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Environmental exposure of Atlantic horseshoe crab (*Limulus polyphemus*) early life stages to essential trace elements

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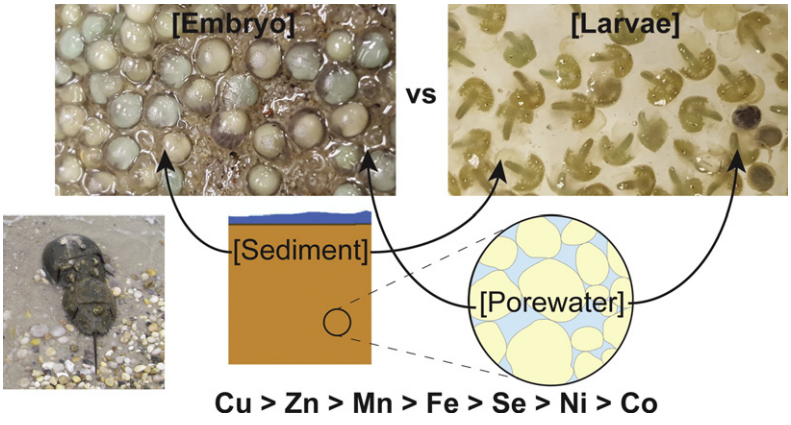
HIGHLIGHTS

- All essential trace elements were accumulated in embryo and larvae stages.
- Essential trace element accumulation resulted from sediment and water.
- Concentration of Cu, Fe, and Sn increased from egg to embryo to larvae.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the accumulation Co, Cu, Fe, Mn, Ni, Se, and Zn in Atlantic horseshoe crab (*Limulus polyphemus*) early life stages (egg, embryo and larvae) and compared the concentrations to the concentration of each element in sediment, pore water and overlying water for 5 sites across Long Island, NY. For the majority of the sites, all essential trace elements accumulated in the embryos and larvae. However, many of the embryos and larvae at specific sites presented different concentration patterns which had no apparent relationship with the local habitat sediment and water values. Generally, Cu, Fe, and Se sequentially increased from egg stage through larval stages for the majority of sites, while Co, Mn, and Ni only did for a few sites. Zinc also showed an increase across sites from embryo to larval stage, however was the only one to show a decrease in concentration from egg to embryo stage at all sites. Interestingly, Mn at Manhasset Bay presented embryo and larval stages to be 50 fold greater than all other sites while the egg stage showed similar values to other sites; this high degree of uptake could be due to a high concentration in the overlying water. All essential trace elements can be accumulated from the environment but greater concentrations may be influenced by abiotic factors and the predominant uptake route (aqueous versus diet) at each life stage. Future laboratory experiments are required to investigate factors that influence essential trace element accumulation and loss in horseshoe crab early life stages.

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1. Introduction

Trace element exposure to early life stages is an important factor in reducing reproductive success for many marine organisms because early life stages exhibit higher vulnerability to these pollutants compared to adults (Weis and Weis, 1991). The uptake of essential trace elements, including Cu, Fe, and Zn is important during embryonic and larval development in marine invertebrates. For example, Cu and Zn have been shown to be used for the synthesis of metallothioneins and metalloproteins used in cellular regulation for marine organisms (Kägi, 1991). Exposure routes consist of two different mechanisms: firstly, the maternal transfer from adult to offspring during egg development (Conley et al., 2009; Lavradas et al., 2014; Bakker et al., in review) and secondly the uptake through dissolved and particulate sources from the surrounding habitat (Wang and Fisher, 1999; Marsden et al., 2003). The absorption of dissolved particles occurs from exposure to essential trace elements in pore water (water found within the sediment) and overlying water (water that covers the sediment surface), while particulates are associated with ingested food or sediment (Wang and Fisher, 1999; Marsden and Rainbow, 2004). However, these sources can be affected by trace element partitioning among habitat components such as sediment and pore water and trace element bioavailability (Simpson and Batley, 2007). For many marine organisms, identifying the effects from these sources has important implications with understanding trace element bioaccumulation and defining uptake pathways as the hazard of each element are different and influences from the environment are complex (Luoma and Rainbow, 2005). Compared to nonessential trace elements which are toxic at low concentration, essential trace elements are required as a micronutrient, but can exert toxic effects at high concentration (Rainbow, 1985). For example, exposure to an elevated level of Se has resulted in physical deformities, including spinal curvature in freshwater fish (Lemly, 2014).

