EFFECT OF LOW SALINITY ON CADMIUM ACCUMULATION AND CALCIUM HOMEOSTASIS IN THE SHORE CRAB (CARCINUS MAENAS) AT FIXED FREE Cd²⁺ CONCENTRATIONS

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Abstract—Increased Cd toxicity at low salinity has been attributed to increased free Cd²⁺ ion concentration ([Cd²⁺]ₐ), but transfer to dilute seawater also stimulates physiological ionic regulation in crabs. In this study, Cd accumulation and Ca homeostasis in the shore crab (Carcinus maenas) were explored at fixed [Cd²⁺]ₐ to reveal the physiological events during sublethal Cd exposure. Crabs were exposed to 3.4 or 34 µg/L [Cd²⁺]ₐ in both 100% seawater (SW) and 33% SW for up to 10 d and sampled for hemolymph composition as well as gill and hepatopancreas Ca, Cd, and Ca-ATPase activity. Cadmium exposure ameliorated the expected fall in hemolymph osmotic pressure and NaCl at low salinity and generally protected tissue Ca from decline. Cadmium exposure alone (within salinity) inhibited Ca-ATPase, but this was offset by stimulation of Ca-ATPase at low salinity. The Ca-ATPase activity in the anterior and posterior gills showed different responses to Cd/low salinity stress. Crabs were more sensitive to a 10-fold increase in [Cd²⁺]ₐ at low salinity. Overall, we conclude that exposure to a fixed sublethal [Cd²⁺]ₐ reveals a compensatory physiological response that is driven primarily by salinity rather than Cd²⁺ free ion concentration. Physiological responses are therefore important during low-level Cd exposure in dilute seawater.

Keywords—Carcinus, Cadmium, Salinity, Free ion concentration, Calcium ATPase

INTRODUCTION

Cadmium (Cd) toxicity to aquatic biota is well known [1], and estuarine crustacea have received particular attention [2,3], partly because Cd toxicity increases with decreasing salinity [4–8]. This increased Cd toxicity at low salinity is associated with elevated Cd accumulation in crustacea [2], suggesting increased Cd bioavailability at low salinity. Cadmium forms a variety of chloride complexes in seawater (SW), including CdCl⁰, CdCl⁻, CdCl₂, and these dominate in 100% SW so that free Cd²⁺ concentrations ([Cd²⁺]ₐ) are low (around 2.5%; [9–11]). With decreasing salinity, the concentration of Cl⁻ ions declines, and this results in a higher proportion of Cd²⁺ ions (around 20% in 1/3 SW), which is assumed to be available for uptake [11]. However, physicochemical events alone do not explain salinity-dependent Cd toxicity because calculation of median lethal concentrations on the basis of [Cd²⁺]ₐ does not fit the expected salinity trend [12]. Alternatively, physiological status may be important in Cd toxicity [13–15].

Many estuarine crustaceans ionoregulate in response to salinity changes and are able to maintain relatively stable osmotic pressure in the hemolymph by increasing or decreasing active ion uptake from the medium [16,17]. In the case of the shore crab (Carcinus maenas), ionoregulation includes increased uptake of Ca at low salinity, which has been correlated with Cd accumulation [4,18]. Indeed, the process of Cd uptake on the Ca-transport system has been well characterized, especially in fish gills, where about a third to a half of the total Cd uptake is as free Cd²⁺ [19]. This Cd²⁺ competes with Ca²⁺ for entry into the gill cells via apical Ca channels, and, once accumulated in the cells, may inhibit basolateral Ca-ATPase to reduce Ca influx [20]. Similar mechanisms operate in crustacean gills, with additional Cd²⁺ entry via stimulation of apical and/or basolateral Na/Ca exchangers [21–23]. Stimulation of these Ca⁻⁻⁻ influx pathways by low salinity therefore might cause incidental uptake of Cd²⁺.

However, the relative importance of modified ionoregulatory physiology compared with metal speciation effects in the seawater for Cd accumulation has not been clarified for most crustaceans. In this experiment, we explore Cd accumulation and Ca homeostasis in the shore crab (Carcinus maenas) at normal and low salinity. Additionally, unlike previous studies on the shore crab, we fix the [Cd²⁺]ₐ at all salinities used so that the physiological contribution to Cd uptake is revealed. Furthermore, we explore these events using low sublethal Cd concentrations so that Ca homeostasis, as measured by Ca-ATPase activity, remains physiologically responsive. We also explore Cd²⁺ concentration effects on these revealed physiological components. Finally, for the first time, we demonstrate that Ca homeostasis in the anterior (respiratory) and posterior (ionoregulatory, e.g., [24]) gills differ in response to Cd exposure, confirming the notion of an ionoregulatory-driven process for Cd accumulation at low salinity.

