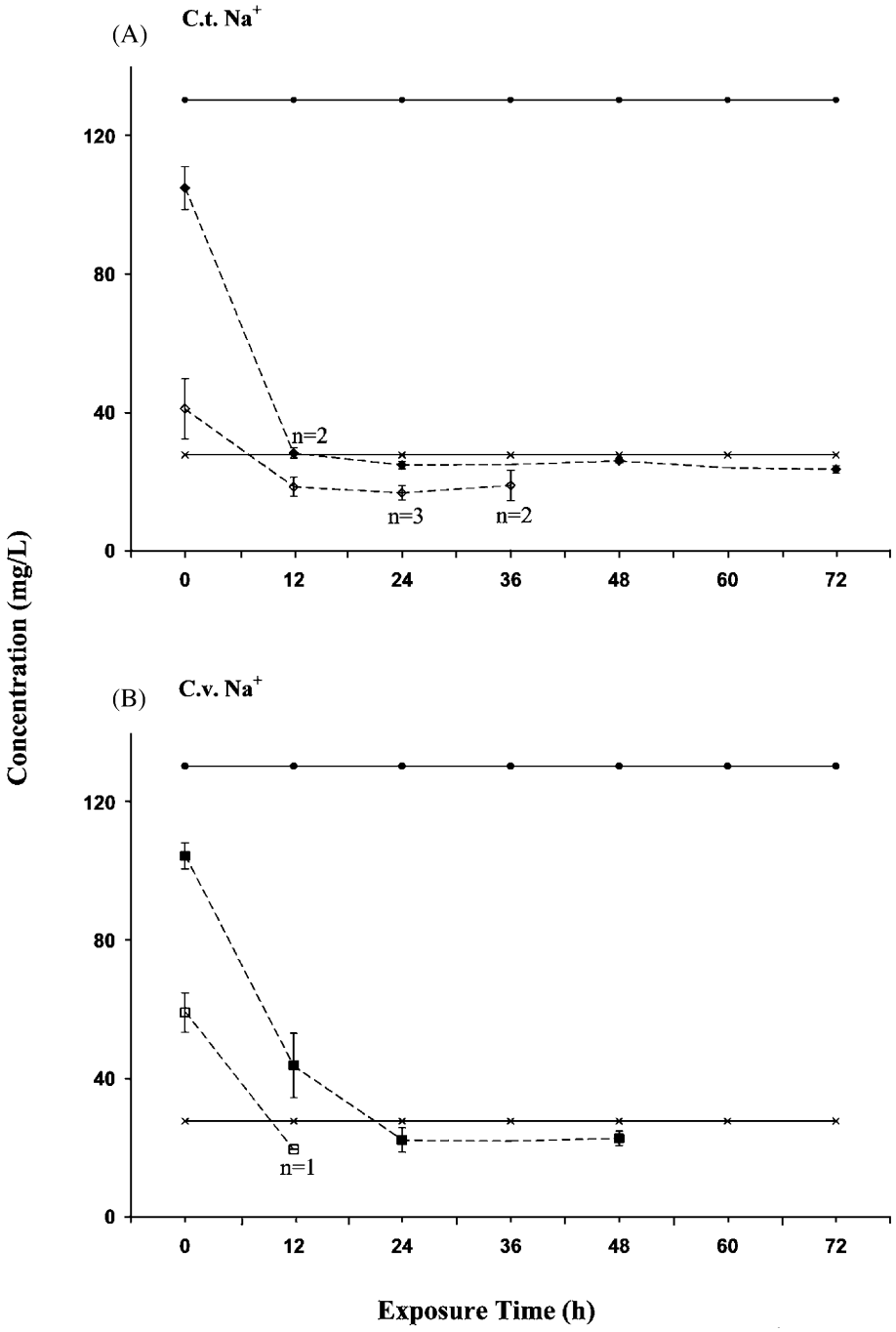

Fig. 4. Haemolymph sodium concentration in A: *C. taeniatus* (♦) and B: *C. virescens* (■) exposed to 8‰ SW at 15 °C (solid) and 35 °C (open) for up to 72 h. The concentration of sodium in 36‰ (●) and 8‰ SW (x) are given for comparison. Bars represent 1 standard error. Each point represents the mean of four replicates, except where indicated. Initial points represent live hermit crabs, as do the final points for *C. taeniatus*.

