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The Role of the Carapace in the Accumulation of Metals from Seawater in the Green Crab (*Carcinus maenas*): Studies with Radio-labeled Calcium, Zinc, and Nickel

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Abstract

The role of the carapace in the uptake and storage of newly accumulated metals was investigated in the green crab exposed to environmentally relevant concentrations of calcium ($[Ca] = 389 \text{ mg L}^{-1}$ or 9.7 mmol L^{-1}), zinc ($[Zn] = 82 \text{ } \mu\text{g L}^{-1}$ or $1.25 \text{ } \mu\text{mol L}^{-1}$), and nickel ($[Ni] = 8.2 \text{ } \mu\text{g L}^{-1}$ or $0.14 \text{ } \mu\text{mol L}^{-1}$) in 12°C seawater, using radio-tracers (^{45}Ca , ^{65}Zn , ^{63}Ni). After 24-h exposure, carapace exhibited the highest concentration of newly accumulated Ca, whereas carapace and gills exhibited the highest concentrations of both newly accumulated Zn and Ni relative to other tissues. For all three metals, the carapace accounted for $> 85\%$ of the total body burden. Acute temperature changes (to 2°C and 22°C) revealed the highest overall temperature coefficient Q_{10} (2.15) for Ca uptake into the carapace, intermediate Q_{10} for Ni (1.87) and lowest Q_{10} (1.45) for Zn. New Ca uptake into the carapace continued linearly with time for 24h, new Zn uptake gradually deviated from linearity, whereas Ni uptake reached a plateau by 6h. Attachment of a rubber membrane to the dorsal carapace, thereby shielding about 20% of the total crab surface area from the external water, eliminated both new Zn and Ni incorporation into the shielded carapace, whereas 36% of new Ca incorporation persisted. When recently euthanized crabs were exposed, new Zn uptake into the carapace remained unchanged, whereas Ca and Ni uptake were reduced by 89% and 71%, respectively. We conclude that the carapace is a very important uptake and storage site for all three metals. All of the uptake of new Zn and new Ni, and most of the uptake of new Ca into this tissue comes directly from the external water. For Zn, the mechanism involves only physicochemical processes, whereas for Ca and Ni, life-dependent processes make the major contribution.

Keywords: exoskeleton, newly accumulated metals, hepatopancreas, cuticle, mineralization, hemolymph

1. INTRODUCTION

The uptake of trace metals from the external sea water in marine decapod crustaceans has been intensively studied, with an almost exclusive focus on the gills as the primary route of entry (reviewed by Griffin et al., 2024; Henry et al., 2012; Rainbow, 1997, 1995, 1985). Much of this work has been performed on the green crab *Carcinus maenas*, a successful invader in many parts of the world (Frederich and Lancaster, 2024; Yamada, 2001; Yamada et al., 2005), one that has become a model species in decapod physiology and ecotoxicology (Leignel et al., 2014; Rodrigues and Pardal, 2014; Weihrauch and McGaw, 2023). In general, it is believed that trace metals sourced via the gills are transported via the hemolymph to the various tissues, including the carapace. This is the recognized pathway by which the major element calcium enters the carapace (Greenaway, 1985; Neufeld and Cameron, 1993; Wheatly et al., 2002), which is mainly composed of calcium and magnesium carbonates, chitin and proteins. As opposed to calcium, reported concentrations of trace metals in the carapace are generally lower than those in the internal tissues (Bryan, 1968; Depledge, 1989) but the opposite may occur in polluted areas (Wright, 1976). Regardless, since the carapace is the highest percentage of the wet body weight (Blewett et al., 2015; Chan and Rainbow, 1993a; Jennings and Rainbow, 1979) the contribution of the carapace to the total body metal burden may be substantial. The traditional view is that metals stored in the carapace are not biologically available (Rainbow, 1988; Rainbow et al., 1990). Nevertheless, the carapace is

sometimes used directly as a tissue in biomonitoring for metal contamination (e.g. Wright, 1976; Shaiek et al., 2018; Saadati et al., 2020), and contributes substantially when the whole crab is digested (e.g. Yeh et al., 2009), though more commonly the hepatopancreas is recommended as the tissue of choice for pollution monitoring (Rodrigues et al., 2022).

The whole surface area of the carapace is exposed to the external water, and while we are aware of no direct measurements, rough calculations based on typical gill surface area measurements (Gray, 1957; Johnson and Rees, 1988) suggest that total carapace surface area may be about 2-fold the gill surface area. Total gill surface area in a typical 56-g crab would be about 40 cm² and our measurements from 2-D photographs (by Image J) yield a total carapace surface area of about 80 cm². It is therefore possible that some of the metals that accumulate in the carapace may actually enter directly from the sea water through the external carapace surface rather than via the gills, and even that metal taken up by this route may move to internal tissues in the body. While these possibilities have been generally overlooked, early studies using radio-labeled cadmium and zinc suggested that adsorption to the carapace might occur, and that upon return to clean water, direct desorption from the carapace might also occur (Chan and Rainbow, 1993a, 1993b; Vilquin et al., 1975; Wright, 1977; Wright and Brewer, 1979). Indeed, Jennings and Rainbow (1979) estimated that 59-80% of radiolabeled cadmium taken up from the sea water was sequestered in the carapace of *Carcinus maenas*, and Chan and Rainbow (1993b, 1993a) provided very similar estimates (61-82%) for radio-labeled zinc distribution in the same species. However, while adsorption and desorption were sometimes suggested, none of these studies experimentally addressed the route(s) or

mechanism(s) by which the radio-labeled cadmium and zinc entered the carapace during exposure, or left the carapace during depuration. More recently, Blewett and Wood (2015) and Blewett et al. (2015) revisited this issue for another metal, and reported that > 80% of radio-labeled nickel uptake from the seawater was sequestered in the carapace of the same species, regardless of salinity or exposure concentration. Blewett et al. (2015) also performed the first mechanistic study on this topic, observing that radio-labeled nickel incorporation into the carapace from external sea water was reduced by 50% when recently euthanized crabs were compared to live crabs. This suggests that only about half of the uptake of nickel from the environment into the carapace was dependent on life processes such as transport from the gills via the hemolymph.

In the present study, our goal was to illuminate this topic using simple experimental approaches so as to dissect the processes responsible for the incorporation of newly accumulated nickel, zinc and calcium into the carapace of *Carcinus maenas*. To this end, using radioactive tracers for all three metals (^{63}Ni , ^{65}Zn , ^{45}Ca), we have examined the time course of metal incorporation into the carapace, the effect of recent euthanasia on this incorporation, the temperature dependence of metal appearance in the carapace, and the influence of sealing off the dorsal surface of the carapace from contact with the external seawater. A second goal was to assess whether the patterns of tissue-specific accumulation and their dependence on temperature were similar or not amongst the three metals. Calcium was examined as it is well established that this metal is mainly taken up by the gills and transported to the carapace via the hemolymph (Greenaway, 1985; Neufeld and Cameron, 1993; Wheatly et al., 2002). Zinc and nickel were selected as metals of environmental concern that are often found in coastal environments at

concentrations that exceed toxicity thresholds for marine life (Blewett and Leonard, 2017; Hogstrand, 2011; Pyle and Couture, 2011; USEPA, 1995). Zinc is essential and can act as a calcium analogue and antagonist (Hogstrand, 2011) and nickel is probably essential and can act as a magnesium analogue and antagonist (Blewett and Leonard, 2017; Pyle and Couture, 2011). Calcium and magnesium are the two major cationic components of the crab carapace (Compère et al., 1993, 1992; Greenaway, 1985; Travis, 1963).