MATERIALS AND METHODS

Experimental design

Shore crabs, Carcinus maenas (mean wt ± standard error [SE] was 45.6 ± 1.15; n = 68), were collected from clean open seawater sites off Plymouth, United Kingdom. The animals were not fed prior to or during exposure. Crabs were kept in recirculating, filtered seawater at 14°C ± 1°C for at least 48 h before experiments started. The main purpose of the experiment was to determine Cd accumulation while ionoregulatory physiology was stimulated by transfer to dilute sea-
water, without interference from the increasing [Cd\textsuperscript{2+}]\textsubscript{sw} normally associated with reduced salinity. Therefore, crabs were maintained in 100% seawater (32 ppt SW) or diluted 33% seawater (10.5 ppt SW) for 10 d, and at both salinities, the [Cd\textsuperscript{2+}]\textsubscript{sw} was fixed at 3.4 μg/L (designated low Cd\textsuperscript{2+}), achieved by adding 1.0 or 0.261 mg/L total Cd as CdCl\textsubscript{2} to 100% or 33% SW respectively. To explore the effect of increased free metal ion concentration at each salinity, the experiments were also performed with a 10-fold increase in [Cd\textsuperscript{2+}]\textsubscript{sw}, 34 μg/L (designated high Cd\textsuperscript{2+}), achieved by adding 1.0 or 0.261 mg/L Cd as CdCl\textsubscript{2} to 100% or 33% SW respectively. Total Cd concentrations were achieved by carefully pipetting the appropriate volume of a 1-g/L CdCl\textsubscript{2} stock solution into each tank containing 8 L of the appropriate seawater (e.g., 8 ml of Cd stock solution to achieve a working concentration of 1 mg/L in 8 L of seawater, 0.6 ml for 0.1 mg/L, and so on). The [Cd\textsuperscript{2+}]\textsubscript{sw} was calculated using MINEQL\textsuperscript{+} (default settings, pH 8.0, [Cl\textsuperscript{-}] 500 mM, salinity at 32 or 10.5 ppt). Negative controls included crabs held in 100 or 33% SW, respectively, and tissues from the left were used for Ca\textsuperscript{2+} and K\textsuperscript{+} content measurements that contributed to osmotic pressure. NaCl in the presence of Cd compared with no-added-Cd controls, with declines in osmotic pressure of 22 and 27% in low (3.4 μg/L [Cd\textsuperscript{2+}]\textsubscript{sw}) and high Cd\textsuperscript{2+} (34 μg/L [Cd\textsuperscript{2+}]\textsubscript{sw}), respectively, in 33% SW. The protective effect of free Cd\textsuperscript{2+} on osmotic pressure within 24 h was due partly to a relative preservation of plasma Na\textsuperscript{+} in the presence of Cd compared with no-added-Cd controls at low salinity and partly to the mobilization of other unmeasured osmoles that contributed to osmotic pressure. NaCl accounts for 85 to 87% of the osmotic pressure in the presence of Cd, compared with 96% in the 33% SW control without Cd at 24 h (Table 1). The apparently beneficial effect of micromolar Cd\textsuperscript{2+} on electrolyte status at low salinity persisted until the end of the experiment (Table 1). Furthermore, a post-