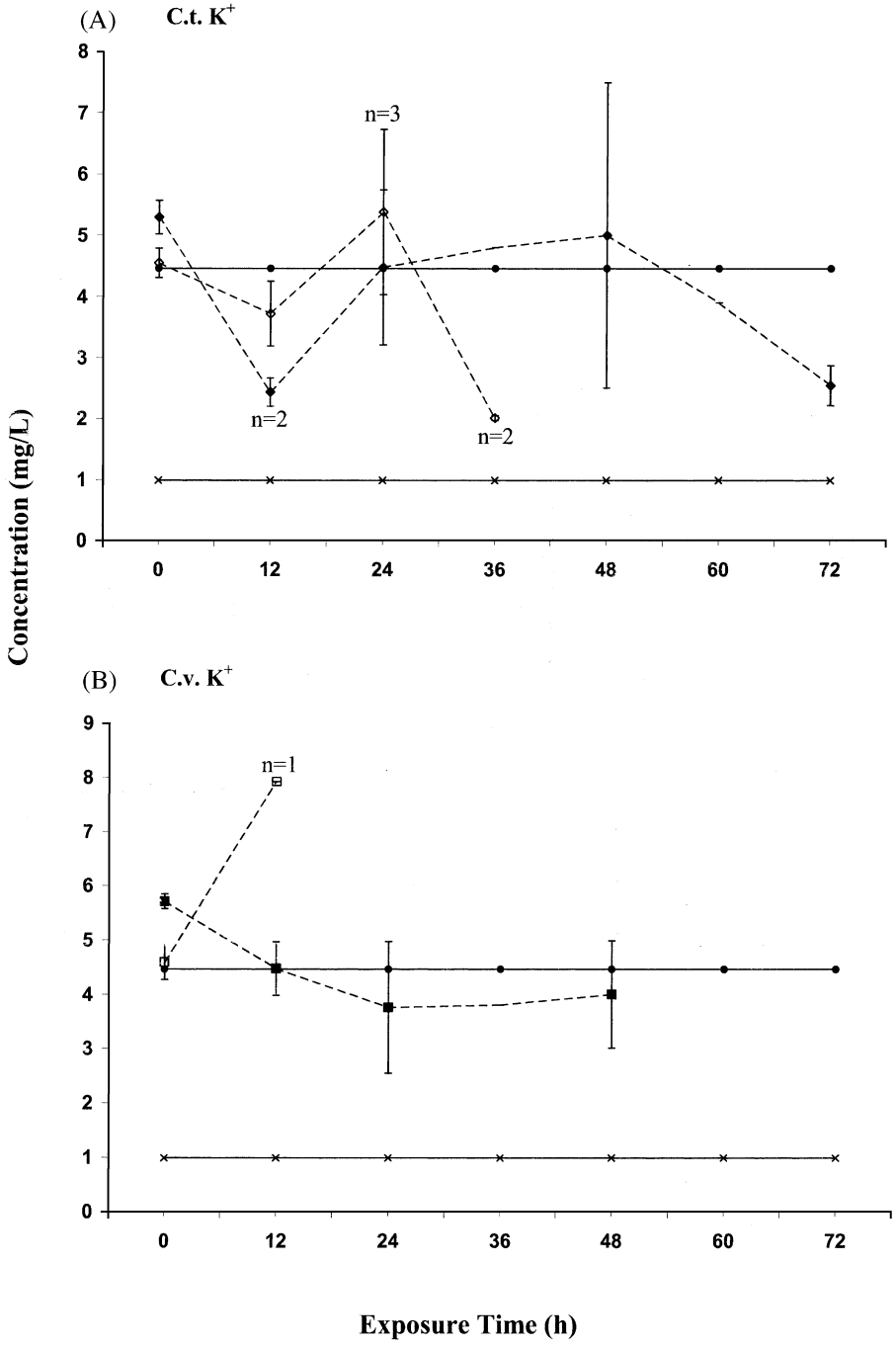

Fig. 5. Haemolymph potassium concentration in A: *C. taeniatus* (♦) and B: *C. virescens* (■) exposed to 8‰ SW (x) at 15 °C (solid) and 35 °C (open) for up to 72 h. The concentration of potassium in 36‰ (●) and 8‰ SW (x) are given for comparison. Bars represent 1 standard error. Each point represents the mean of four replicates, except where indicated. Initial points represent live hermit crabs, as do the final points for *C. taeniatus*.