The purpose of the kinetic experiment was to inform whether saturation of uptake into the carapace would occur differentially for the three metals. The euthanasia experiment was designed to extend the findings of Blewett et al. (2015) to all three metals, to assess whether life-dependent processes are involved in the incorporation of the metals into the carapace. Similarly, the objective of the temperature experiments was to compare the temperature sensitivity of the relative appearance rates of the three metals in the carapace versus other tissues. Whenever a transport process powered by biological energy (i.e. ATP) is involved, the rate usually has a higher temperature dependence, as expressed by the Q_{10} . This can be used to distinguish physicochemical processes (low Q_{10}) such as adsorption from biologically mediated processes (high Q_{10}) such as carrier-mediated transport (Hoar, 1983). Sealing off the dorsal surface of the carapace evaluated whether metal uptake occurred directly into the carapace from the external seawater, and whether carapace-absorbed metal could move to other tissues in the crab.

2. MATERIALS AND METHODS

2.1. Animal collection

Green shore crabs (*Carcinus maenas*) (300 male individuals, mean weight \pm SEM = 55.87 ± 1.4 g) were collected in July 2015 and 2016 by baited traps from Pipestem Inlet ('N49.01,57.66" W125.19'21.22") in Barkley Sound (BC, Canada). Collections were conducted under licences (XR2772015 and XR2032016) from Fisheries and Oceans Canada. In compliance with licence requirements regulating the spread of this invasive species, only male crabs were transported from the collection site to Bamfield Marine Sciences Centre (BMSC, Bamfield, BC, Canada). Crabs were transported in coolers and, upon arrival at BMSC (~within 1 h of collection), transferred to outdoor tanks with flow-through seawater at 12°C under a natural photoperiod (10 h:14 h dark:light). This natural seawater (used in all experiments in this study) was pumped from 20 m deep in the Bamfield inlet and filtered through various mesh sizes (finest filtration at 0.32 cm) prior to research use. Its composition was characterized by Blewett et al. (2015) 1-2 years prior to the experiments conducted in the present study: 32 ppt salinity, pH 8.1; 475 mM Na⁺, 11 mM K⁺, 9.7 mM Ca²⁺, 47 mM Mg²⁺, 515 mM Cl⁻, 2.9 mg/L dissolved organic carbon (DOC). Crabs were fed every two days with salmon heads, and food was withheld 48h prior to any experimentation. Crabs were acclimated to holding conditions for at least one week prior to experiments, and only inter-moult individuals were used.

2.2 Test solutions

Exposure solutions were prepared by spiking metals into sea water 24h prior to the experiment. For the Ca test solution, sea water was spiked with ⁴⁵Ca (Amersham Biosciences, Little Chalfont, U. K.) to achieve a final activity concentration of 9 μ Ci L⁻¹ while the natural sea water Ca concentration (i.e. 389 mg L⁻¹ or 9.7 mmol L⁻¹) was

unchanged. For Ni, a stock solution with non-radioactive Ni ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, ACS grade, Sigma-Aldrich, St. Louis, M, USA) and radio-active ^{63}Ni (Eckert and Ziegler, Valencia, CA, USA) was spiked into sea water to obtain a final Ni concentration of $8.2 \mu\text{g L}^{-1}$ ($0.14 \mu\text{mol L}^{-1}$) and an activity concentration of $4 \mu\text{Ci L}^{-1}$. For Zn, a stock solution with non-radioactive Zn ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, ACS grade, Fisher Scientific, Toronto, Canada) and radio-active ^{65}Zn (Amersham Biosciences) was spiked into sea water to obtain a final Zn concentration of $82 \mu\text{g L}^{-1}$ ($1.25 \mu\text{mol L}^{-1}$) and an activity concentration of $7 \mu\text{Ci L}^{-1}$. The total Zn and Ni concentrations were chosen to be environmentally realistic for moderately polluted coastal sea water (Hogstrand, 2011; Pyle and Couture, 2011) and correspond to the current US EPA recommended chronic water quality criteria for the protection of marine life (USEPA, 1995). British Columbia adopted this US EPA Ni guideline (BC MOECC, 2021), but uses a lower Zn guideline of $10 \mu\text{g L}^{-1}$ (BC MOECC, 2022). These selected Ni and Zn concentrations were not expected to cause significant toxicity to *C. maenas*. Indeed, previous studies observed toxicity only at Zn and Ni concentrations two orders of magnitude higher than the selected concentrations (Blewett and Wood, 2015; Elumalai et al., 2007).

2.3 Metal exposures

Crabs were exposed to each test solution at 2°C (cold), 12°C (control) and 22°C (warm) to evaluate the temperature effects on 24-h metal distribution in crabs (carapace, gills, muscles, hepatopancreas, hemolymph). The 12°C treatment corresponded to the baseline sea water condition at the research station, and the warm and cold treatments ($\pm 10^\circ\text{C}$ from the control) are representative of winter and summer temperatures that this

eurhythmic crab can encounter and tolerate in its habitat (Tepolt, 2024). For 2°C and 12°C, crabs were acutely transferred (i.e. without thermal acclimation) from their acclimation temperature of 12°C. For the 12°C treatment only, a separate series was run in which a small piece of carapace (about 1cm x 1cm) was collected at 6h, 12h, and 24h to characterize metal accumulation kinetics in the carapace. Furthermore, two other series of 24-h exposures were conducted at 12°C after i) shielding the dorsal surface of the carapace from the water using a rubber membrane (dental dam), and ii) euthanizing the crabs prior to exposure. For the carapace shielding experiment, the crab was briefly removed from the sea water, towel-dried, and held on a restraint board while the membrane was applied using cyanoacrylate tissue cement (Vetbond™, 3M Corporation, St. Paul, MN, USA), so as to cover the whole dorsal surface of the carapace (Supplementary Figure). The crab was returned to the sea water and the radio-labeled metal exposure was started. A 2D analysis using Image J software indicated that the covered area represented about 20% of the entire surface area of the crab. For the experiment with dead crabs, cryo-euthanasia was conducted by putting the crabs in a freezer (at -20°C) for 1h, and the experiment was started a few hours following death.

For each treatment, crabs were exposed for 24h to 15-L test solution in 20-L plastic containers (with n= 6-7 individuals) under constant aeration and natural photoperiod (10 h:14 h dark:light). No water renewal was conducted throughout the exposure, but water mixing was ensured by a small submersible pump and constant aeration. To control water temperature, test containers were set in a water bath at either 12°C (control temperature, achieved with running sea water), 2°C (cold treatment, achieved with a chiller) or 22°C (warm treatment, achieved with heaters). For the metal-

carapace loading kinetics, carapace pieces (about 1 cm x 1 cm) were gently excised from the dorsal surface over the branchial chambers with a drill (Dremel, Konijnenberg, Netherlands). The holes were then covered with glued dental dam as described above, then crabs were quickly placed back into their exposure containers. Subsequent carapace samples were taken from different sites over the branchial chambers. At the end of the experiment, crabs were quickly rinsed in clean sea water, then in a 10 mmol L⁻¹ EDTA solution (disodium dihydrate EDTA salt, ACS grade, Anachemia, Richmond, BC, Canada), and finally transferred to a ten-fold higher non-radioactive metal concentration than the corresponding exposure solution so as to remove loosely-bound radioisotope. Hemolymph was collected with a disposable syringe and 21-gauge needle from the arthroal membrane of a posterior walking leg after cryo-anesthesia on ice. Then crabs were euthanized at -20°C and thawed for sampling of carapace, anterior gill 5, posterior gill 8, flank muscle and hepatopancreas (HP). Not all samples were obtained from all animals, as explained in supplementary Table SI.1. Notably, the decision to collect muscles was only made later in the project so that the Zn and Ca datasets (which were collected first) are missing most of the muscle data. In shielded crabs, the carapace samples were taken from an area completely covered by the latex dam, after removing the dam using scissors and forceps. Water samples were also collected at the start and end of the 24h exposure, and filtered (<0.45 µm, nylon membrane filter) for analyses of dissolved metal concentration (20-mL samples), radioactivity (2-mL samples), and ammonia concentrations (1-ml samples).