Blood chemistry, tissue Ca\textsuperscript{2+}-ATPase, and tissue Cd:Ca content

Six crabs (3 from each duplicate tank) were collected from each treatment after 24 h and 10 d of Cd exposure, for hematology to assess osmotic status, tissue Ca\textsuperscript{2+}-ATPase, and tissue metal content (see below). Six of the initial stock animals were also sampled. The hemolymph was collected into heparinized syringes, via the basal arthrodial membrane of the first walking leg, and centrifuged at 7,000 rpm for 1 min (Micro Centaur MSE Sanyo Gallenkamp, Loughborough, UK) to recover plasma. The plasma was frozen at −80°C for up to two weeks until analyzed for Cl\textsuperscript{-} (Chemlab CCM1 chloride meter, Essex, UK), osmotic pressure (Wescor 5100c vapor pressure osmometer), then Na\textsuperscript{+} and K\textsuperscript{+} (Corning 480 flame photometer; Corning, New York, NY, USA). The animals were weighed and their carapace widths measured and then were pithed via the central ganglia. Anterior gills, posterior gills, and hepatopancreas were quickly dissected from the animals. Tissues from the right side of the body were used for metal analysis, and tissues from the left were used for Ca\textsuperscript{2+}-ATPase. Tissues for Ca\textsuperscript{2+}-ATPase were immediately homogenized on ice, in 2.5 ml of hypotonic Tris buffer (20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.0) using a 5-ml hand-held Potters homogenizer. Tissues were stored at −80°C for up to three weeks until analyzed for Ca-ATPase (in triplicate). Ca-dependent phosphate liberation was measured at 660 nm in solutions with and without ethylenediaminetetraacetic acid, according to Jones and Besch [25], but with the basal assay medium modified to 10 mM KCl. Tissue protein was determined by the Hartree method [26]. Trace metal analysis followed Handy et al. [27]. Briefly, tissues were dried to constant weight, digested in nitric acid, and analyzed for total Ca\textsuperscript{2+} (by flame-atomic absorption spectrophotometry; Varian SpectraAA 600, Walnut Creek, CA, USA) or total Cd (by inductively coupled plasma-atomic emission spectrophotometry (Perkin-Elmer Liberty 200; Norwalk, CT, USA). Matrix-matched standards were used (e.g., matrix for Cd standards contained 500 mM NaCl, 50 mM MgSO\textsubscript{4}, and 10 mM Ca\textsuperscript{2+} to reflect salinity of samples) that were previously validated against certified material (see [27] and references therein). The Ca\textsuperscript{2+} and Cd content of seawater were similarly measured. Measured detection limits for Cd derived from three times the standard deviations of the blanks (blanks contain matrix only, no Cd) were 13 μg/L (by inductively coupled plasma-atomic emission spectrophotometry). Thus, a typical tissue digestion containing 5 ml of solution and about 0.5 g tissue had a detection limit equivalent to 0.1 μg/g dry weight.

Statistics

Statistical analyses used Statgraphics 4.0 Plus\textsuperscript{®} software (Statistical Graphics, Rockville, MD, USA). After initial descriptive statistics, data were analyzed for time or treatment effects using one-way analysis of variance followed by the least squares difference multiple-range test. For data that were nonparametric, the Kruskal–Wallis test was applied; the Mann–Whitney W test was used to compare treatments within a time point. All analyses used α set at p = 0.05.

RESULTS

Osmoregulatory response to combined low salinity and Cd exposure

Crabs showed the expected hemodilution after 24 h in 33% SW compared with the 100% SW control, in the absence of Cd (Table 1). This was characterized by a 36% decline in osmotic pressure, attributed mainly to declining NaCl in the hemolymph. Hemodilution persisted throughout the 10-d experiment, indicating that shore crabs (as expected) survive in 33% SW but tolerate some dilution of the body fluids. Animals treated with Cd also showed hemodilution in dilute seawater (Table 1), but the disturbance to osmotic pressure was less marked compared with no-added-Cd controls, with declines in osmotic pressure of 22 and 27% in low (3.4 μg/L [Cd\textsuperscript{2+}]\textsubscript{sw}) and high Cd\textsuperscript{2+} (34 μg/L [Cd\textsuperscript{2+}]\textsubscript{sw}), respectively, in 33% SW. The protective effect of free Cd\textsuperscript{2+} on osmotic pressure within 24 h was due partly to a relative preservation of plasma Na\textsuperscript{+} in the presence of Cd compared with no-added-Cd controls at low salinity and partly to the mobilization of other unmeasured osmoles that contributed to osmotic pressure. NaCl accounts for 85 to 87% of the osmotic pressure in the presence of Cd, compared with 96% in the 33% SW control without Cd at 24 h (Table 1). The apparently beneficial effect of micromolar Cd\textsuperscript{2+} on electrolyte status at low salinity persisted until the end of the experiment (Table 1). Furthermore, a pos-

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### Table 1. Osmotic disturbance in the plasma of shore crabs held at fixed free Cd$^{2+}$ concentrations ([Cd$^{2+}]_{sw}$) in 33 or 100% seawater for 10 d. Low Cd$^{2+}$ = [Cd$^{2+}$] of 3.4 μg/L at both salinities. High Cd$^{2+}$ = [Cd$^{2+}$] of 34 μg/L at both salinities. Control = no added Cd at salinity indicated.

<table>
<thead>
<tr>
<th>Plasma parameter</th>
<th>100%, control</th>
<th>33%, control</th>
<th>100%, low Cd$^{2+}$</th>
<th>33%, low Cd$^{2+}$</th>
<th>100%, high Cd$^{2+}$</th>
<th>33%, high Cd$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h Osmotic pressure</td>
<td>946 ± 33</td>
<td>606 ± 27</td>
<td>971 ± 16</td>
<td>740 ± 35</td>
<td>946 ± 20</td>
<td>693 ± 43</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>433.3 ± 15.2</td>
<td>285.0 ± 16.3</td>
<td>413.3 ± 17.1</td>
<td>301.6 ± 17.0</td>
<td>424.0 ± 9.8</td>
<td>308.0 ± 9.6</td>
</tr>
<tr>
<td>K$^+$</td>
<td>8.7 ± 0.2</td>
<td>6.7 ± 0.6</td>
<td>8.5 ± 0.3</td>
<td>5.9 ± 0.2</td>
<td>7.9 ± 0.2</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>490 ± 19</td>
<td>302 ± 14</td>
<td>432 ± 15</td>
<td>330 ± 31</td>
<td>467 ± 15</td>
<td>300 ± 13</td>
</tr>
</tbody>
</table>