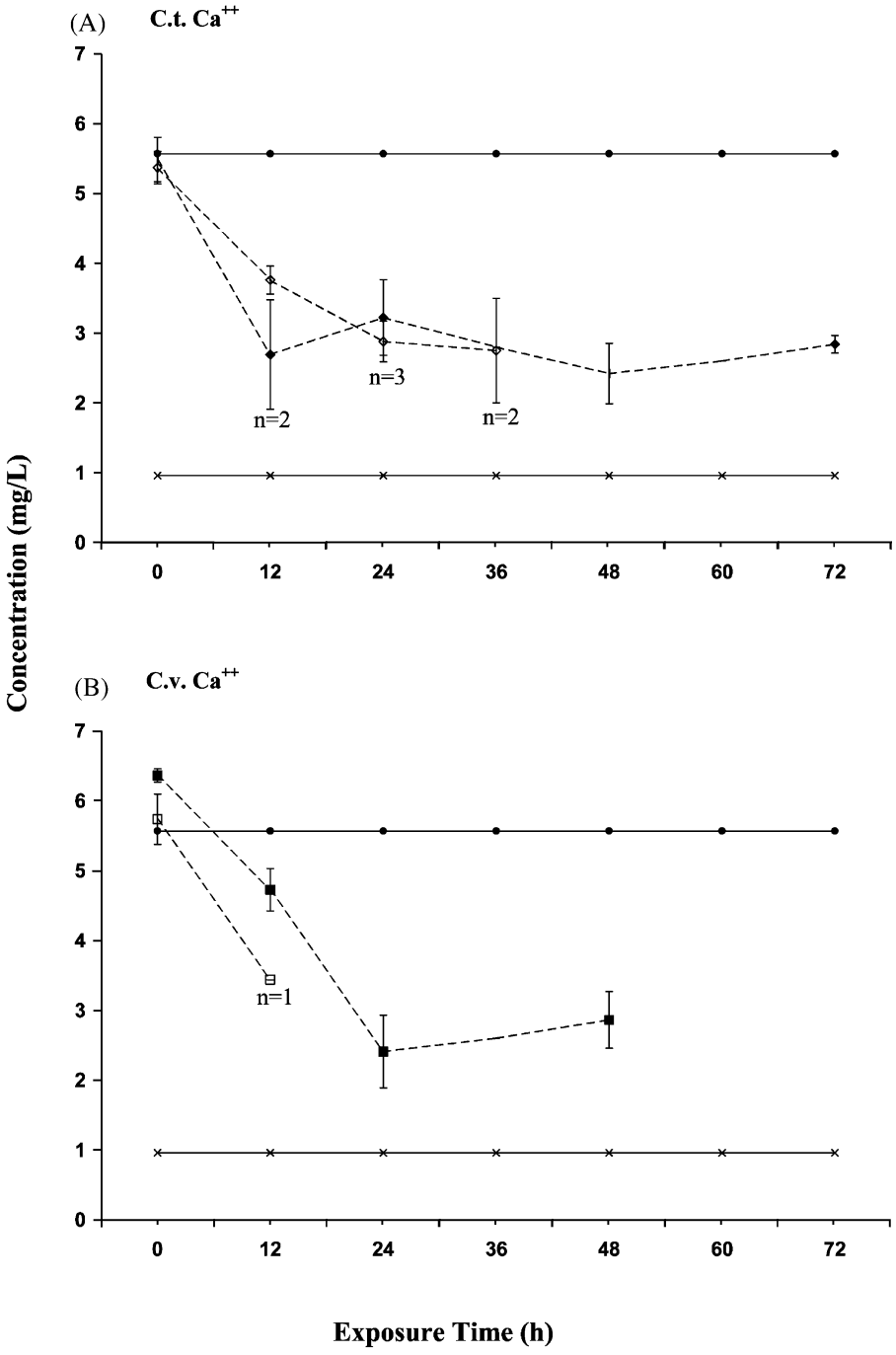

Fig. 6. Haemolymph calcium concentration in A: *C. taeniatus* (♦) and B: *C. virescens* (■) exposed to 8‰ SW (x) at 15 °C (solid) and 35 °C (open) for up to 72 h. The concentration of calcium in 36‰ (●) and 8‰ SW (x) are given for comparison. Bars represent 1 standard error. Each point represents the mean of four replicates, except where indicated. Initial points represent live hermit crabs, as do the final points for *C. taeniatus*.

