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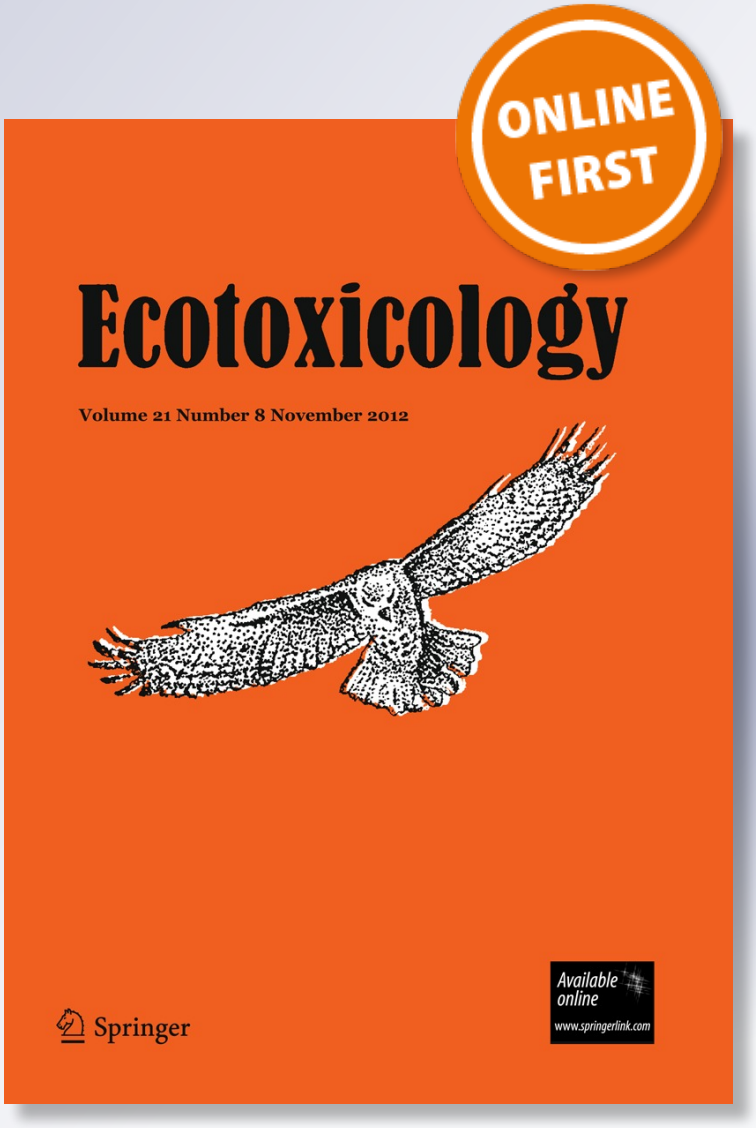

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The role of native salinity regime on grass shrimp (*Palaemonetes pugio*) sensitivity to cadmium

Andrew Y. Oguma · Paul L. Klerks

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Abstract In euryhaline crustaceans, sensitivity to toxic trace metals may be linked to osmoregulation and salinity conditions. This study investigated if grass shrimp (*Palaemonetes pugio*) populations from different salinity regimes differed in sensitivity to cadmium (Cd). Grass shrimp were collected in May 2011 from two marsh sites with average salinities of ~3.0 ppt and 24.0 ppt. Groups were acclimated for 3–32 weeks in either their respective native salinity (3.0 ppt → 3.0 ppt and 24.0 ppt → 24.0 ppt), or the average of the salinities of the two collection sites (3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt). After acclimation, groups were exposed to equivalent free-ion Cd concentration (4.8 ± 0.3 mg/L, Cd^{2+}) in their respective acclimated salinity to compare survival among salinity treatments. Results of Kaplan–Meier survival analysis indicated that 3.0 ppt → 3.0 ppt shrimp were more sensitive to Cd^{2+} than any other group ($p < 0.0001$). Additionally, 3.0 ppt → 13.5 ppt shrimp were less sensitive to Cd^{2+} than were 24.0 ppt → 13.5 ppt shrimp ($p = 0.0013$). These results suggest that sensitivity of grass shrimp to Cd is dependent upon the salinity during exposure, and the salinity regime from which the tested population originated. The implication is that toxicity studies and risk assessments using euryhaline crustaceans should consider the salinity of test population collection sites when interpreting and comparing results.