| 10 d Osmotic pressure | 975 ± 42 | 569 ± 11 | 911 ± 32 | 691 ± 13 | 1108 ± 224 | 641 ± 38 |
| Na$^+$ | 393.3 ± 8.8 | 262.0 ± 5.8 | 415.0 ± 15.7 | 308.0 ± 7.3 | 410.0 ± 10.0 | 326.0 ± 12.8 |
| K$^+$ | 6.8 ± 0.3 | 7.1 ± 0.6 | 7.7 ± 0.2 | 5.9 ± 0.3 | 7.7 ± 0.1 | 6.0 ± 0.7 |
| Cl$^-$ | 489 ± 24 | 323 ± 6 | 441 ± 40 | 385 ± 15 | 482 ± 7 | 419 ± 30 |

*Significant difference between 100% seawater (SW) and 33% SW controls without Cd (analysis of variance [ANOVA], p < 0.01).

*Significant difference between 100% and 33% SW within Cd treatment (ANOVA, p < 0.01).

*Significant difference between no-added-Cd control and Cd treated within salinity (ANOVA, p < 0.05).

*Significant effect of exposure time within treatment (ANOVA, p < 0.05).

*Significant difference between low- and high-Cd$^{2+}$ treatments (free ion concentration effect) within salinity (ANOVA, p < 0.05).

### Table 2. Tissue cadmium concentrations (μg/g dry wt) in shore crabs following exposure to fixed free Cd$^{2+}$ concentrations ([Cd$^{2+}]_{sw}$) in 33 or 100% seawater for 10 d. Low Cd$^{2+}$ = [Cd$^{2+}$] of 3.4 μg/L at both salinities. High Cd$^{2+}$ = [Cd$^{2+}$] of 34 μg/L at both salinities. Control = no added Cd at salinity indicated. Data are means ± standard error (n = 6). Tissue-Cd concentration is expressed in μg/g dry weight.

<table>
<thead>
<tr>
<th>Treatment (salinity/[Cd$^{2+}]_{sw}$)</th>
<th>100%, Control</th>
<th>33%, Control</th>
<th>100%, low Cd$^{2+}$</th>
<th>33%, low Cd$^{2+}$</th>
<th>100%, high Cd$^{2+}$</th>
<th>33%, high Cd$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h Anterior gill</td>
<td>2.37 ± 1.15</td>
<td>&lt;0.1</td>
<td>0.99 ± 0.547</td>
<td>0.80 ± 0.515</td>
<td>24.18 ± 14.4</td>
<td>5.96 ± 1.96</td>
</tr>
<tr>
<td>Posterior gill</td>
<td>1.76 ± 1.53</td>
<td>&lt;0.1</td>
<td>1.06 ± 0.514</td>
<td>2.66 ± 2.06</td>
<td>11.21 ± 5.65</td>
<td>17.42 ± 4.01</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>0.34 ± 0.218</td>
<td>&lt;0.1</td>
<td>0.32 ± 0.24</td>
<td>1.17 ± 0.136</td>
<td>1.94 ± 1.51</td>
<td>19.72 ± 6.22</td>
</tr>
</tbody>
</table>

| 10 d Anterior gill | 2.2 ± 0.61 | 0.54 ± 0.549 | 58.21 ± 4.9 | 32.61 ± 22.7 | 77.20 ± 31.1 | 75.01 ± 28.8 |
| Posterior gill | 0.38 ± 0.334 | 0.70 ± 0.7 | 44.46 ± 3.4 | 12.34 ± 2.2 | 112.35 ± 66.3 | 67.74 ± 35.5 |
| Hepatopancreas | 2.50 ± 0.575 | <0.1 | 16.60 ± 3.64 | 5.08 ± 3.19 | 61.82 ± 30.02 | 54.04 ± 17.21 |

*Significant difference between anterior and posterior gill within time point and treatment (t-test, p < 0.05).

*Significant difference between 100% seawater (SW) and 33% SW within Cd treatment (analysis of variance [ANOVA], p < 0.01).

*Significant difference between no-added-Cd control and Cd treated within salinity (ANOVA, p < 0.05).

*Significant effect of exposure time within treatment (ANOVA, p < 0.05).