2.4 Sample analyses

Ammonia levels in test waters were checked with the colorimetric assay developed by Verdouw et al. (1978). Temperature checks were conducted every 6-12 hours during experiments, using glass thermometers.

The measurements of total dissolved Ca, Zn and Ni concentrations in water samples were performed by inductively coupled plasma mass spectrometry (quadrupole ICP-MS, Agilent 7700×, Santa Clara, CA, USA). Standards (SCP Science, Baie-D'Urfe, QC, Canada) and samples were in a matrix of 1% HNO₃ (v/v) (Trace Metal grade, Fisher Scientific) with 10 µg L⁻¹ Indium (SCP Science). Calibration was checked with a certified reference water (TM-25.4, Natural Resources Canada, Ottawa, ON, Canada).

The radioactivity of ⁶⁵Zn (CPM·mL⁻¹) in collected water and weighed crab samples (CPM·g⁻¹ ww) was measured by gamma counting (Triathler LSC, Hidex, Mississauga, Canada). The radioactivities of ⁴⁵Ca and ⁶³Ni concentration in water samples (CPM·mL⁻¹) were measured by liquid scintillation counting (LS6500, Beckman Coulter) after addition of scintillation cocktail (Optiphase, PerkinElmer) at a ratio 2.5:1 cocktail:water. For ⁴⁵Ca and ⁶³Ni analyses in crab samples (CPM·g⁻¹ ww), samples were weighed, digested in 4 N nitric acid (ACS grade, Fisher Scientific) for two days at 65°C, mixed with scintillation cocktail (Ultima Gold ABTM, PerkinElmer, Waltham, MA, USA) at a ratio of 20:1 cocktail:digest, then measured by liquid scintillation counting (LS6500, Beckman Coulter, Brea, CA, USA). For these latter analyses, quench correction was performed using different volumes of acid digests and the external standard method to correct for the counting efficiency differences with that of sea water.

2.5 Data calculations and statistical analyses

The concentrations of newly accumulated Ca, Zn and Ni in the various crab tissues ($[M]$, in $\text{nmol}\cdot\text{g}^{-1}$ wet weight) were calculated based on radioactivity in the digested tissues and the inverse of the mean specific activity ($\text{CPM}\cdot\text{mL}^{-1}/\text{nmol}\cdot\text{mL}^{-1}$) of each radioisotope in the sea water during the exposure:

$$[M] = a \cdot (b \cdot c^{-1})^{-1} \quad \text{Eq. 1}$$

where a is the radioisotope concentration in the tissue (in $\text{CPM}\cdot\text{g}^{-1}$ wet weight), b is the radioisotope concentration in the test water (in $\text{CPM}\cdot\text{mL}^{-1}$) and c is the total dissolved metal concentration in the sea water (in $\text{nmol}\cdot\text{mL}^{-1}$).

Dry tissue (dw) weights were not measured in the present study, so all metal tissue concentrations are presented on a ww basis in the main manuscript. However, many studies only report metal concentrations on a dw basis, so we also provide estimates of dw concentrations (as Supplementary Information) that may be used for comparison purposes with these latter studies. These estimates were obtained using wet:dry ratios measured in previous studies with *Carcinus meanas*: 1.5 for carapace, 9.5 for gills, 4.1 for muscle, 13 for hemolymph (Bjerregaard and Delpledge, 2002) and 4.1 for hepatopancreas (Bjerregaard, 1990).

Calculations of the speciation of metals in sea water were performed using Visual MINTEQ 4.0. For these calculations, we used the sea water composition given in section 2.1, with a temperature of 12°C , a nominal alkalinity of 115 mg/L CaCO_3 and a nominal $[\text{SO}_4^{2-}]$ of 28 mM . Metal complexation with dissolved organic matter (DOM) was modelled with the NICA-Donnan model, considering DOM is 50% carbon in weight,

100% fulvic acid, and only 65% of this fulvic acid is active in binding metals (Stockdale et al., 2015).

Whole-body percent distributions of newly accumulated metals were calculated in each tissue, based on measured metal tissue concentrations and the relative percentage contribution of each tissue to total crab mass. These latter proportions were 56.4% for the carapace, 1.21% for the gills, 6.96% for the muscles, 3.08% for the HP, and 32.2% for the hemolymph. We calculated these values from the relative proportions for carapace, gill, muscles, and hepatopancreas measured by Blewett et al. (2015) on green crabs collected in the same area at close to the same time. Blewett et al. (2015) did not measure hemolymph volume directly. Therefore we used the average hemolymph proportion measured on this species by four previous studies, which are remarkably uniform, ranging from 31 to 33% (Harris and Andrews, 1982; Martin and Rainbow, 1998; Robertson, 1960; Zanders, 1980).

Temperature coefficients (Q_{10}) were calculated on newly accumulated metal incorporation over 24h into the various crab tissues with the following equation:

$$Q_{10} = \left(\frac{[M]_2}{[M]_1} \right)^{\left(\frac{10}{T_2 - T_1} \right)} \quad \text{Eq. 2}$$

where $[M]_1$ and $[M]_2$ are newly accumulated metal concentrations in a crab tissue (in $\text{nmol} \cdot \text{g}^{-1}$ wet weight) (as calculated by Equation 1) at temperature T_1 and T_2 respectively (with $T_1 < T_2$).

Data are reported as means \pm SEM (n), where n refers to number of crabs. For statistical analyses, newly accumulated metal concentrations in the various tissues were first evaluated for normality and homoscedasticity using the Shapiro-Wilk test and the Brown-Forsythe test, respectively. For each metal, tissue comparison of metal

concentrations were assessed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. Further, for each metal, arcsin-transformed percent total body burden in the control experiment were assessed using the parametric Brown-Forsythe ANOVA test followed by Dunnett's T3 multiple comparisons test (for Ca and Ni) or using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test (for Zn). For a given tissue and metal, temperature effects on metal accumulation were assessed by comparing the 2°C, 12°C and 22°C data using a one-way ANOVA with Tukey's multiple comparison test for parametric data, or a Kruskal-Wallis test with Dunn's multiple comparisons for non-parametric data. Time effects on metal accumulation in the carapace were assessed with a Friedman test and Dunn's multiple comparisons test for Ca (non-parametric data), and with a repeated measures one-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test for Zn and Ni (parametric data). For a given metal, the effects of carapace-shielding and euthanasia on accumulation in the carapace were assessed by comparing unshielded (control) with shielded or dead crabs with a Kruskal-Wallis test and Dunn's multiple comparisons test. Furthermore, the effects of carapace-shielding on metal accumulation in gills and internal tissues were assessed by comparing unshielded (control) with shielded crabs with multiple t-tests with FDR (false discovery rate) (Ca) or multiple Mann-Whitney tests with Holm multiple comparison test (Zn and Ni).