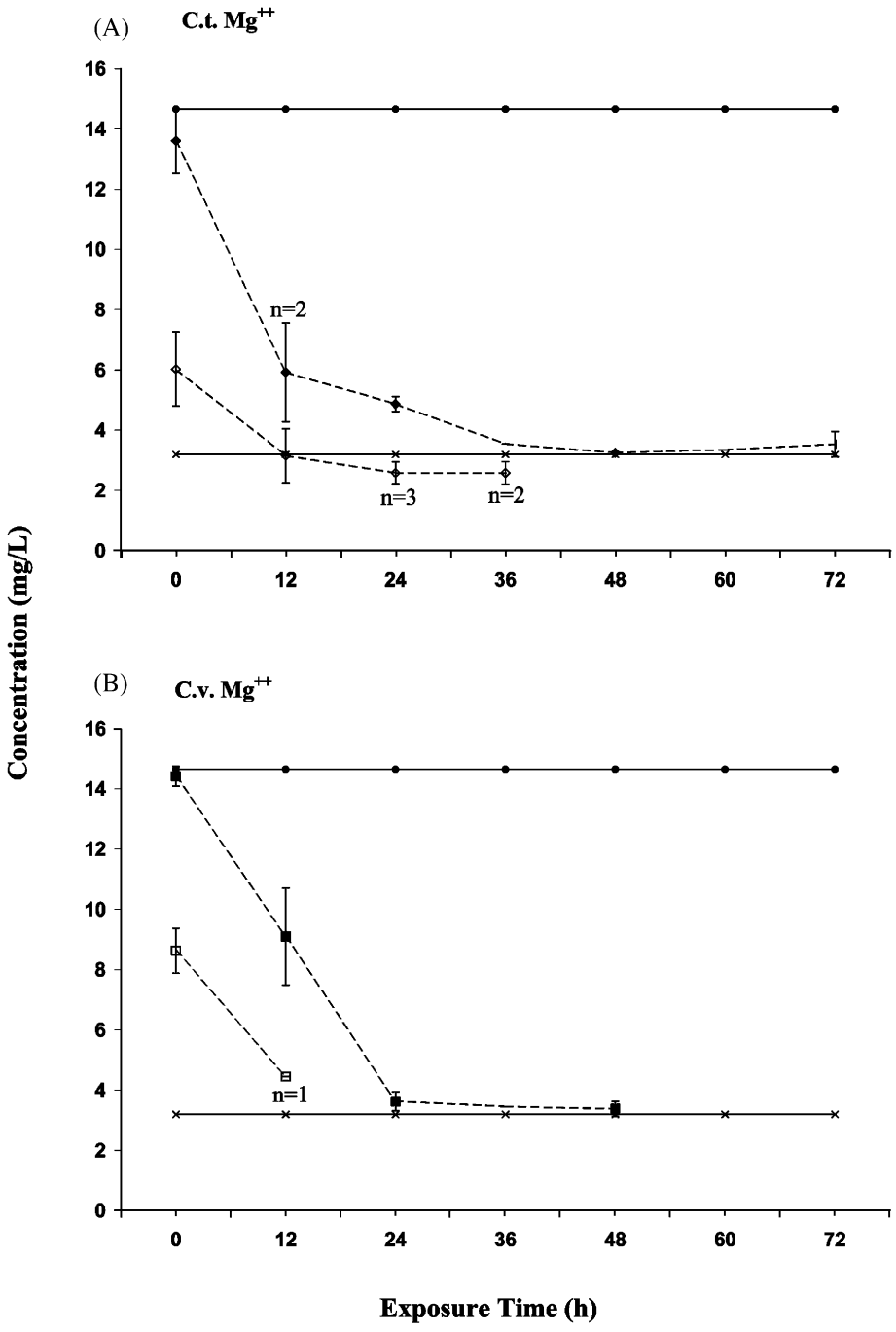

Fig. 7. Haemolymph magnesium concentration in A: *C. taeniatus* (♦) and B: *C. virescens* (■) exposed to 8‰ SW (x) at 15 °C (solid) and 35 °C (open) for up to 72 h. The concentration of magnesium in 36‰ (●) and 8‰ SW (x) are given for comparison. Bars represent 1 standard error. Each point represents the mean of four replicates, except where indicated. Initial points represent live hermit crabs, as do the final points for *C. taeniatus*.