Keywords Palaemonetes · Osmoregulation · Cadmium · Salinity · Free-ion concentration

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Introduction

Many crustacean species of coastal marshes are adapted to persist throughout a gradient of fresh to euhaline waters. Osmoregulating crustaceans such as the grass shrimp, *Palaemonetes pugio*, maintain relatively stable hemolymph ion concentrations throughout this gradient (Kirby and Knowlton 1976; Knowlton and Kirby 1984; Rowe 2002). Euryhaline organisms generally achieve this by actively taking up water and excreting ions in hyperosmotic conditions to compensate for osmotic water loss; in hyposmotic conditions, they produce dilute urine to compensate for inward osmosis of water and actively take up ions to compensate for those lost in urine (Potts and Parry 1964; Rankin and Davenport 1981; Krogh 1965). Since uptake mechanisms of toxic metal ions, such as the divalent cadmium ion (Cd^{2+}), can use the same biochemical pathways as essential ions such as the divalent calcium ion (Ca^{2+}), the salinity at which a euryhaline crustacean is exposed may affect metal toxicity (Burke et al. 2003). Individuals from areas with different salinities may also differ genetically with respect to their ability to handle stressful salinities, as has been reported for coastal fish (Purcell et al. 2008), which in turn could affect metal tolerance due to the potential for uptake mechanisms being shared between iono- and metal-regulation (Pedersen and Bjerregaard 1995). Thus, the main objective of this study was to determine whether differences in sensitivity to metal toxicity exist between two populations of grass shrimp from environments that differ in mean annual salinities.

Palaemonetes pugio is an abundant estuarine omnivore that plays an important role in the ecology of coastal marshes from Nova Scotia to Texas (Vernberg and Piyatiratitivorakul 1996; Welsh 1975). The physiology, bioenergetics and ecological implications of osmoregulation

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in *P. pugio* are well studied (Knowlton and Kirby 1984; Vernberg and Piyatiratitivorakul 1996; Manyin and Rowe 2009; Rowe 2002; Alon and Stancyk 1982; McKenney and Neff 1979; Sunda et al. 1978; Welsh 1975). These grass shrimp are able to maintain their hemolymph salinity at 12 ppt in ambient salinities ranging from 1 to 35 ppt (Rowe 2002; Knowlton and Kirby 1984; Kirby and Knowlton 1976). Although differences in metal sensitivity when exposed in different salinities have been demonstrated in *P. pugio* (Sunda et al. 1978), the salinity regime of the habitat from which the study population was collected was not considered.

Cadmium is a toxic, non-essential metal and a common contaminant in coastal habitats (Manyin and Rowe 2009). Major anthropogenic sources include non-ferrous metal smelting and refining, domestic wastewater, chemical manufacturing, fossil fuel combustion and waste incineration (Nriagu and Pacyna 1988). Dependent on the chemical species present, Cd can form a variety of complexes in solution. For example, when Cd is present in seawater it forms a variety of chloride (Cl^-) complexes that vary in abundance with salinity. Since the Cd^{2+} form is the main bioavailable form in waterborne exposures for crustaceans, Cd toxicity is generally inversely related with salinity due to the relative increase of Cd^{2+} concentration ($[Cd^{2+}]$) at lower salinity (Blust et al. 1992; Burke et al. 2003; DeLisle and Roberts 1988; Roast et al. 2001b; Wright 1977; Zanders and Rojas 1996; Sunda et al. 1978; Barbieri and Paes 2011). This makes it difficult, when determining toxicity of Cd at different salinities, to separate the effect of salinity on biological processes from the effect of salinity on Cd chemical speciation (e.g. Guerin and Stickle 1995). One solution, used in the current study and elsewhere, is to use a fixed $[Cd^{2+}]$ across a range of salinity treatments (Burke et al. 2003; DeLisle and Roberts 1988; Roast et al. 2001a). By this method, the effects of salinity on Cd toxicity to the organism, rather than on the chemical speciation of the metal, can be tested.

To examine the effect of native salinity on *P. pugio* sensitivity to Cd, a static time-to-death (TTD) exposure design was used. This approach allows for the comparison of time-dependent event data among different treatment groups, while accounting for potential effects of covariates (Newman and Aplin 1992). Thus, the TTD test was well suited for this study's comparison of Cd-sensitivity among shrimp differing in their history of salinity exposure. Shrimp were collected from two sites of differing mean annual salinities, acclimated in water of either their native salinity or the mean salinity of the two sites, and then exposed to a lethal $[Cd^{2+}]$ at each of the acclimation salinities. Acclimating shrimp collected from each site to the mean salinity of the two sites prior to exposure, and then exposing shrimp to equivalent lethal $[Cd^{2+}]$ in that salinity, allowed us to examine the effect of native salinity on Cd sensitivity. The null hypothesis tested was that grass shrimp survival time during lethal Cd exposure did not differ between shrimp from two coastal marsh habitats with different salinity regimes. To further investigate the effect of salinity during Cd exposure, grass shrimp were also exposed to equivalent fixed $[Cd^{2+}]$ in their native salinities. In order to gain some insight into the cause of inter-population differences in tolerance (genetic adaptation vs. phenotypic plasticity), exposures were repeated after shrimp had been maintained in a common environment for an additional 29 weeks.