*Significant difference between low- and high-Cd$^{2+}$ treatments (free ion concentration effect) within salinity (ANOVA, p < 0.05).
of exposure (no statistically significant differences) due to proportionally more Cd accumulation in the 100% SW cohorts and increased interanimal variability in the high-Cd$^{2+}$ treatments by the end of the experiment.

**Effects of low salinity and cadmium exposure on tissue calcium**

Control animals (no added Cd) showed the expected hypocalcemia as salinity (and thus external [Ca]) was lowered (Table 3). This pattern persisted throughout the experiment, even though temporal increases in tissue [Ca] in control crabs in 100% SW indicated some redistribution of tissue-Ca stores. Cadmium exposure in 100% SW generally did not cause major disturbances to tissue Ca, compared with unexposed controls, apart from a statistically significant increase in Ca in the anterior gill, a trend of Ca depletion in the posterior gill, and variable responses in the hepatopancreas. However, low salinity combined with Cd exposure prevented declines (or caused increases) in tissue Ca, indicating that Cd protects against salinity-induced hypocalcemia compared with the no-added-Cd controls. The protective effect of Cd to prevent tissue-Ca loss at low salinity also may have masked salinity effects within Cd$^{2+}$ treatments because no statistically significant effects were observed other than a fivefold increase in [Ca] of the posterior gill in crabs treated with high Cd$^{2+}$ in 33% compared with 100% SW at the end of the experiment (Table 3). Concentration effects of Cd exposure alone at fixed salinity were particularly evident in the hepatopancreas, with 3.7- and 10.8-fold increases in tissue-Ca content associated with a 10-fold increase in Cd$^{2+}$ exposure in 100 and 33% SW, respectively. The increases in [Ca] in the hepatopancreas coincided with a decline in [Ca] of the posterior gill, suggesting redistribution of Ca to the hepatopancreas during Cd exposure.

**Ca-ATPase**

The Ca-ATPase activity in the crude tissue homogenates from control animals in 100% SW initially was approximately 1.3 µM/mg/h. Reductions in salinity caused slight increases in Ca-ATPase activity within 24 h, with most notable increases in Ca-ATPase in the hepatopancreas (2.8-fold; Table 4). Cadmium exposure alone (within salinity treatments) compared with unexposed controls caused a statistically significant reduction in Ca-ATPase activity in the anterior gill at all salinities and in the hepatopancreas of animals held in 33% SW. However, the combined effect of reduced salinity with Cd exposure had no effect compared with Cd exposure in 100% SW except in the anterior gill, where a transient decrease in Ca-ATPase activity at 24 h was replaced by a sustained increase in Ca-ATPase activity by the end of the experiment. Furthermore, both anterior and posterior gill Ca-ATPase activity in Cd-exposed crabs correlated with [Ca] in these tissues at low salinity ($r^2 = 0.4$ and 0.46 for anterior and posterior gills, respectively, $n = 12$), but not in 100% SW ($r^2 = 0.05$ and 0.02, respectively). This finding suggests that salinity-dependent restoration of Ca-ATPase activity was important in maintaining tissue Ca during Cd exposure. However, elevation of [Cd$^{2+}$]$_{sw}$ at each salinity tended to decrease Ca-ATPase activity, with the anterior gill showing a statistically significant decline by the end of the experiment compared with the equivalent low Cd$^{2+}$ treatment (Table 4).

**DISCUSSION**

We performed this experiment at fixed [Cd$^{2+}$]$_{sw}$ so that the physiological effects of Cd at low salinity could be explored without the physicochemical influences of salinity on Cd availability. Overall, the experiment revealed some beneficial physiological changes in osmoregulation and Ca homeostasis that illustrate the importance of physiological compensation over physicochemical considerations during sublethal Cd exposure at low salinity.

**Osmorregulatory response to combined low salinity and Cd exposure**

Relatively few studies have measured hematological disturbances in *Carcinus* during Cd exposure [28–31], and while hemolymph totals [Cd] have been recorded at different salinities in crabs (e.g., [4,7]), changes in hemolymph Na$^{+}$, K$^{+}$, Cl$^{-}$, and osmotic pressure have not been measured during combined salinity and Cd exposure. Shore crabs osmoregulate in dilute seawater, and tolerate some internal dilution in 33% SW [16]. The crabs used here showed this response to low salinity exposure alone. However, sublethal concentrations of Cd also appeared to partly protect crabs from internal dilution (Table 1). This can be explained by a reduction of apparent

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**Table 3. Tissue calcium concentrations (mg/g dry wt) in shore crabs following exposure to fixed free Cd$^{2+}$ concentrations ([Cd$^{2+}$]$_{sw}$) in 33 or 100% seawater for 10 d. Low Cd$^{2+}$ from control animals in 100% SW initially was approximately 1.3 µM/mg/h.**