The Atlantic horseshoe crab (*Limulus polyphemus*) is a marine invertebrate found on the eastern coast of the United States between Maine and Florida (Shuster et al., 2004). Early life stages are semi-immobile (i.e. are unable to change locations within the sediment without external forces such as sediment shift or tidal forces) and are therefore exposed to pollutants, including elevated levels of essential trace elements, from their immediately surrounding habitat. Females spawn on intertidal beaches during spring high tides from April to August where the eggs develop over three to four weeks within the beach sediment and then hatch into (first instar) larvae (Botton, 2000). These stages of development can therefore be inadvertently exposed to essential trace elements in surrounding pore water and sediment which could have negative consequences if concentrations are high (Rainbow, 1985). A series of laboratory studies showed that Cu and Zn can cause developmental abnormalities, including deformed eyes and legs, and decreased survivorship in these embryo and larvae stages (Botton et al., 1998; Itow et al., 1998a, b). However, the embryos and larvae showed a high tolerance to Cu and Zn, although embryos were more susceptible to effects at lower concentrations than larvae (Botton et al., 1998). To our knowledge this is the first field study to investigate the accumulation of essential trace elements into horseshoe crab embryos and larvae due to environmental exposure from sediment, pore water and overlying water in situ.

The objective of the present study was to investigate (i) the concentrations of Co, Cu, Fe, Mn, Ni, Se, and Zn in egg, embryo and larval stages of developing horseshoe crabs collected from five beaches on Long Island, NY and (ii) determine the potential for absorption of essential elements from pore water, overlying water, and sediment in the habitat to the developing embryo and larvae. The chosen elements are all considered to be essential because although they have not been confirmed to be essential for horseshoe crabs, they are essential for other invertebrates such as Fe for fruit flies (Nichol et al., 2002), and Cu and Zn for decapods and crustaceans (Rainbow, 1993). These elements are of particular concern as they are found at elevated concentrations in heavily populated and industrialized coastal areas due to anthropogenic activities including coal-fired power plants, industrial activities, urban and agricultural runoff, boating activities and wastewater treatment plants. Furthermore, Cu, Se, and Zn are listed as priority pollutants by the United States Environmental Protection Agency (USEPA). In the present study, we assess uptake and excretion patterns of essential trace elements during development by comparing levels in unspawned eggs within females to early developing stages within the sediment (i.e. embryos) to later developing stages in the sediment (i.e. larvae).

2. Methods

2.1. Study sites and horseshoe crab collection

Horseshoe crab eggs, embryos and larvae were collected from five beaches on Long Island, NY during June to July 2015. Collections were made at two locations on the north shore of Long Island [Beekman Beach, Oyster Bay (OBY; 40°52′34.3″ N, 73°32′26.3″ W) and a private beach in Manhasset Bay (MBY; 40°50′01.9″ N, 73°43′37.2″ W)] and three locations on the south shore of Long Island [Plumb Beach, Jamaica Bay, Brooklyn (JBY; 40°34′53.3″ N, 73°54′52.0″ W); West Jones Beach Coast Guard Station, Wantagh (WJB; 40°35′24.5″ N, 73°32′57.2″ W); and Pikes Beach, Westhampton Beach (WHB; 40°46′55.0″ N, 72°42′19.3″ W)]. These sites present a unique opportunity to investigate beach habitats close to urban and industrialized areas where horseshoe crabs are potentially confronted by elevated levels of essential trace elements. Sites were also chosen based off high spawning density locations for Long Island. Site differences were not assessed because they are independent of each other, as pollution sources will differ between them, and this was outside the scope of this study. All appropriate federal and state permits were acquired prior to the collection of eggs, embryos and larvae.

In the present study, eggs were extracted from within the carapace and were used as a natural 'baseline' for the trace element concentrations they receive from maternal transfer (see Bakker et al., in review). As a result, any uptake or excretion of trace elements that occur during the embryonic stages can be determined by comparison to the levels embryos' had at spawning (i.e. received via maternal transfer). An increase in trace element concentration from egg to embryo would indicate absorption from water, while increase from embryo to larvae could either be absorption from pore water, overlying water, or ingestion of contaminated food or sediment particles from the surrounding habitat. Similarly, a decrease in an element's concentration between stages would indicate effective excretion, growth dilution, or no uptake of that element.