Materials and methods

Shrimp collection and salinity acclimation

At the University of Louisiana at Lafayette Biology Department's aquatic lab, 1 week prior to shrimp collection, four glass aquaria were fitted with under-gravel filters and gravel substrate, and filled with 90 L of water at salinities of 3.0 ppt, 13.5 ppt, and 24.0 ppt. The salinity of 3.0 ppt was chosen as it approximates the average annual salinity as measured from August 2008 to May 2011 by the United States Geological Survey (USGS) Coastwide Reference Monitoring System (CRMS) at site 0662 (USGS 2010). This monitoring station was located near the low-salinity collection site (Fig. 1). The salinity of 24.0 ppt was chosen on the basis of this being the average of all direct measurements taken at the high-salinity collection site during the spring and summer of both 2010 and 2011 (Fig. 1) in the absence of any nearby monitoring station. The third salinity, 13.5 ppt, was the average of the low- and high-salinities. All aquarium water was prepared by dilution of filtered seawater (0.5 μm mesh, Galveston Bay, TX) with deionized water, and salinities were checked daily with an YSI® model 30 meter (Yellow Springs, OH, USA). When necessary, evaporated water was replaced with deionized water to maintain salinity and volume.

Grass shrimp were collected by dip net from vegetated margins of open water pools in low- and high-salinity marshes in the Chenier Plain near Sabine Lake in southwest Louisiana (30°2.812′N, 93°42.471′W and 29°43.579′N, 93°48.953 W respectively, Fig. 1) on 5 and 6 May, 2011 respectively. The marshes near Sabine Lake were selected as shrimp collection sites because the location and hydrology of the lake provides marsh habitat for grass shrimp through a large range of their salinity tolerance. Classification of these areas as oligohaline (low-salinity) or polyhaline (high-salinity) marshes was based on vegetation type (Visser et al. 2000; USGS 2010) in addition to salinity data mentioned earlier. Netted shrimp were sorted on site to remove non-target species such as fish, crabs and

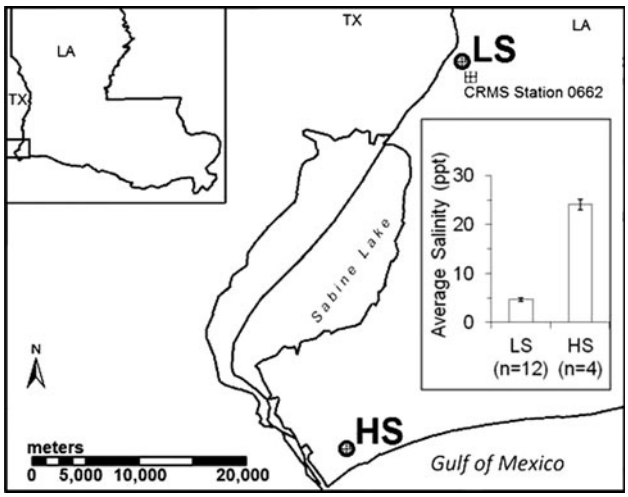

Fig. 1 Collection sites for grass shrimp of low- (LS) and high-salinity (HS) habitat types. Sites were over 48 km apart by water (37.2 km straight linear distance). The *inset* graph shows mean salinities at each site with LS site data being from a monitoring station (12 measurements taken August 2008–May 2011) and HS site data from 4 field measurements using a YSI meter (2 in October 2010, 1 in April 2011 and 1 in May 2011)

penaeid shrimp. Shrimp from each site were immediately placed in separate 45.4 L (48 qt Rubbermaid®) aerated coolers with ~40 L of ambient water. An excess of 180 grass shrimp were collected from each site, and transported in these coolers to the prepared aquaria within 36 h of collection. An excess of 90 shrimp from the low-salinity site were placed in either a 3.0 ppt salinity tank (3.0 ppt → 3.0 ppt) or a 13.5 ppt salinity tank (3.0 ppt → 13.5 ppt). Similarly, an excess of 90 shrimp from the high-salinity site were placed into each of the tanks with a salinity of 24.0 ppt (24.0 ppt → 24.0 ppt) and 13.5 ppt salinity (24.0 ppt → 13.5 ppt). *Palaemonetes pugio* have been shown to physiologically acclimate to extreme fluctuations in salinity within 8 h as shown by monitoring respiration during acclimation (Rowe 2002). Therefore, the transition from either native salinity (3.0 ppt or 24.0 ppt) to the mean salinity (13.5 ppt) was not likely to cause irreversible physiological damage to the shrimp. Grass shrimp were held in aquaria with a 14 h photoperiod, at 20 (±0.09) °C, and fed Tetramin® flakes ad libitum in equal portions to all tanks, for 20 days prior to the first cadmium exposure experiment, and between subsequent exposures. Two male and two female shrimp from each holding tank were placed in vials with tank water, anesthetized by brief placement in a freezer at 0 °C, fixed with 10 % neutral buffered formalin, and preserved in 80 % ethanol. These specimens were identified as *Palaemonetes pugio* using a taxonomic key (Williams 1984), and archived should further taxonomic confirmation be desired.