Table 1
Comparison of mean oxygen consumption (μl O_2/h/g) between *C. taeniatus* and *C. virescens* in 8, 20 and 36‰ SW at 15, 25 and 35 °C

	Salinity (‰)		
	8	20	36
C. taeniatus			
15 °C	47.2±12	151.6±13	169.9±32
25 °C	244.7±26	236.0±32	190.9±39
35 °C	230.2±28	242.2±26	191.1±16
C. virescens			
15 °C	57.9±12	165.3±17	122.1±29
25 °C	148.0±30	263.1±64	237.5±27
35 °C	236.3±60	313.0±19	305.0±22

Means are reported with ± standard error.

1. Results of Model I, three-way ANOVA for oxygen consumption data corrected for shell controls showed that there was no significant difference between species ($F_{1,55}=1.198$, $P>0.05$), but salinity and temperature both had a significant effect (salinity: $F_{2,55}=7.327$, $P<0.005$; temperature: $F_{2,55}=30.560$, $P<0.001$) on oxygen consumption. There were no interactions between independent factors.

Results of pairwise a posteriori comparisons showed that at 15 °C the oxygen consumption of *C. taeniatus* in 8‰ was significantly lower than in 20 and 36‰ (Table 1). At temperatures of 25 and 35 °C *C. taeniatus* showed no significant difference in oxygen consumption between salinities tested. In contrast, oxygen consumption in *C. virescens* appeared lower in 8‰ compared with both 20 and 36‰ at all three temperatures, although these differences were not significant.

In general, oxygen consumption of *C. virescens* was higher than that of *C. taeniatus*, except in 8‰ at 25 °C (*C. taeniatus*: 244.7±26 μl O_2/h/g; *C. virescens*: 148.0±30 μl O_2/h/g) and in 36‰ at 15 °C (*C. taeniatus*: 169.9±32 μl O_2/h/g; *C. virescens*: 122±29 μl O_2/h/g).

Oxygen consumption was compared between temperatures in each experimental salinity (Table 2). Between 25 and 35 °C, there were no significant differences in oxygen consumption for either species of crab in any of the salinities tested. For *C. taeniatus* oxygen consumption was significantly lower at 15 °C than at either 25 or 35 °C in 8‰. For *C. virescens*, there were no significant differences in oxygen consumption between 15 and 25 °C in 8 and 20‰ water. However, in the acclimation salinity, oxygen consumption at 15 °C was significantly less than at 25 °C (Table 2). Oxygen consumption by *C. virescens* was significantly lower at 15 °C, compared with 35 °C in all three salinities.

4. Discussion

4.1. Survival in low salinity

Results of laboratory experiments on the survival of both species in a constant medium of 8‰ at three different temperatures demonstrated a significant difference in survival between species. These results suggest that *C. taeniatus* is able to tolerate long-term exposure to low salinity water significantly better than *C. virescens*. Dunbar et al. (2003) also demonstrated significantly better survival of *C. taeniatus* over *C. virescens* when both species were translocated to an estuarine environment and exposed to tidal salinity ranges between 7 and 12‰ for up to 48 h. Furthermore, in the present study, survival of both species was reduced at high and low temperatures. Biggs and McDermott (1973) and Young (1991) also found that temperature stress reduced tolerance to low salinity in the hermit crab *P. longicarpus*. These results are consistent with field experiments by Dunbar (2001) showing that *C. taeniatus* survives low salinity conditions better than *C. virescens*, and with the geographical distributions of the two species. *C. taeniatus* is able to tolerate prolonged seawater dilution, as may occur during tropical rainstorms or flood events, and thus can inhabit and survive high shore conditions. Indeed, Dunbar (2001) and Dunbar et al. (2003) have reported

Table 2
Results of Tukey pairwise comparisons of mean oxygen consumption (μl O_2/h/g) of *C. taeniatus* and *C. virescens* between treatment temperatures (°C) at 8, 20 and 36‰ SW

	Salinity (‰)	Pairwise comparisons of temperature (°C)		
		15–25	25–35	15–35
C.t.	8	*	N.S.	*
C.t.	20	N.S.	N.S.	N.S.
C.t.	36	N.S	N.S.	N.S.
C.v.	8	N.S.	N.S.	*
C.v.	20	N.S.	N.S.	*
C.v.	36	*	N.S.	*

*: $P\leqslant 0.05$, N.S.: $P>0.05$

that *C. taeniatus* is found in higher relative abundances than *C. virescens* where habitats are influenced by freshwater.