3. RESULTS

3.1. Exposure conditions

Measured dissolved Ni concentrations in test solutions were $6.97 \pm 0.54 \mu\text{g/L}$ ($n = 14$), i.e. 85% of the nominal concentration of $8.2 \mu\text{g/L}$. Measured dissolved Zn concentrations in test solutions were $78.9 \pm 9.4 \mu\text{g/L}$ ($n = 12$), i.e. 96% of the nominal concentration of $82 \mu\text{g/L}$. Clean seawater had $0.29 \pm 0.07 \mu\text{g/L}$ of Ni and $5.84 \pm 0.23 \mu\text{g/L}$ of Zn. The modelled speciation of the metals in the exposure sea water is reported in Table SI.2 in the supplemental information. For all three metals, the free cationic metal (M^{2+}) predominated (55-71%), followed either by the cationic chloride complex (MCl^+) for Ca and Zn, or the neutral sulphate complex (MSO_4) for Ni. Temperature measurements were within $\pm 1^\circ\text{C}$ of targeted values (2, 12 and 22°C) throughout the experiments. Ammonia water concentrations remained $\leq 100 \mu\text{M}$ during the experiments. No crabs died during the exposures.

3.2 Metal distribution in the tissues of the green shore crab

As detailed in Supplementary Table SI.1, all experiments were conducted in 2015, except for the Ni exposures at 2°C and 22°C which were conducted in 2016. Further, all control/baseline experiments (12°C uncovered and live crabs) and the shielded-crab Ni experiment were fully repeated in 2015 and 2016. As repeatability was high, the 2015 and 2016 data were pooled.

All metal tissue concentrations are presented in molar concentrations and on a wet weight basis (in $\text{nmol g}^{-1} \text{ww}$, all values compiled in Supplementary Table SI.3). Molarity was used over atomic mass concentrations (which are more often used in toxicological studies) to enable concentration comparison between the three metals of interest. Wet tissue weights were used (over dry tissue weights) to present metal concentrations

occurring in fresh live crabs. If of interest to the reader, mass concentrations and dry-weight concentration estimates are provided in the supplementary file (metal tissue concentrations in mg kg^{-1} ww in Table SI.4, in nmol g^{-1} dw in Table SI.5, and in mg kg^{-1} dw in Table SI.6).

Concentration of newly accumulated Ca, Zn and Ni in various tissues of crabs from the control experiment (24-h exposure of live and unshielded crabs at 12°C) are presented in Fig. 1 (and Supplementary Table SI.3). This baseline experiment was repeated three times over a 12-month period for each metal and similar data were obtained in each repeated experiment. Thus, we report the combined results in this Fig. 1. Also, metal concentrations in posterior gills (primarily engaged in ion transport) and anterior gills (primarily engaged in respiration and excretion) (Henry et al., 2012) were not significantly different, so an overall gill measurement is given for each of the three metals.

The concentration of newly accumulated metal in the green shore crab followed this order: $\text{Ca} > \text{Zn} > \text{Ni}$. More precisely, after 24 h of exposure, the whole-body concentration of newly accumulated Ca was > 3 orders of magnitude (i.e. $> \sim 1,000\text{x}$) and > 4 orders of magnitude (i.e. $> \sim 10,000\text{x}$) greater than the newly accumulated whole-body concentrations of Zn and Ni, respectively (Fig. 1). Note however that the differences in exposure concentrations were close to 4 ($\text{Ca} > \text{Zn}$) and 5 ($\text{Ca} > \text{Ni}$) orders of magnitude respectively. The metal bioaccumulation factors (BAF) in the carapace, corresponding to the ratio of metal carapace concentrations to metal water concentrations, were 4.8 for Ca, 10.2 for Zn, and 5.8 for Ni for these control conditions (24-h exposure of live and unshielded crabs at 12°C).

Differences in the tissue accumulation of metals were observed between the three metals. Calcium concentrations were the highest in the carapace, then the hemolymph, and the smallest in the HP, muscle and gills (Fig. 1A). On the other hand, Zn and Ni concentrations were the highest in both the carapace and gills, while both were much lower in the other tissues (Fig. 1B and 1C). Nevertheless, because the carapace represents ~56% of the whole-body weight of green shore crabs, it was the main site of all newly accumulated metals (Table 1). Indeed, $89 \pm 4\%$ of total Ca, $86 \pm 4\%$ of total Zn and $98 \pm 1\%$ of total Ni were in the carapace after 24h of exposure (Table 1). The second most important site of accumulation was the hemolymph for Ca ($10 \pm 4\%$ of total Ca) and Zn ($9.7 \pm 2.9\%$ of total Zn) and the gills for Ni ($1.3 \pm 0.4\%$ of total Ni) (Table 1).

3.3 Temperature effects on metal distribution in the tissues of the green shore crab

Temperature had a significant effect on the accumulation of all metals in almost all the measured tissues ($p < 0.05$, one-way ANOVA or Kruskal-Wallis test) (Fig. 2 and Supplementary Table SI.3). The only non-significant thermal effect was observed for Ni accumulation in muscle (Fig. 2M) (note that Ca and Zn concentrations were not analyzed in the muscle at 2 and 22°C). The concentration of all three metals increased by similar extents when temperature increased from 2 to 22°C (on average by about 3-fold from 2 to 22°C, though there was considerable variation in patterns). The Q_{10} values for these data are given in Table 2.

3.4 Kinetics and pathways of metal accumulation in carapace of the green shore crab

New Ca accumulation in the carapace increased linearly with exposure time from 6h to 24h (Fig. 3A). New Zn accumulation in the carapace gradually deviated from linearity over time (Fig. 3B). New Ni concentration did not increase from 6h to 24h (Fig. 3C).

Shielding the carapace from contact with the external sea water led to a 64% decrease in new Ca accumulation in the shielded area (Fig. 4A) and to a complete elimination of new Zn and new Ni accumulation (Fig. 4B and 4C) in this compartment. In other analyzed tissues, shielding of the dorsal carapace only led to a weakly significant reduction in newly accumulated Ca in the gills, while there was no change in the other tissues for all three metals (Table 3).

Euthanasia led to a decrease in new Ca accumulation in the carapace by 89% (Fig. 4A). A similar but not significant trend was observed for Ni, with a 71% decrease in dead animals (Fig. 4C). In marked contrast, there was no difference in new Zn accumulation in the carapace of live *versus* recently dead crabs (Fig. 4B).

4. DISCUSSION

4.1 Patterns of new metal incorporation into tissues

Very clearly, the carapace is by far the most important site on a quantitative basis for incorporation of newly accumulated Ca, Zn, and Ni in *Carcinus maenas*, accounting for over 85% of the total body burden after 24h of exposure to environmentally relevant concentrations of these three metals (Fig. 1, Table 1). Our combination of rinsing with seawater, then with EDTA, then with 10-fold greater non-radioactive metal, ensured that

this was not just metal loosely bound to the surface, but rather metal that is in some way incorporated into the carapace structure (Cresswell et al., 2017). This confirms earlier reports on the same species using radio-labeled Zn (Chan and Rainbow, 1993b, 1993a), Ni (Blewett et al., 2015; Blewett and Wood, 2015), and Cd (Jennings and Rainbow, 1979; Wright and Brewer, 1979) and extends this finding to radio-labeled Ca distribution. It may not be surprising that all of these trace metals mimic Ca in this regard, because all of them can be considered as analogues and antagonists of Ca (Wood, 2011), including Ni (Lee et al., 1999), although the role of Ni as a mimic of Mg, the other major cation in the carapace, is more frequently cited (Blewett and Leonard, 2017; Pyle and Couture, 2011). However, this preference for accumulation by the carapace may not be confined to just Ca and Mg analogs; radio-labeled Cu (a Na analog) also seems to preferentially accumulate in the carapace of the blue crab (Martins et al., 2011); C. Martins and C.M. Wood, unpubl. data). One explanation may be the unusually high pH and $[\text{HCO}_3^-]$ of the interstitial fluid phase in the crab carapace. In *Callinectes sapidus*, Wood and Cameron (1985) measured these to be about 8.23 and 12 mmol L^{-1} respectively, well above both hemolymph and seawater levels for these two critical determinants of precipitation. These conditions would promote the precipitation of metal carbonates (e.g. Druckenmiller and Maroto-Valer, 2005; Kim and O'Neil, 1997) thereby maintaining a concentration gradient for further entry of dissolved metal across both the internal and external surfaces of the carapace.