Collection site cadmium analysis

In order to ascertain that ambient Cd levels did not differ at shrimp collection sites, water samples were collected at each site. Ambient water samples were collected in brown, acid-washed, 25 mL Nalgene® bottles near each collection area, but away from any disturbed sediment. Field blanks were also prepared on site in identical bottles using nano-pure water (Barnstead water purifier model D11901, Thermo Scientific, Dubuque, IA, USA) for quality assurance/quality control. Analysis of [Cd] in water samples was conducted using a Perkin Elmer model 1100B atomic absorption spectrophotometer with acetylene flame ionization. Standard curves were generated using a certified Cd reference standard solution (Fisher Scientific, Fair Lawn, NJ, USA). Results indicated that total [Cd] of field water samples taken at each site were below the detection limit of 0.001 mg/L, as were field and equipment blanks.

Cadmium exposures

A static TTD test was conducted to compare the Cd^{2+} sensitivity of the shrimp from the low- and high-salinity sites, acclimated to previously described salinity treatments (3.0 → 3.0, 3.0 ppt → 13.5 ppt, 24.0 ppt → 24.0, and 24.0 ppt → 13.5 ppt). An initial range-finding experiment conducted with a subset of shrimp from the 3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt treatments, revealed that a concentration just above 4 mg/L [Cd^{2+}] was well suited in order to observe 100 % mortality, and variation in survival time, within a 24 h exposure period. The range-finding experiment tested shrimp mortality rate in the range of 0.25–4.00 mg/L [Cd^{2+}]. Total Cd concentrations required to achieve equivalent [Cd^{2+}] in all three salinities were calculated using chemical equilibrium modeling software (Visual MINTEQ version 3.0) (Gustafsson 2010). Similar software has been used in previous studies in which fixed free-ion metal levels were required (Burke et al. 2003; DeLisle and Roberts 1988; Blust et al. 1992), and one of these verified the [Cd^{2+}] outputs of such software using a Cd ion-selective electrode (Blust et al. 1992). Shrimp were exposed in Cd-spiked solutions prepared from anhydrous $CdCl_2$ (ACS certified, CAS 10106-64-2, Fisher Scientific, Fair Lawn, NJ, USA) in two 2.5 L batches for each treatment, at each respective salinity (3.0 ppt, 13.5 ppt, and 24.0 ppt). Subsamples from each batch were taken for analysis of total [Cd]. Analysis of [Cd] in test solutions was conducted using flame atomic absorption spectrophotometry similarly to the methods used in analysis of field samples. All glassware and containers used in preparation of exposure solutions and subsampling had been acid washed, and equipment blanks

were prepared as a measure of quality assurance in all atomic absorption analyses.

For experiment 1, 18–20 similar sized, non-brooding, shrimp from each holding tank were individually exposed in food-grade plastic cups with 250 mL of a 5 mg/L Cd^{2+} solution at the same salinities to which the shrimp had been acclimated. Cups were systematically interspersed to avoid the potential confounding effects of environmental gradients (e.g., light, temperature) in the exposure area. Shrimp were transferred individually into exposure cups. Translucent plastic sheets (those designed to cover fluorescent light fixtures) were placed over the cups whenever possible to prevent escape by jumping shrimp. Sample sizes varied (18–20) due to removal of shrimp that jumped from cups during loading. Survival of shrimp was assessed every 30 min by observed movement of whole shrimp, antennae or scaphognathites, or only when necessary, by observation of any movement upon gentle prodding with a glass rod. Shrimp were considered dead if no movement was detected. Once a shrimp was determined to be dead, length (determined from tip of rostrum to tip of telson) was measured. During the exposure, temperature and light cycle were maintained as during acclimation; however, lights were turned on as necessary when checking survival.

A second static TTD test (experiment 2) was conducted to compare the sensitivity of shrimp from treatment groups 3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt with increased sample size ($n = 40$) for each group. This exposure was conducted 22 days after experiment 1 by the same methods as previously described, with one exception; to reduce variability of [Cd] among exposure solutions, all Cd exposure solutions were prepared in one (22 L) batch.