When closely related species, such as *C. taeniatus* and *C. virescens*, tolerate osmotic stress differently, particularly when coupled with temperature stress, it is important to ask whether this is based on differences in osmoregulatory ability or some other mechanism.

4.2. Osmoregulation in low salinity

No marked differences in osmoregulation were observed between *C. taeniatus* and *C. virescens*. Both species were equally capable of maintaining hyperosmotic haemolymph relative to the medium throughout the range of salinities tested. Both *C. taeniatus* and *C. virescens* maintained haemolymph osmolarity well above the osmolarity of the medium in 4–36‰, and especially below 25‰. However, Castillo et al. (1988) found that another species of *Clibanarius*, *C. erythropus*, was not a good osmoregulator in dilute seawater. Castillo et al. (1988) found this species able to survive salinities between 23 and 78‰, however in 5‰, mortality reached 100% within 72 h. Nevertheless, Castillo et al. (1988) worked with shell-less hermit crabs and the osmoregulatory ability of shell-less hermit crabs is probably compromised (Shumway, 1978). We view experiments with shell-less hermit crabs as unrealistic from the perspective of ecological physiology.

Osmoregulation is correlated with tolerance of diluted seawater in other crustaceans and/or gill preparations (Davenport, 1972; Jury et al., 1994; Piller et al., 1995; Castilho et al., 2001). The intertidal hermit crab, *C. vittattus* was much more tolerant of diluted seawater than the subtidal species, *P. longicarpus* and *P. pollicaris* (Young, 1979) and appeared to be less affected by the interaction of temperature and osmolarity stresses (Young, 1980). *C. vittattus* is an osmoregulator while *Pagurus* spp. are osmoconformers (Young, 1979, 1980, 1991), and this has been suggested as the reason for the greater tolerance of *C. vittattus* to low salinity (Young, 1991). While *C. vittattus* belongs to the superfamily Coenobitoidea, the *Pagurus* spp. belongs to the Paguroidae. However, results reported by Castillo et al. (1988) for shell-less *C. erythropus* are at least suggestive that a mechanism other than osmoregulation is involved in the tolerance of a hermit crab to diluted seawater. Similarly, the greater tolerance of diluted seawater by juvenile, compared with adult female red king crabs did not appear to be based on osmoregulatory ability (Thomas and Rice, 1992). Instead, these authors suggested that significantly better volume regulation of juveniles over adults could possibly explain differences in survival when exposed to both gradual and acute reductions in salinity.

For both *C. taeniatus* and *C. virescens*, the ability to hyperosmoregulate declined as temperature increased, particularly in more dilute seawater (Fig. 3A–C). Therefore, although low salinities and high temperatures may be stressful as independent factors, the combination of the two appears to be especially stressful and results in greater dilution of body fluids. Combined low salinity and high temperature are most likely to occur in the upper shore of tropical intertidal areas influenced by freshwater. *C. taeniatus* tends to be in greater relative abundance than *C. virescens* in both the upper intertidal shore and those intertidal areas that are influenced by freshwater (Dunbar, 2001; Dunbar et al., 2003).

4.3. Volume changes in low salinity

Volume changes in *C. taeniatus* and *C. virescens* were inversely related to the salinity of the medium. However, no overall difference existed in the abilities of these species to regulate volume as measured by changes in wet weight. Increases in volume can be explained by the osmotic gradient maintained between haemolymph and external medium by both species over the range of salinity dilutions tested.

Although Mackay and Prosser (1970) reported that adult red king crabs that survived acute exposure to dilute seawater maintained hyperosmotic haemolymph, our data showed that while *C. taeniatus* and *C. virescens* did not differ in ability to maintain hyperosmotic haemolymph, they differed significantly in tolerance to prolonged low salinity.

4.4. Ionic regulation in low salinity

Our studies on ionic regulation in *C. taeniatus* and *C. virescens* did not reveal striking differences between the two species (Figs. 4–7). For both *C. taeniatus* and *C. virescens* in diluted seawater (8‰) the haemolymph concentrations of Na^+ and Mg^{++} were quickly reduced to that of the medium

while the haemolymph concentrations of K^+ and Ca^{++} remained high; K^+ near that of undiluted seawater and Ca^{++} about midway between the concentration of Ca^{++} in 8 and 36‰ seawater (Figs. 5 and 6).