For all sites except WJB, the egg data presented here is data taken from our previous study investigating maternal transfer in horseshoe crabs (Bakker et al., in review). WJB samples were collected using a "drill-siphon" technique. On spawning nights, females were collected and a hole was drilled into the carapace with a 5/8th bit. Using a trace-metal clean 60 cm^3 syringe attached to a 3/16 vinyl tube, eggs were siphoned from the cavity. The hole was then covered with a disinfected rubber square using a non-toxic adhesive (ZAP®). Females were then released back to the water and the eggs were transported to Hofstra University on ice to be processed. Embryo and larvae collections for all sites were made directly from the sediment. South shore collections were made by using random plots spread 1–3 m apart at the linear high water. Clutches were less abundant at north shore locations due to low spawning densities and therefore sample plots were approximately 0.5 m apart. Stages were collected using a similar technique to Botton et al. (2006) using plastic utensils and sieves to separate embryos and larvae from debris and sediment. Since early stages are small (Botton, 2000) and to achieve an adequate sample weight for analysis, samples were pooled together by the clutch in which they were found. Samples were then transferred into a trace metal clean tube, placed on ice for transport to Hofstra University, rinsed with

Milli-Q water, and then separated by stage using a dissection scope. Embryos were separated into late stages (stage 20-1) by identifying the loss of the outer chorion egg shell layer (Botton et al., 2010). All samples were then dried at 60 °C for 48 h, homogenized into a fine powder using a mortar and pestle, and sent to the Trace Element Analysis Core Lab at Dartmouth College (Hanover, NH) for closed vessel acid digestion and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis. All samples were collected and processed using a trace metal clean technique to avoid contamination. The percentage water content in each life stage [91% (SD = 1.57; $N = 35$) for embryo and 87% (SD = 6.42; $N = 16$) for larvae] was calculated to allow for a conversion between wet weight and dry weight. Eggs from the previous maternal transfer study were comprised of 73% water [SD = 0.03; $N = 30$ (Bakker et al., in review)].

2.2. Sediment and water collection

Sediment samples ($N = 3$) were collected at all sites on random plots using a trace metal clean technique. First, 5 cm of sediment was removed, then the next 15–20 cm (depth) of sediment was gathered using plastic shovels and placed into Ziploc® bags. The first 5 cm was removed because horseshoe crab embryo and larvae typically develop in layers between 15 and 25 cm in depth (Loveland et al., 1996). Samples were then transferred to Hofstra University, dried at 60 °C for 48 h and passed through a 1 mm sieve. Since sediment samples were composed of sand and granules at every site, pore water samples were collected as seep water through excavated troughs at embryo depths (15–20 cm in depth) and sampled once tidal water seeped through the sediment. Pore water and overlying water samples ($N = 3$) were taken during embryo collections for all sites using trace metal clean 50 mL tubes and immediately placed on ice and transferred to Hofstra University. During water collections, the pH, temperature and salinity values were recorded and are reported in Table 1. All water samples were filtered using a trace metal clean syringe and 0.45 μm filters (Whatman CAT No. 6970-2504) and preserved with 50 μL of trace metal grade nitric acid. All sediment (<1 mm diameter) and water samples were sent to Dartmouth College for acid digestion (sediment only) and ICP-MS analysis.

2.3. Trace element analysis

All of the microwave digestion and ICP-MS analysis was carried out using a trace element clean technique to avoid contamination of the samples. To summarize, the embryo and larvae digestion procedure, 0.25 g of homogenized tissue was digested in 5 mL of acid (9:1 nitric acid:hydrochloric acid) in a CEM MarsXpress microwave (Matthews, NC) for 55 min (20 min ramp time to 210 °C, followed by 15 min hold time at 210 °C and 20 min cool down). The digested sample was then diluted with 45 mL of deionized water for a total tissue sample volume of approximately 50 ml (dilution factor ~ 200). To digest the sediment samples, 0.25 g of homogenized sediment was digested in 5 mL of acid (9:1 nitric acid:hydrochloric acid) using an open vessel microwave digestion procedure. Samples were ramped to 90 °C in 15 min and held at that temperature for a further 45 min. The digested samples were then diluted with 45 mL of deionized water, resulting in a total digested sediment sample volume of approximately 50 mL (dilution factor = ~200). All of the tissue and sediment samples were further diluted 10-fold before trace element analysis using ICP-MS (Agilent 7700×). All of the horseshoe crab and sediment data in this study is reported as μg/g dry weight.