Experiment 2 was repeated (experiment 3) with the remaining shrimp (3.0 ppt → 13.5 ppt, $n = 16$; 24.0 ppt → 13.5 ppt, $n = 19$) 203 days later (225 days after experiment 1, 245 days after collection) to examine if any changes in sensitivity may have disappeared after a longer time in the common environment (in other words, being physiological rather than genetic). Additionally, shrimp from each acclimation tank (3.0 ppt → 13.5 ppt, $n = 3$; 24.0 ppt → 13.5 ppt, $n = 5$) were included as no-Cd controls to assess whether oxygen depletion in exposure cups, or other potential confounding variables, may have affected shrimp during exposure. Dissolved oxygen (DO) was measured in control cups at the beginning of the exposure and after the last exposed shrimp died.

Statistical analyses

Kaplan–Meier survival analysis (SAS PROC LIFETEST) (SAS Enterprise Guide 2006) was used to estimate survival functions which were compared among groups using the Wilcoxon test. The analysis also allowed us to test for association between the quantitative covariates of length and exposure [Cd^{2+}] (as the latter varied slightly within treatments in experiment 1), and TTD. The Kaplan–Meier method also estimates the 25, 50, and 75th percentile lethal times (LT_{25}, LT_{50}, LT_{75}) with confidence intervals (CIs) for each group. Pairwise comparisons between different groups could then be made by examining the CIs of LT_{25}s, LT_{50}s, and LT_{75}s. In all statistical hypothesis tests, alpha was set a priori at 0.05.

Results

Experiment 1

Results of Cd quantification of exposure solutions and subsequent calculations of [Cd^{2+}] using visual MINTEQ software (Gustafsson 2010) were included as covariates in analysis (Table 1). Kaplan–Meier survival analysis revealed that neither shrimp length nor within-treatment variability in exposure [Cd^{2+}] had significant associations with TTD (Wilcoxon test $X^2 = 1.2$, $p = 0.2653$ and $X^2 = 0.29$, $p = 0.5895$ respectively, $n = 75$). Additionally, we rejected the null hypothesis that survival functions did not differ among treatment groups (Fig. 2). By comparing survival function plots and LT_{50}s along with their 95 % confidence intervals, it was clear that survival time in the shrimp from the 3.0 ppt salinity marsh, acclimated and exposed in their native salinity (3.0 ppt → 3.0 ppt) was significantly shorter than that in all other treatment groups (Fig. 3). However, no differences were detected among the other groups (3.0 ppt → 13.5 ppt, 24.0 ppt → 13.5 ppt, 24.0 ppt → 24.0 ppt; Figs. 2 and 3).

Experiment 2

For this experiment, the Cd free-ion concentration was a bit higher than it was for experiment 1 (Table 1). However, the [Cd^{2+}] was not included in the analysis of the results for experiment 2 since both groups of shrimp were exposed to the exact same water. Kaplan–Meier survival analysis revealed that length was positively associated with TTD (Wilcoxon test $X^2 = 4.00$, $p = 0.0454$, $n = 80$). The analysis rejected the null hypothesis of no difference in TTD between groups 3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt (Fig. 2). Examination of Kaplan–Meier survival functions and LT_{50}s, showed that survival time was shorter in shrimp from the 24.0 ppt salinity marsh acclimated to the mean salinity (24.0 ppt → 13.5 ppt) than it was in shrimp from the 3.0 ppt salinity marsh acclimated to the mean salinity (3.0 ppt → 13.5 ppt, Fig. 3).

Table 1 Salinity, pH, total cadmium [Cd], free-ion cadmium [Cd^{2+}] concentrations (±standard error with sample sizes for chemical analyses in parentheses), and concentrations of the major ions in seawater, in each treatment solution used in experiments

Experiment	Treatment	Salinity (ppt)	pH	[Cd] (mg/L)	[Cd^{2+}] (mg/L)	Seawater solution ions (mM)					
						[Cl^-]	[Na^+]	[SO_4^-]	[Mg^{2+}]	[Ca^{2+}]	[K^+]
1	3.0 ppt → 3.0 ppt	**3.0**	**8.11**	**13.00 ± 0.83**	*4.08 ± 0.26 (n = 2)*	46.00	39.59	2.42	4.63	0.91	0.68
	3.0 ppt → 13.5 ppt	**13.5**	**7.71**	**41.00 ± 0.37**	*4.59 ± 0.04 (n = 2)*	207.01	178.13	10.91	20.83	4.10	3.08
	24.0 ppt → 13.5 ppt	**13.5**	**7.71**	**41.36 ± 0.54**	*4.63 ± 0.06 (n = 2)*	207.01	178.13	10.91	20.83	4.10	3.08
	24.0 ppt → 24.0 ppt	**24.0**	**8.01**	**72.09 ± 6.43**	*4.47 ± 0.40 (n = 2)*	368.03	316.68	19.39	37.03	7.29	5.47
2	3.0 ppt → 13.5 ppt 24.0 ppt → 13.5 ppt	**13.5**	**7.71**	**53.20 ± 0.20**	*5.90 ± 0.02 (n = 5)*	207.01	178.13	10.91	20.83	4.10	3.08
3	3.0 → 13.5 ppt 24.0 ppt → 13.5 ppt	**13.5**	**8.07**	**46.93 ± 0.55**	*5.20 ± 0.06 (n = 5)*	207.01	178.13	10.91	20.83	4.10	3.08