Sabourin and Stickle (1980) reported that in *C. vittatus* the haemolymph concentration of Na^+ was essentially isoionic to a medium of 30‰, while K^+ was regulated well above its concentration in the medium. These authors found that when *C. vittatus* was acclimated in 20 and 10‰ seawater, haemolymph concentrations of Na^+, Mg^{++} and K^+ were elevated relative to the water. They concluded that high concentrations of K^+ in haemolymph indicated that the concentration of this ion was maintained independently of ambient seawater concentration in low salinities.

The concentration of Ca^{++} also remained high in *C. taeniatus* and *C. virescens* haemolymph relative to ambient concentration. When Greenaway (1976) acclimated the shore crab, *Carcinus maenas* to dilute seawater, he found that this species maintained haemolymph calcium concentration significantly higher than that of the external medium, with the difference between haemolymph and medium concentrations inversely related to salinity. In media of less than 50% SW, Greenaway (1976) found a marked electrochemical gradient leading to calcium loss from the haemolymph, strongly suggesting a mechanism other than passive regulation for high Ca^{++} concentrations maintained in the haemolymph. While Greenaway (1976) did not show direct evidence for increased calcium uptake from the medium, he did suggest that active transport would be the only way to regulate haemolymph calcium concentration against an electrochemical gradient. In agreement with the findings of Greenaway (1976), Neufeld and Cameron (1992) found that the haemolymph calcium concentration of *Callinectes sapidus* in 2‰ salinity was also regulated at significantly higher concentrations than the surrounding medium. They also found that electrochemical gradients favoured the loss of calcium from the haemolymph to the medium through gill epithelia. Despite this transepithelial gradient, there was a net uptake of calcium implicating active transport of calcium from the external medium to haemolymph. There is evidence that the transport of calcium across the gill epithelium of crustaceans in dilute water involves: (i) a Na^+/Ca^{++} antiporter kept active by the Na^+ gradient maintained by Na^+/K^+ATPase and (ii) active uptake by Ca^{++}ATPase (for details, see review by Wheatly, 1997). In *Carcinus* spp. Ca^{++}ATPase has been reported to have a high affinity and capacity for Ca^{++} in 50% SW. The Na^+/Ca^{++} antiporter has also been shown to have a higher affinity for Ca^{++} in dilute, than in normal seawater and is most likely the main mechanism for basolateral movement of Ca^{++} into the haemolymph (Wheatly, 1997).

4.5. Oxygen consumption

No significant differences in whole animal oxygen consumption were found in this study between *C. taeniatus* and *C. virescens* when subjected to low salinity conditions in the laboratory. Oxygen consumption of both species was generally lower in 8 than in 20‰ at all three temperatures tested, but only significantly so for *C. taeniatus* at 15 °C (Table 1). These data are consistent with those of Shumway (1978) who reported a marked temporary increase in the oxygen consumption of *Pagurus bernhardus* with shells, upon exposure to 75% SW. However, Shumway (1978) concluded that this temporary increase was unlikely due to osmoregulatory work, since *P. bernhardus* is an osmoconformer. Rather, she speculated that it was due to increased muscular activity during escape responses to low salinity. We observed no difference in motor activity between *C. taeniatus* and *C. virescens* in 8‰ compared with 20‰ seawater.

Although osmotic regulation, volume regulation, ionic regulation and overall respiratory response did not appear to differ between species, clearly, *C. taeniatus* was able to survive prolonged exposure to dilute seawater better than *C. virescens*, at the temperatures tested. We suggest that the dilution of body fluids may have a more detrimental effect on *C. virescens* than on *C. taeniatus*. We do not dismiss the idea that there may be alternative explanations for differences in survival between these species. For example, *C. taeniatus* may be able to recruit stress response proteins that protect cell membrane structures (Somero, 1995; Willmer et al., 2000) better than *C. virescens*. However, we suggest that even this would be a mechanism for tolerating body fluid dilution. We have also considered that tolerance of diluted body fluids may be due to intracellular regulation, perhaps based on amino acid concentrations (Gilles and Pequeux, 1983). Nevertheless, we have not yet investigated either of these factors. The mecha-

nism, or mechanisms, involved in survival differences remain unknown. In any case, our work indicates that between closely related crustaceans, physiological mechanisms other than ionic, osmotic or volumetric regulation may exist to produce differences in tolerance to diluted seawater. An interesting area of future research would be the identification and study of such mechanisms.

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