Despite the similar preferential accumulation of Ca, Zn, and Ni in the carapace, there were marked differences in their handling by other tissues. Most notably, new metal accumulation in the gills was relatively low for Ca (Fig. 1A), whereas for both Zn (Fig.

1B) and Ni (Fig. 1C), it was relatively high, and equal in concentration to that of the carapace. This is in accord with studies on perfused crab gills where a sustained, vigorous net uptake of Ca into the perfusate occurred for many hours, with no evidence of Ca buildup in the gill tissue (Lucu, 1994; Pedersen and Bjerregaard, 1995). In contrast, for Zn and Ni, only a very small fraction of the labelled metal taken up appeared in the perfusate (< 2% for Zn, Niyogi et al. (2016); 3-10% for Ni, Blewett et al. (2015)); the vast majority remained bound in the gill tissue. The other major difference was the relatively low incorporation of newly acquired Ni into the hemolymph (Fig. 1C) relative to Ca (Fig. 1A) and Zn (Fig. 1B). As explained subsequently, this may reflect time course differences in the uptake of Ni relative to the other two metals.

It should be noted that, in their natural environment, crabs are exposed to trace metals from both the water phase (dissolved metal exposure) and from their food (dietary metal exposure). For inorganic metals such as Zn and Ni, much fewer studies have looked into metal assimilation from this latter route. For *C. maenas*, some studies have found this route to be dominant (Bjerregaard et al., 2005; Miramand et al., 1981), while other studies have not (Jennings and Rainbow, 1979; Renfro et al., 1975; Ünsal, 1983), emphasizing that the relative importance between the two pathways depends on the species, metal, diet composition, and a variety of other factors (e.g. salinity, temperature, life stage) (Wang and Fisher, 1999). Regardless of its relative contribution, the metal distribution patterns observed in our study would probably be different if a dietary metal pathway had also been considered. Indeed, since metal associated with food may be assimilated in internal tissues via the gut but not directly in the carapace, the relative

proportion of new metal in the carapace would likely be lower with this additional metal uptake pathway.

4.2 Temperature and time dependence of new metal incorporation into tissues

The temperature studies were primarily directed at factors affecting new metal accumulation in the carapace. The goal was to separate purely physicochemical processes (e.g. diffusion, physical and chemical adsorption, and other chemical reactions), where Q_{10} values close to 1.0 are expected, from those dependent on biological processes (e.g. transporters, enzymes, channels), where Q_{10} values closer to 2.0 or higher are expected (Hoar, 1983). The results suggest that for new Ca incorporation into the carapace (Fig. 2A, Table 2), biological processes dominated over the entire range from 2°C to 22 °C. However, in marked contrast, for new Zn incorporation (Fig. 2F, Table 2), physicochemical processes appeared to dominate from 2°C to 12 °C, but biological processes took over from 12°C to 22 °C. Exactly the opposite was seen for new Ni incorporation (Fig. 2K, Table 2), with biological processes heavily involved from 2°C to 12 °C, but only physicochemistry from 12°C to 22 °C. Thus, the overall Q_{10} values from 2°C to 22 °C, were lowest for new Zn, intermediate for new Ni, and highest for new Ca (Table 2). It is also apparent that the temperature pattern in the carapace was largely duplicated in the hepatopancreas for all three metals (Figs. 2D, 2I, 2N). The hepatopancreas is generally considered the most important internal storage organ for environmental contaminants (e.g. Bryan, 1968; Griffin et al., 2024; Rodrigues and Pardal, 2014). However, these data must be interpreted with caution, as many factors that confound this simple interpretation of physicochemical versus biological processes as

rate determinants have been known for almost a century, perhaps most importantly the time scales and the temperature ranges over which rates are measured (Běhrádek, 1930).

In this regard, the kinetic experiment was instructive. Whereas new Ca incorporation into the carapace continued linearly (Fig. 3A), and new Zn incorporation somewhat less linearly up to 24 h (Fig. 3B), new Ni incorporation had reached saturation by 6 h (Fig. 3C). This time scale experiment was performed only at 12°C, but it suggests that if rates had been measured over a much shorter time period for Ni, it is possible that the apparent lack of effect of temperature between 12°C and 22°C (Fig. 2K) might not have occurred, whereas the lack of temperature effect on new Zn incorporation between 2°C and 12 °C (Fig. 2F) would still have been seen.

4.3 Dependence of new metal incorporation into tissues on life processes and on the contact of the carapace with external sea water

The decrease in new Ca (by 64%) and new Zn and Ni (by 100%) in the dorsal carapace following its shielding (Fig. 4) suggests that only 36% of the new Ca and none of the new Zn and new Ni accumulating in the carapace over 24 h at 12°C had entered from the inside via the hemolymph. The live *versus* dead crab experiment revealed that 89% of new Ca uptake (Fig. 4A), 0 % of new Zn uptake (Fig. 4B), and 71% of new Ni uptake (Fig. 4C) into the carapace were blocked when life processes were removed, the latter in reasonable agreement with Blewett et al. (2015) who reported a 50% decrease in a comparable experiment. The conclusions that can be drawn for new Zn uptake into the carapace are clear: all of it enters from the sea water by processes that do not depend on life; this fits in well with the low Q_{10} at 2 °C to 12°C, and the fact that Zn exhibited the

lowest overall Q_{10} (2 °C to 22 °C) of the three metals from (Table 2). This also agrees well with the conclusion of Bryan and Cole (1997) that Zn absorption by crustaceans may be a completely passive process. However, for new Ni uptake, again all of it enters from the external environment, but most of this uptake (71%) depends on life processes. This fits with the intermediate overall Q_{10} at 2 °C to 22 °C (Table 2). And for new Ca, the great majority (89%) of incorporation into the carapace depends on life processes, but since Ca enters from both external (64%) and internal pathways (36%), it is likely that both of these pathways are contributing to this 89%. Regardless, the highest overall Q_{10} (2 °C to 22 °C; Table 2) suggests that biological mediation plays the dominant role, in accord with current understanding of Ca delivery to the exoskeleton (Greenaway, 1985; Neufeld and Cameron, 1993; Wheatly et al., 2002).

Regardless of whether a metal enters the carapace from the external environment by simple physicochemical processes or biological transport, it could potentially move to other tissues. For example, Ca is known to move from carapace to hemolymph prior to moulting (Greenaway, 1985; Roer, 1980). The measurements in Table 3 showing a general trend for reduction of newly accumulated metal concentrations in gills (for Ca and Ni) and some internal tissues (for all three metals), despite the fact that only about 20% of the entire surface area of the carapace was shielded, are suggestive that such internal mobility may occur. However, these data must be interpreted with caution. Most of the changes were not significant and were not seen consistently in all tissues.

4.4 Future Directions

Thus, there was no conclusive evidence that metals, taken up directly through the carapace from the external sea water, can move into internal tissues. Yet, this question should be further explored. This may become especially important just prior to moulting, because we know that much of the Ca in the carapace is reabsorbed into internal tissues at this time (Greenaway, 1985; Neufeld and Cameron, 1993; Roer, 1980; Wheatly et al., 2002). Does the same occur for potentially toxic metals such as Zn and Ni, where most of the body burden is stored in the exoskeleton? The study of Bergey and Weisz (2007) on the fiddler crab (*Uca pugnax*) suggests that this does occur for Zn, Cu, and Pb, but these workers did not identify the original uptake pathways of the metal reabsorbed from the carapace.