Seawater solution ions were estimated by using the stable relative proportions of each ion in natural seawater. Boldface values were measured directly (salinity by YSI model 30, pH by Fisher Scientific model 915 Accumet pH meter, and total Cd by flame ionized AA spectrophotometry, Perkin Elmer model 1100B). Italicized values were the outputs from Visual Minteq chemical equilibrium software (Gustafsson 2010)

Experiment 3

Kaplan–Meier survival analysis revealed that shrimp length was positively associated with TTD (Wilcoxon test $X^2 = 4.46$, $p = 0.0346$, $n = 35$). The analysis failed to reject the null hypothesis of no difference in TTD between groups 3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt (Fig. 2). However, Kaplan–Meier survival functions and LT_{50}s were qualitatively in agreement with the results of experiment 2 (Fig. 3). The no-Cd control shrimp showed 100 % survival after 30 h containment in treatment cups. Additionally, salinity-adjusted DO levels were 10.0 (± 0.04) and 9.2 (± 0.12) mg/L in control treatment cups ($n = 8$) at the initiation of exposure, and at the time when the last exposed shrimp died, respectively.

Discussion

Experiment 1 revealed that grass shrimp from a low salinity marsh exposed in 3.0 ppt were more sensitive to Cd^{2+} than shrimp from the same marsh exposed at 13.5 ppt. Under hypotonic conditions, ions are lost with urine as it is excreted to counteract the osmotic influx of water. As essential ions such as Ca^{2+} are lost, they must be recovered through active uptake from the environment. Due to the similar charge and ionic radii of Ca^{2+} and Cd^{2+} (114 and 109 pm respectively), Cd^{2+} can be taken up through the same ATPase pumps used to move Ca^{2+} against its concentration gradient (Rainbow 1995). This uptake route could explain why shrimp exposed in a hypotonic solution showed increased sensitivity relative to those exposed in nearly isotonic or hypertonic solutions. However, shrimp from the high salinity marsh exposed at 24.0 ppt salinity (24.0 ppt → 24.0 ppt) showed no difference in sensitivity to Cd^{2+} when compared to the same population exposed at a lower salinity (24.0 ppt → 13.5 ppt). At mean (13.5 ppt) and high (24.0 ppt) salinities, environmental conditions were either nearly isotonic or hypertonic relative to an internal salinity of 12 ppt (Kirby and Knowlton 1976; Knowlton and Kirby 1984; Rowe 2002). Thus, in isotonic and hypertonic conditions the production of excess urine to maintain constant cell volumes is not necessary, essential ions such as Ca^{2+} are not depleted, and Ca^{2+} ATPase pumps are not activated (Rainbow 1995; Potts and Parry 1964; Krogh 1965). Therefore, when [Cd^{2+}] was held constant for exposures in 13.5 ppt and 24.0 ppt salinities, shrimp were likely not utilizing Ca^{2+} ATPase pumps, thus reducing Cd^{2+} uptake and its consequent toxic effects. This pattern has been studied and discussed in great detail in studies using other euryhaline crustaceans (Chan et al. 1992; Burke et al. 2003; Sunda et al. 1978; Zanders and Rojas 1996) and copper-exposed euryhaline fish (Adeyemi et al. 2012). However, the difference in Cd sensitivity between the two populations of shrimp when acclimated and exposed in the mean salinity were the main focus of this study, and cannot readily be explained in terms of individual-level physiological effects under different osmotic conditions.