<table>
<thead>
<tr>
<th>Treatment (salinity/[Cd$^{2+}$]$_{sw}$)</th>
<th>100%, Control</th>
<th>33%, Control</th>
<th>100%, low Cd$^{2+}$</th>
<th>33%, low Cd$^{2+}$</th>
<th>100%, high Cd$^{2+}$</th>
<th>33%, high Cd$^{2+}$</th>
</tr>
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<tbody>
<tr>
<td><strong>24 h</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior gill</td>
<td>4.99 ± 0.65</td>
<td>0.54 ± 0.11</td>
<td>5.77 ± 1.53</td>
<td>7.36 ± 1.69</td>
<td>20.05 ± 9.54</td>
<td>6.42 ± 1.31</td>
</tr>
<tr>
<td>Posterior gill</td>
<td>48.98 ± 97.90</td>
<td>0.29 ± 0.07</td>
<td>53.36 ± 16.78</td>
<td>64.02 ± 13.91</td>
<td>53.53 ± 22.49</td>
<td>98.46 ± 20.85</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>0.33 ± 0.10</td>
<td>0.26 ± 0.03</td>
<td>0.99 ± 0.59</td>
<td>0.60 ± 0.05</td>
<td>2.81 ± 2.36</td>
<td>0.63 ± 0.11</td>
</tr>
<tr>
<td><strong>10 d</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior gill</td>
<td>9.09 ± 1.38</td>
<td>0.42 ± 0.05</td>
<td>9.99 ± 0.87</td>
<td>7.62 ± 1.48</td>
<td>15.97 ± 3.73</td>
<td>11.30 ± 1.37</td>
</tr>
<tr>
<td>Posterior gill</td>
<td>135.60 ± 45.71</td>
<td>0.41 ± 0.08</td>
<td>109.56 ± 25.35</td>
<td>156.83 ± 73.32</td>
<td>42.47 ± 18.49</td>
<td>225.22 ± 72.02</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>5.16 ± 3.04</td>
<td>1.37 ± 0.14</td>
<td>4.74 ± 1.38</td>
<td>2.40 ± 0.84</td>
<td>22.15 ± 17.15</td>
<td>2.40 ± 0.53</td>
</tr>
</tbody>
</table>

- *Significant difference between 100% seawater (SW) and 33% SW controls without Cd (Kruskal–Wallis, $p < 0.05$).
- *Significant difference between no-added-Cd control and Cd treated within salinity (Kruskal–Wallis, $p < 0.05$).
- *Significant difference between low- and high-Cd$^{2+}$ treatments (concentration effect) within salinity (Kruskal–Wallis, $p < 0.05$).
- *Significant difference between 100 and 33% SW within Cd treatment (Kruskal–Wallis, $p < 0.05$).
- *Significant effect of exposure time within treatment (Kruskal–Wallis, $p < 0.05$).

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Table 4. Tissue Ca-ATPase activity μM/mg protein/h in shore crabs following exposure to fixed free Cd$^{2+}$ concentrations ([Cd$^{2+}$]$_{in}$) in 33 or 100% seawater for 10 d. Low Cd$^{2+}$ = [Cd$^{2+}$] of 3.4 μg/L at both salinities. High Cd$^{2+}$ = [Cd$^{2+}$] of 34 μg/L at both salinities. Control = no added Cd at salinity indicated. Data are means ± standard error (n = 5–6). Ca-ATPase-specific activity is expressed in μM/mg protein/h.

<table>
<thead>
<tr>
<th>Treatment (salinity/[Cd$^{2+}$]$_{in}$)</th>
<th>100%, Control</th>
<th>33%, Control</th>
<th>100%, low Cd$^{2+}$</th>
<th>33%, low Cd$^{2+}$</th>
<th>100%, high Cd$^{2+}$</th>
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<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior gill</td>
<td>1.10 ± 0.05</td>
<td>1.16 ± 0.17</td>
<td>1.56 ± 0.37</td>
<td>0.84 ± 0.28$^a$</td>
<td>0.80 ± 0.12</td>
<td>0.54 ± 0.13$^b$</td>
</tr>
<tr>
<td>Posterior gill</td>
<td>1.30 ± 0.12</td>
<td>1.47 ± 0.07</td>
<td>1.57 ± 0.16</td>
<td>1.49 ± 0.18</td>
<td>1.52 ± 0.22</td>
<td>1.33 ± 0.17$^b$</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>1.32 ± 0.20</td>
<td>1.90 ± 0.26</td>
<td>0.93 ± 0.12</td>
<td>1.32 ± 0.20$^b$</td>
<td>1.02 ± 0.16</td>
<td>1.05 ± 0.13$^b$</td>
</tr>
<tr>
<td>10 d</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anterior gill</td>
<td>2.87 ± 0.04</td>
<td>0.83 ± 0.30$^d$</td>
<td>1.72 ± 0.33$^b$</td>
<td>2.09 ± 0.36$^b$</td>
<td>1.16 ± 0.04$^b$</td>
<td>2.92 ± 1.19$^{ae}$</td>
</tr>
<tr>
<td>Posterior gill</td>
<td>2.50 ± 0.23$^e$</td>
<td>1.89 ± 0.22</td>
<td>2.38 ± 0.33$^e$</td>
<td>2.08 ± 0.20</td>
<td>2.31 ± 0.26</td>
<td>2.60 ± 0.50$^b$</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>0.94 ± 0.24</td>
<td>2.62 ± 0.83$^d$</td>
<td>0.94 ± 0.17</td>
<td>0.73 ± 0.16$^b$</td>
<td>0.87 ± 0.11</td>
<td>0.97 ± 0.26$^b$</td>
</tr>
</tbody>
</table>