The present study has shown that direct metal uptake from the sea water into the carapace occurs largely “passively” for Zn (i.e. not mediated by life-dependent processes), with “active” biologically mediated uptake dominating for Ca and Ni. With respect to “passive” uptake, it is noteworthy that finely ground crab carapaces are now used in industrial water remediation to remove Zn (Lu et al., 2007) and Ni (Pradhan et al., 2005) from solution. The high content of chitin in the carapace, a natural polysaccharide composed of (1–4)-2-acetamido-2-deoxy-d-glucose units, appears to be critical, and the processes of adsorption, micro-precipitation, chelation and ion-exchange all appear to contribute (Lu et al., 2007; Pradhan et al., 2005). Whether the same complexity is true *in vivo* remains unknown and should be investigated.

With respect to “active” processes, it is noteworthy that crab carapace contains many different types of cells, most importantly epidermal cells which send protoplasmic extensions termed “pore canals” all the way through the basal endocuticle, and overlying

exocuticle to the thin epicuticle at the surface (Green and Neff, 1972; Travis, 1963). These pore canals serve as important sites of calcite formation and also act as pathways to provide key materials to the various layers of the exoskeleton. Travis (1963) estimated their abundance at $4 \times 10^6 \text{ mm}^{-2}$ of carapace surface area! Clearly these could provide direct routes for external metals to enter the carapace for either “active” or “passive” incorporation into the carapace, though this remains to be demonstrated.

To obtain further insights into the movement and distribution of metals within the carapace of decapod crustaceans, future studies could employ microchemical methods that can measure elements in solid samples at the micrometer scale, such as Laser Ablation Inductively Coupled Plasma Mass Spectrometry. Finally, it will be important to know whether the carapace can serve as a direct pathway for metal depuration. Specifically, can the carapace excrete newly incorporated metals from its own matrix, and /or from internal tissues into the external sea water? If so, what is the time course? Crémazy et al. (in prep) address these questions in a parallel study.

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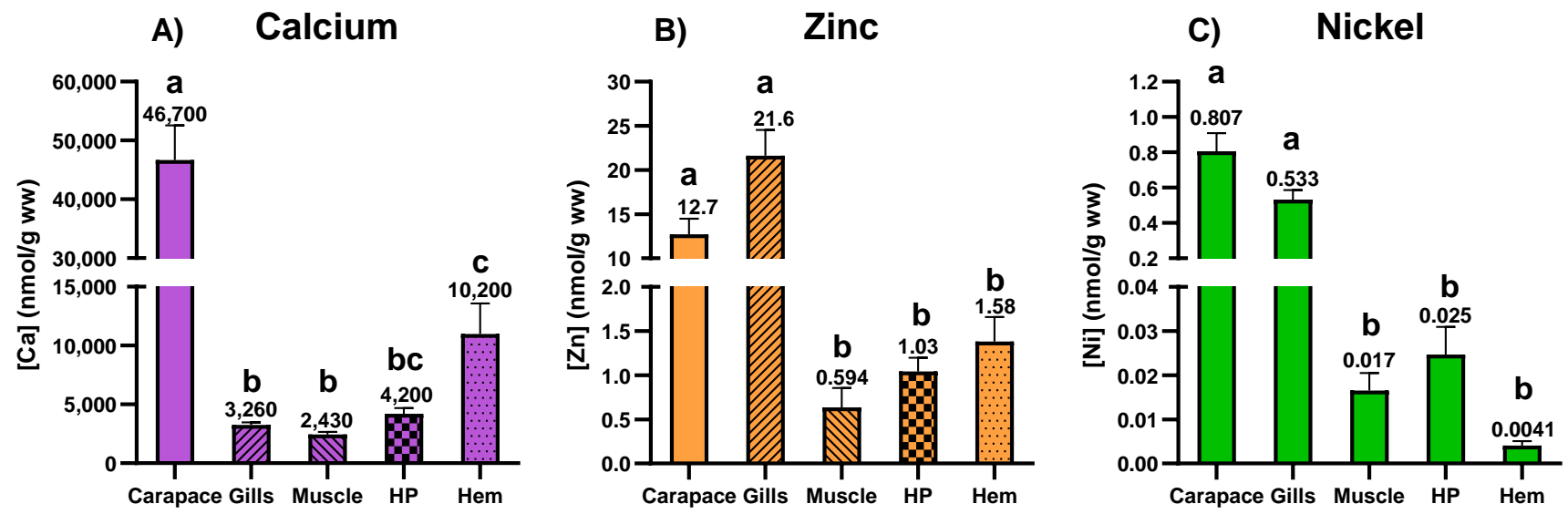


Figure 1: Concentration of newly accumulated **A) Ca**, **B) Zn** and **C) Ni** in various tissues of the green shore crab, after a 24 h exposure to respectively Ca (389 mg L⁻¹) Zn (82 µg L⁻¹) or Ni (8.2 µg L⁻¹) at 12°C. Values are mean ± SEM (n values in Supplementary Table SI.3). Mean values plotted above bars. For a given metal, bars not sharing a common letter are significantly different (p < 0.05, Dunn's test).

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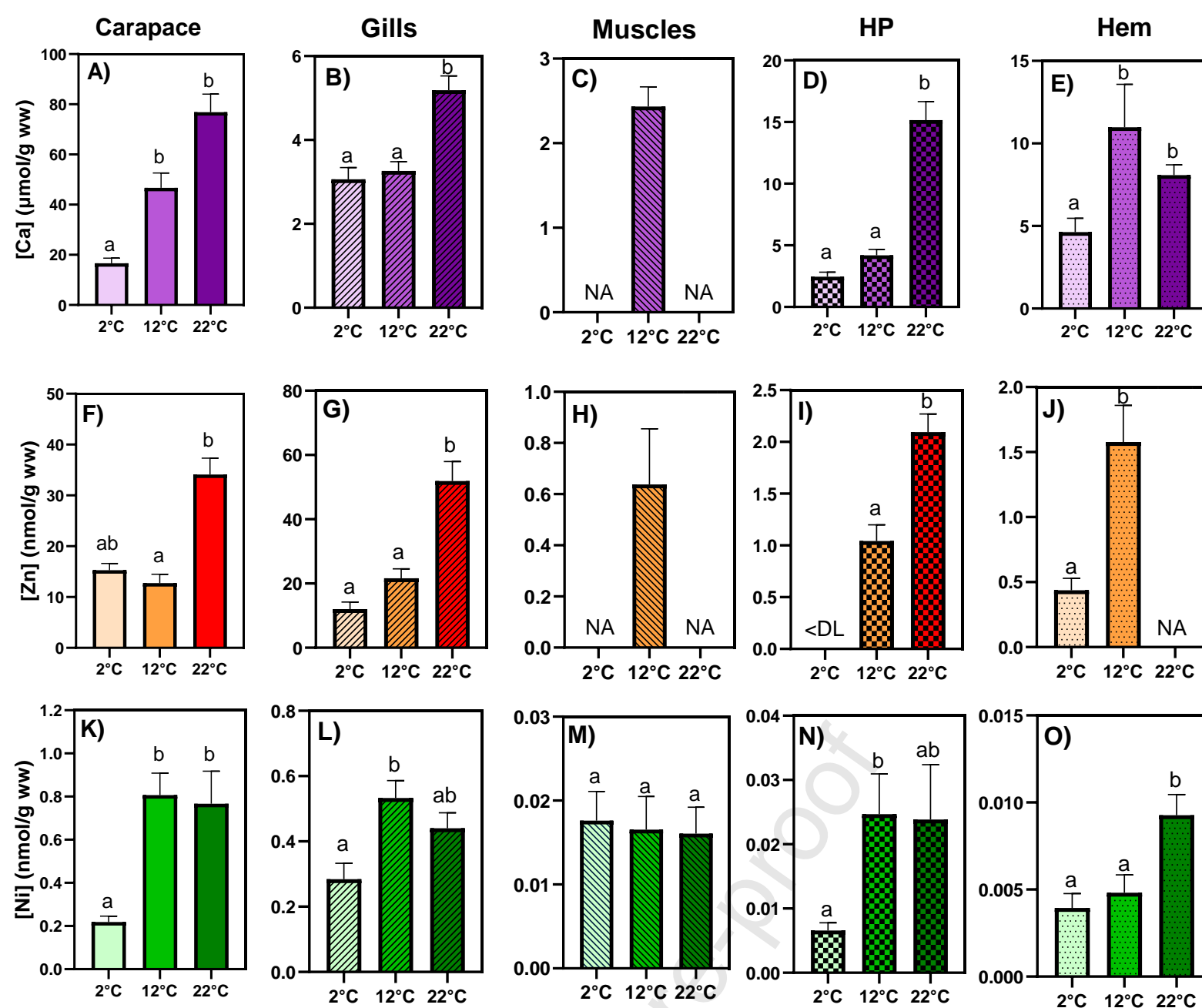