Experiments 2 and 3 were designed to focus on potential differences in Cd sensitivity between two populations from areas with different average salinity regimes, while controlling for the effect of salinity during exposure. When each population of shrimp was exposed to Cd in the same salinity (13.5 ppt), shrimp from the high salinity marsh (24.0 ppt → 13.5 ppt) were more sensitive to Cd than those from the low salinity marsh (3.0 ppt → 13.5 ppt). Qualitatively similar results have been shown in a study using crab (*Carcinus maenus*) populations from different native salinities (Chan et al. 1992). The results of that study

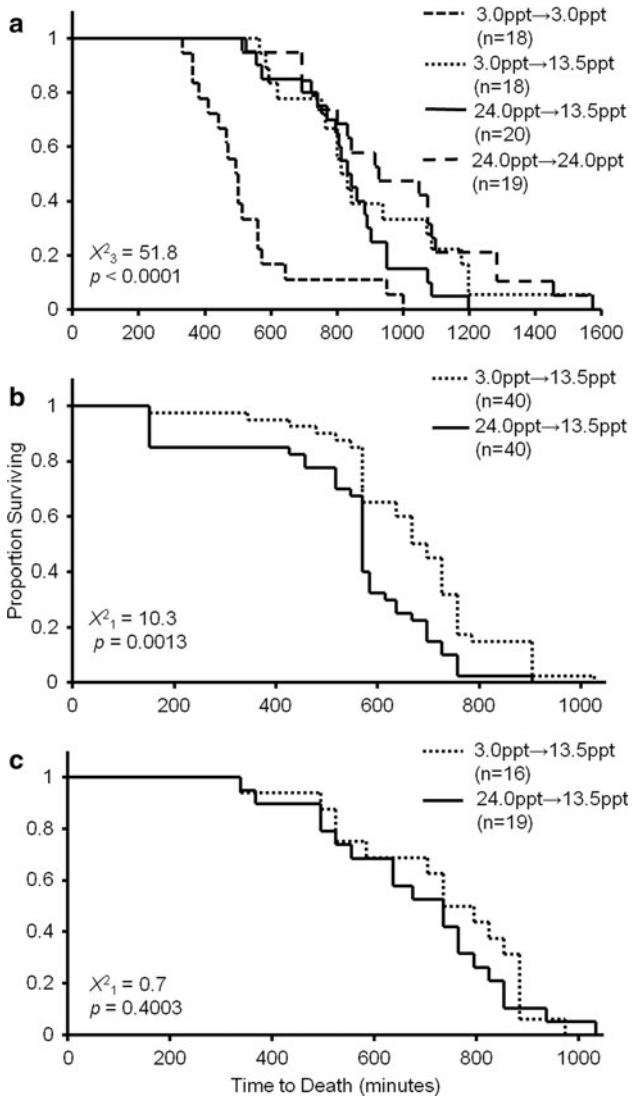

Fig. 2 Kaplan–Meier survival functions for different treatment groups exposed to Cd, in experiments 1, 2 and 3 (panels *a*, *b*, and *c* respectively). Treatment groups consisted of grass shrimp acclimated and exposed to equivalent Cd^{2+} in either native salinities (3.0 ppt → 3.0 ppt and 24.0 ppt → 24.0 ppt) or in the mean salinity of the two collection sites (3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt)

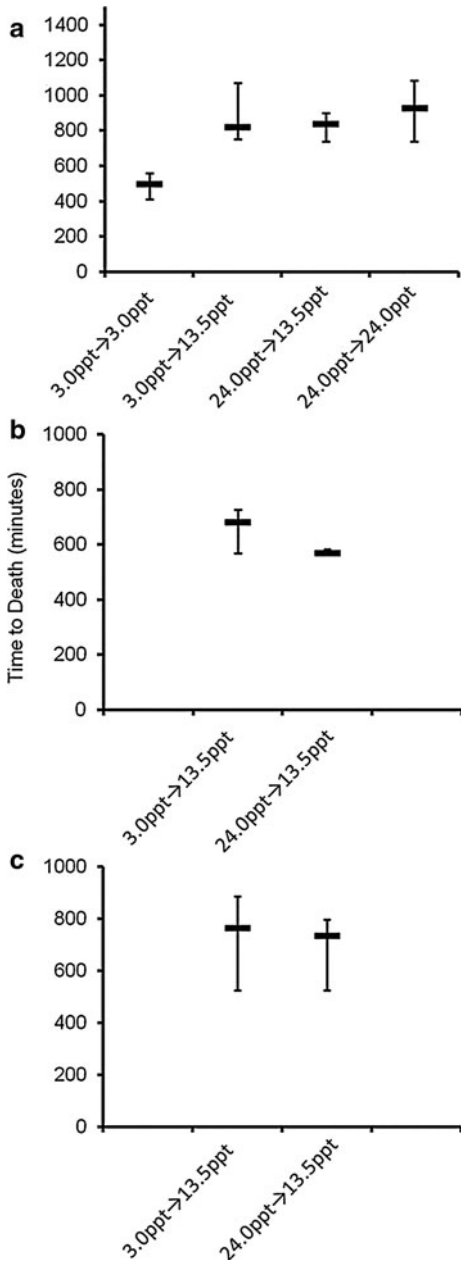

Fig. 3 The median lethal times (LT_{50}s) for each treatment group with *bars* indicating 95 % confidence intervals for experiments 1, 2 and 3 (panels *a*, *b*, and *c* respectively)