$^a$Significant difference between 100% seawater (SW) and 33% SW within Cd treatment (Kruskal–Wallis or analysis of variance [ANOVA], p < 0.05).
$^b$Significant difference between no-added-Cd control and Cd treated within salinity (t test, p < 0.05).
$^c$Significant difference between anterior and posterior gill within time point and treatment (t test, p < 0.05).
$^d$Significant difference between 100 and 33% SW controls without Cd (Kruskal–Wallis or ANOVA, p < 0.05).
$^e$Significant effect of exposure time within treatment (Kruskal–Wallis or ANOVA, p < 0.05).
$^{ae}$Significant difference between low- and high-Cd$^{2+}$ treatments (free ion concentration effect) within salinity (Kruskal–Wallis or ANOVA, p < 0.05).

Water permeability of the gills during Cd exposure [32] and the ability of Cd$^{2+}$ to reduce passive Na$^+$ permeability of epithelia [33]. These effects presumably are mediated via Cd$^{2+}$ binding to tight junctions in the gill epithelium, which slows passive ion efflux. As a result, the hemolymph of Cd-exposed crabs had a 9 to 14% higher osmotic pressure compared with that of unexposed low-salinity controls (Table 1). However, this beneficial effect on hemolymph osmotic pressure is reduced as [Cd$^{2+}$]$_{in}$ was increased from 3.4 to 34 μg/L due to the loss of solutes other than NaCl. The additional solute leak was probably not Ca because the gills showed elevated Ca at low salinity in the presence of Cd (Table 3).

Effects of salinity on Cd accumulation

Cadmium accumulates especially in the hemolymph, gills, and hepatopancreas of crabs, with the hepatopancreas probably acting as the central compartment for Cd storage/excretion mechanisms [4,29,30]. This general pattern of accumulation in target organs was not particularly altered during Cd exposure at low salinity [4]. However, whole-body Cd uptake rates may have increased at low salinity [4], perhaps due to elevation of apparent water permeability of the animal [14]. In this study, we also observed that Cd accumulation generally increased with falling salinity, especially in the first 24 h of exposure (Table 2). However, unlike previous studies, we can attribute increased Cd accumulation at low salinity to physiological events because [Cd$^{2+}$]$_{in}$ is constant. For example, we collected data on both the anterior and posterior gills because they are attributed different physiological functions (respiration and osmoregulation, respectively). In the posterior (osmoregulatory) gill, a salinity-dependent Cd accumulation occurs (Table 2, first 24 h), which is associated with an initial 56 to 283% increase in Ca-ATPase activity in posterior compared with anterior gills at low salinity during Cd exposure (Table 4). Thus, upregulation of Ca absorption mechanisms is associated with increased absorption of Cd and implies increased incidental Cd uptake on the Ca-transport systems of the gills. This physiological process also is dynamic. At the end of the experiment, the difference in posterior versus anterior gill tissue Ca-ATPase activity was small because the rate of induction of the anterior gill Ca-ATPase was initially slower. However, anterior gill Ca-ATPase activity eventually caught up with that of the posterior gill (Table 4). Thus, the posterior versus anterior gill difference in [Cd] at low salinity also was small by the end of the experiment (Table 2). The physiological significance of this inducible Ca-ATPase in the anterior gill is not yet clear but suggests that the respiratory gill filaments can take on the osmoregulatory role of Ca absorption during a crisis. Alternatively, inducible Ca-ATPase in the respiratory gill may be simply to preserve the cellular physiology of the respiratory cells, regardless of events elsewhere in whole-body Ca homeostasis.