Figure 2: Effect of temperature (2, 12 and 22°C) on metal concentration in the various tissues of the green shore crab after a 24 h exposure to respectively Ca (389 mg L^{-1}), Zn ($82 \text{ } \mu\text{g L}^{-1}$) or Ni ($8.2 \text{ } \mu\text{g L}^{-1}$). Values are mean \pm SEM (n values in Supplementary Table SI.3). For a metal in a given tissue, bars not sharing a common letter are significantly different ($p < 0.05$, t-test for Zn HP, Tukey's or Dunn's test for the rest of the dataset). NA: not analyzed; <DL: below detection limit (DL = 1, 0.001 and 0.00005 $\text{nmol g}^{-1} \text{ ww}$ for Ca, Zn and Ni respectively)

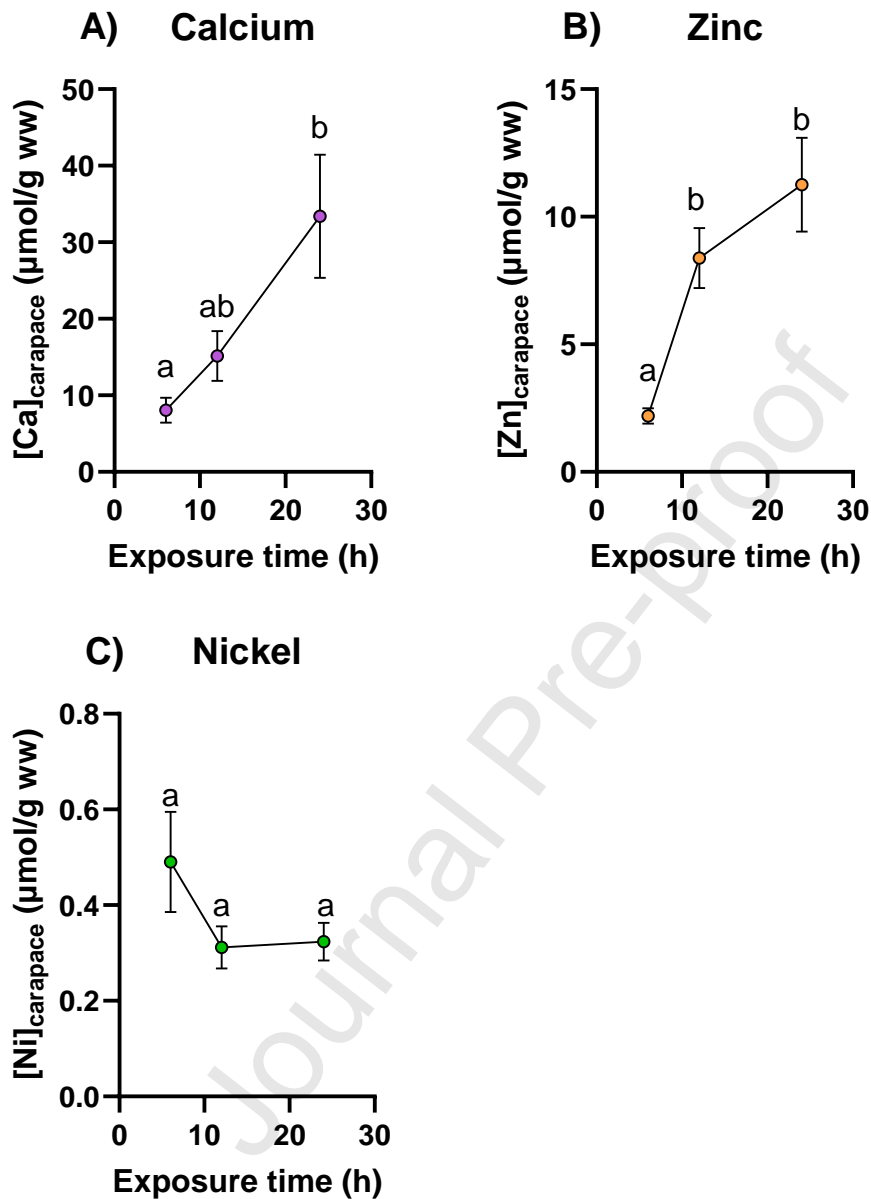


Figure 3: Concentration of newly accumulated **A)** Ca, **B)** Zn and **C)** Ni in the carapace of the green shore crab, after a 6, 12 and 24 h exposure to respectively Ca (389 mg L^{-1}), Zn ($82 \mu\text{g L}^{-1}$) or Ni ($8.2 \mu\text{g L}^{-1}$) at 12°C . Values are mean \pm SEM ($n=6-7$). For a given metal, data points not sharing a common letter are significantly different ($p < 0.05$, Tukey's or Dunn's test).