and the present study suggest two potential hypotheses, although they are not mutually exclusive. First, long-term acclimation of individual shrimp to their respective salinity regimes may have affected Cd ion uptake. Second, population level adaptation to respective native salinity regimes may have imparted an advantage to shrimp of the low salinity marsh in tolerating Cd exposure. Although each possibility invokes a physiological mechanism relating osmoregulation to metal uptake, the first hypothesis does not imply any relevant genetic difference between the populations, where the second hypothesis does. In experiments 1, 2 and 3, shrimp kept at the mean salinity of the two collection sites (13.5 ppt) had been maintained at this common environment for respectively 20, 42, and 245 days. While genetic differences between the populations would have been maintained, one would expect that non-genetic differences would have become less pronounced. Significant differences in Cd-tolerance between the 3.0 ppt → 13.5 ppt and 24.0 ppt → 13.5 ppt groups in experiment 2 and the lack of a significant difference in experiment 3 is consistent with the presence of a non-genetic component. However, sample sizes (and thus

statistical power) differed between the experiments. If we look at the relative differences in LT_{50}s during the Cd exposure for the 24.0 ppt → 13.5 ppt and 3.0 ppt → 13.5 ppt groups for those three experiments, there is no clear trend of a decrease in the difference between the groups over time. For example, where treatment group 24.0 ppt → 13.5 ppt showed a 1.8 % lower LT_{50} relative to group 3.0 ppt → 13.5 ppt in experiment 1, the same comparison in experiment 3 (conducted 225 days later) showed a 3.9 % difference (in the same direction) with a similar number of replicates. Thus, it does not appear that the observed difference weakened over the time frame of this study, as would be expected if the difference in sensitivity to Cd was due to individual-level acclimation.

A selective process to explain the higher Cd-tolerance of the shrimp from the marsh with historically lower salinity is proposed. There exists an evolutionary trend toward reduced permeability in the chitinous crustacean exoskeleton as we trace lineages that have crossed the marine to freshwater ecological boundary (Péqueux 1995). An exoskeleton with reduced permeability would decrease the passive uptake of Cd^{2+} that may be the source of toxic effects at 13.5 ppt salinity. There is likely selection pressure toward reduced permeability in the low salinity marsh, as its salinity is hypotonic to the hemolymph of osmoregulating grass shrimp. Conversely, in the higher salinity marsh, which averages in the isotonic to hypertonic range relative to grass shrimp hemolymph, no such selection pressure exists. Thus, if microevolution is occurring in response to the selection pressure imposed by a lower salinity habitat, this may effectively reduce permeability in the grass shrimp population from the lower salinity marsh in comparison to the population from the higher salinity marsh. The adaptation of an exoskeleton of reduced permeability in response to a low salinity habitat may also confer increased tolerance to ionic contaminants such as Cd^{2+}. In a similar study using *Carcinus maenus*, hemolymph [Cd^{2+}] after Cd exposure was found to be reduced in crabs from the lower salinity habitat relative to those from the higher salinity habitat (Chan et al. 1992). This would suggest that the observed reduction in sensitivity to Cd in crabs from the low salinity habitat was due to reduced permeability. Although the present study did not investigate the physiological mechanisms that may have conferred resistance to Cd in the shrimp of the low salinity marsh, a reduction in permeability is consistent with that previous study (Chan et al. 1992) and with the observed adaptations to hypotonic environments in euryhaline crustaceans (Depledge 1990). To more definitively determine whether the observed difference in Cd sensitivity between shrimp populations from different salinity habitats is due to genetic or non-genetic differences, a similar experiment could be conducted using offspring from each population reared under the same salinity conditions. Similar results with F1s or F2s from these populations would provide evidence that genetic adaptation to a lower salinity regime increased resistance to Cd.

Overall, the results of this study suggest that native salinity regime affects Cd sensitivity. This has implications for toxicity studies using euryhaline species and implications for risk assessment in coastal marshes and other habitats of fluctuating salinity. Toxicity studies should consider the salinity regime of the habitat from which study organisms are collected. This is especially important when comparing toxicity results among studies, as results could be biased by the use of populations from differing salinity regimes. In the context of risk assessment, this study demonstrates that it is important to consider the salinity regime of the habitat being assessed because it may affect biological responses to ionic contaminants.

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Conflict of interest The authors declare they have no conflict of interest.

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