The experimental design we used incorporated a 10-fold increase in [Cd$^{2+}$]$_{in}$ at each salinity, which allowed us to determine whether the free Cd$^{2+}$ ion concentration caused Cd accumulation equally at both salinities. Increasing [Cd$^{2+}$]$_{in}$ from 3.4 (low) to 34 μg/L (high) generally produced the expected increase in tissue-Cd accumulation at each salinity, suggesting that the free Cd$^{2+}$ ion contributes to Cd accumulation. However, by the end of the experiment, there were proportionally larger increases in tissue-Cd levels in the low-salinity groups compared with the 100% SW groups, even though all exposed treatments were subject to the same 10-fold increase in [Cd$^{2+}$]$_{in}$. For example, the Cd$^{2+}$ concentration effect in the hepatopancreas was a 3.8- and a 10.8-fold increase in tissue Cd within the 100 and 33% SW groups, respectively. This result indicates that crabs may have a physiological preference for Cd$^{2+}$ ions at low salinity or that other physiological mechanisms are switched on at low salinity to increase the absorption of other Cd species. The physiological preference for Cd$^{2+}$ might be a consequence of increased Cd$^{2+}$ uptake on upregulated apical Na:Ca exchanger or Ca channels in the gill [21–23], but even in very well-characterized epithelia, such as fish gill, this accounts for less than half of the new Cd uptake [19]. This result implies that other membrane transport mechanisms that use other Cd species must be upregulated, particularly in this experiment, where the availability of Cd$^{2+}$ species in the water is fixed at low salinity. The uptake of anionic Cd species on the Cl$^–$/HCO$_3$ at low salinity is likely, given that anionic Cd species are readily transported by the ubiquitous Cl$^–$/
HCO$_3^-$ exchanger [34], and this exchanger is dramatically upregulated at low salinity [24]. This mode of membrane transport also may explain the correlation between Cd accumulation in the posterior gill and the hemolymph [Cl$^-$] that we observed.

**Combined effects of low salinity and Cd exposure on calcium homeostasis**

Calcium levels in tissues are broadly similar to previous reports for unfed crabs, being in the mg/g range [4,29]. In this study, the tendency of reduced salinity to cause hypocalcemia was ameliorated by the presence of Cd, so that tissue-Ca status was mainly preserved (Table 3). Previous authors also have noted reasonably small effects of Cd on tissue-Ca content. For example, exposure to lethal concentrations of Cd (10 mg/L total [Cd], [35]) caused only moderate hypocalcemia in the hemolymph ([Ca$^{2+}$] declined from ~13 to ~11 mM over 10 d; [28,35]). Exposure to sublethal concentrations of Cd, even in dilute SW, did not alter [Ca$^{2+}$] of hepatopancreas [29], gills, or muscle of *Carcinus* [4]. Thus, while Cd and Ca may share common mechanisms for uptake across the gills [18,20,36,37], it does not necessarily mean that tissue-Ca depletion will occur. Instead, physiological adjustment of Ca homeostasis may preserve the status of tissue Ca. In this study, we also observed (at fixed salinity) [Cd$^{2+}$]$_{sw}$-dependent reductions in [Ca$^{2+}$] in the posterior gill (Table 3), but [Ca$^{2+}$] elevation in the hepatopancreas. This result indicates physiological redistribution of Ca to the hepatopancreas. This may be functionally important, given the role of the hepatopancreas in Cd storage and excretion [29]. The notion of physiological adjustment also is supported by observed changes in tissue Ca-ATPase (Table 4). Cadmium exposure alone, or increasing [Cd$^{2+}$]$_{sw}$ within salinity, caused inhibition of Ca-ATPase. However, at low salinity, this effect was offset by induction of Ca-ATPase. Thus, the combined effect of low salinity/Cd stress is no net change in Ca-ATPase and preservation of tissue Ca.

In conclusion, fixing the free [Cd$^{2+}$]$_{sw}$ enables observation of the physiological responses to sublethal Cd exposure. These physiological responses are compensatory and include the upregulation of Ca-ATPase to preserve tissue Ca, which is driven primarily by lowered salinity rather than Cd exposure per se. However, our study also identified an inhibitory effect of free Cd$^{2+}$ on Ca-ATPase at fixed salinity and perhaps a physiological preference for the accumulation of free Cd$^{2+}$ at low salinity. Uptake of anionic Cd species also might be stimulated at low salinity. From an ecological perspective, this implies that euryhaline crustaceans that show plasticity in Ca homeostasis are more likely to survive Cd exposure than stenohaline species. It also implies that, if the animals lose the ability to upregulate Ca homeostasis, perhaps due to previous pollutant exposures that have resulted in long-term inhibition of Ca transport or life-cycle events that put extra demands on Ca-uptake mechanisms (e.g., moulting), then Cd toxicity may be driven more by physicochemistry rather than by physiology.

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**REFERENCES**