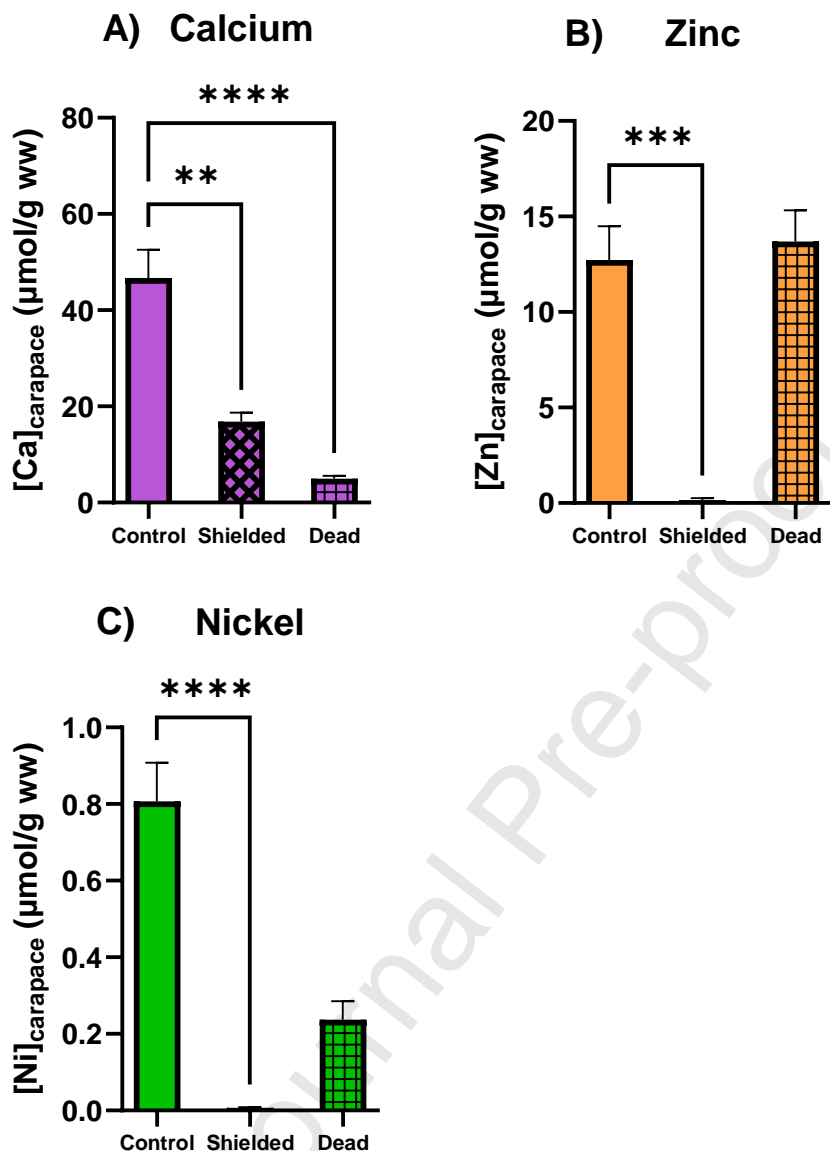


Figure 4: Concentration of newly accumulated **A) Ca**, **B) Zn** and **C) Ni** in the carapace of control crabs (live, unshielded), carapace-shielded crabs and recently deceased crabs, after a 24 h exposure to respectively Ca (389 mg L^{-1}), Zn ($82 \text{ } \mu\text{g L}^{-1}$) or Ni ($8.2 \text{ } \mu\text{g L}^{-1}$) at 12°C . Values are mean \pm SEM (n values in Supplemental Table SI.3). For a given metal, an asterisk indicates a significant difference between a treatment (shielding or euthanasia) and the control (**: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, Dunn's test).

Table 1: Whole-body percent distribution of newly accumulated Ca, Zn and Ni in various tissues of the green shore crab, after a 24 h exposure to respectively Ca (389 mg L⁻¹) Zn (82 µg L⁻¹) or Ni (8.2 µg L⁻¹) at 12°C. Values are mean ± SEM (n). For a given metal, tissues not sharing a letter are significantly different (Dunn's test).

Tissue	Calcium	Zinc	Nickel
Carapace	89 ± 4 % (7) ^a	86 ± 4 % (10) ^a	98 ± 1 % (7) ^a
Gills	0.10 ± 0.02 % (7) ^b	3.4 ± 1.0 % (10) ^{bc}	1.3 ± 0.4 % (7) ^b
Muscle	0.63 ± 0.12 % (7) ^c	0.70 ± 0.39 % (10) ^b	0.33 ± 0.15 % (7) ^b
HP	0.25 ± 0.03 % (7) ^c	0.33 ± 0.14 % (10) ^b	0.067 ± 0.024 % (7) ^b
Hem	10 ± 4 % (7) ^{bc}	9.7 ± 2.9 % (10) ^{ac}	0.36 ± 0.13 % (7) ^b

Table 2: Temperature coefficients (Q_{10}) calculated between 2 and 12°C, 12 and 22°C, and 2 and 22°C data (in Figure 2) for newly accumulated metal incorporation in various tissues over 24h.

Tissue	Calcium			Zinc			Nickel		
	2-12°C	12-22°C	2-22°C	2-12°C	12-22°C	2-22°C	2-12°C	12-22°C	2-22°C
Carapace	2.81	1.65	2.15	0.83	2.68	1.49	3.69	0.95	1.87
Gills	1.06	1.59	1.30	1.80	2.40	2.08	1.87	0.83	1.24
Muscle	-	-	-	-	-	-	0.94	0.97	0.95
HP	1.71	3.61	2.49	-	2.03	-	3.75	0.97	1.90
Hem	2.37	0.74	1.32	4.42	-	-	1.04	2.27	1.53

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Table 3: Newly accumulated metal concentrations (in nmol g⁻¹ ww) in various tissues of control (baseline) and shielded green shore crabs exposed for 24 h to Ca (389mg L⁻¹), Zn (82 µg L⁻¹) or Ni (8.2 µg L⁻¹) in seawater (32 ppt salinity) at 12°C. Values are mean ± SE (n). For a given metal, control vs. shielded crabs comparisons are described by p values from multiple t-tests with FDR correction (calcium) or Mann-Whitney tests with Holm multiple comparison (zinc and nickel).

	Control	Shielded	P Value
<u>Calcium</u>			
Gills	3260 ± 220 (20)	2300 ± 170 (7)	0.042
HP	4200 ± 480 (19)	3130 ± 630 (7)	0.24
<u>Zinc</u>			
Gills	21.6 ± 2.9 (20)	19.3 ± 2.2 (7)	0.65
Hem	1.577 ± 0.28 (14)	0.668 ± 0.070 (6)	0.064
<u>Nickel</u>			
Gills	0.533 ± 0.054 (19)	0.353 ± 0.029 (13)	0.055
Muscle	0.0165 ± 0.0040 (13)	0.0103 ± 0.0039 (7)	0.62
HP	0.0248 ± 0.0063 (13)	0.00995 ± 0.00251 (12)	0.072
Hem	0.00482 ± 0.0010 (11)	0.00466 ± 0.00202 (13)	0.60

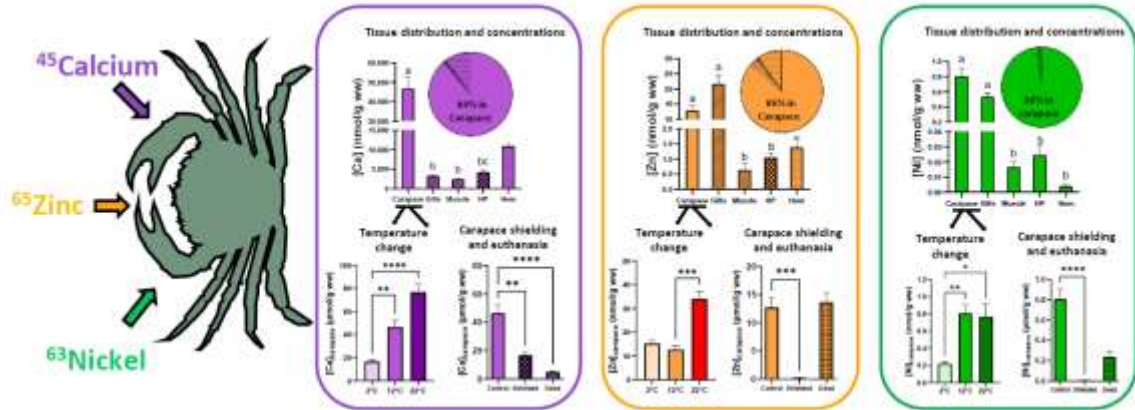
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Graphical abstract



Highlights

- Crabs exposed (24h) to radio-labeled Ca, Zn, or Ni at US EPA chronic criteria levels
- New carapace Ca, Zn, & Ni were >85% of total, with highest Q_{10} for Ca, lowest for Zn
- Carapace shielding eliminated Zn and Ni uptake, but 36% of Ca incorporation persisted
- Death did not alter Zn uptake, but reduced Ca uptake by 89% and Ni uptake by 71%
- Carapace Zn uptake was passive; life processes dominated for Ca and Ni uptake

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