2.4. Quality assurance/quality control (QA/QC)

Blanks, certified/standard reference material (DORM-4 certified fish protein, National Research Council Canada; SRM 2711 Montana II soil, National Institute of Standards and Technology (NIST); SRM 1640a trace elements in natural waters, NIST), duplicate samples and spiked samples were used for quality control ($N = 5$ for embryos and larvae; $N = 3$ for sediment; $N = 2$ for water). The quality control procedure followed the USEPA protocol SW 846 of 1 set of QA/QC per 20 samples. The blanks were BDL (below detection limit) for all elements. For horseshoe crab embryos and larvae, the recovery of DORM-4 ranged between 93 and 115%, the recovery of the spiked samples ranged between 81 and 112%, and the percentage difference between analysis duplicates averaged between 2.7 and 14.7% for all elements. For sediment, the recovery of SRM 2711 ranged between 85 and 103%, the recovery of the spiked samples ranged between 90 and 114%, and the percentage difference between analysis duplicates averaged between 2.7 and 11.4% for all

Table 1
Essential trace element concentrations in sediment (μg/g dry weight; mean ± 1 standard deviation), pore water (μg/L) and overlying water (μg/L). Temperature (°C), pH, and salinity (parts per thousand) values for water are also included. All BDL (below detection limit) values were excluded from the mean and standard deviation calculations. For sediment, BDL = <0.3 μg/g for Ni, <0.08 μg/g for Se, and <4 μg/g for Zn. For water, BDL is <2.10 μg/L for Cu, <4.90 μg/L for Fe, <1.61 μg/L for Ni, and <15.2 μg/L for Zn. $N = 3$ for all elements and samples except for sediment at WJB (Cu, $N = 2$) and in pore water at JBY (Zn, $N = 2$), MBY (Zn, $N = 2$; Cu, $N = 2$), and OBY (Zn, $N = 2$).

	Temperature (°C)	pH	Salinity (‰)	Co	Cu	Fe	Mn	Ni	Se	Zn
Jamaica Bay (JBY)										
Sediment				0.77 ± 0.07	1.68 ± 0.46	1978 ± 151	24.2 ± 0.90	2.49 ± 0.39	BDL	8.78 ± 0.78
Pore water	24.6	7.26	20.5	0.11 ± 0.02	3.81 ± 0.75	BDL	5.44 ± 1.90	BDL	0.65 ± 0.08	32.9 ± 0.46
Overlying water	24.8	8.31	27.1	0.10 ± 0.01	BDL	BDL	15.5 ± 2.33	BDL	0.14 ± 0.02	BDL
West Jones Beach (WJB)										
Sediment				0.16 ± 0.07	1.25 ± 0.29	531 ± 231	5.47 ± 1.34	BDL	BDL	BDL
Pore water	25.4	7.27	35.3	0.03 ± 0.01	BDL	BDL	0.64 ± 0.16	BDL	0.19 ± 0.03	127 ± 96.5
Overlying water	23.7	8.02	34.8	0.05 ± 0.01	BDL	BDL	13.7 ± 2.24	BDL	0.11 ± 0.02	42.8 ± 7.53
Manhasset Bay (MHB)										
Sediment				1.05 ± 0.34	2.99 ± 0.78	2849 ± 885	126 ± 16.7	3.18 ± 0.77	BDL	11.3 ± 2.72
Pore water	28.5	6.92	29.9	0.04 ± 0.01	2.21 ± 0.03	BDL	0.40 ± 0.15	BDL	0.56 ± 0.08	49.8 ± 8.48
Overlying water	27.4	8.3	30.7	0.11 ± 0.01	BDL	BDL	63.8 ± 5.73	BDL	0.15 ± 0.01	BDL
Oyster Bay (OBY)										
Sediment				0.31 ± 0.03	2.15 ± 0.42	1155 ± 139	15.5 ± 4.33	0.74 ± 0.10	BDL	5.86 ± 0.76
Pore water	27.7	6.93	16.3	0.08 ± 0.01	BDL	BDL	2.60 ± 1.61	BDL	0.31 ± 0.09	28.9 ± 9.32
Overlying water	28.5	7.41	9.9	0.11 ± 0.01	BDL	BDL	29.3 ± 2.31	BDL	0.16 ± 0.01	BDL
Westhampton Bay (WHB)										
Sediment				0.14 ± 0.05	1.30 ± 0.28	364 ± 76.9	11.0 ± 2.15	BDL	BDL	BDL
Pore water	25.9	7.09	34.2	0.11 ± 0.01	BDL	BDL	31.7 ± 9.14	BDL	0.43 ± 0.02	BDL
Overlying water	24.2	8.42	33.6	0.06 ± 0.01	BDL	BDL	30.9 ± 1.21	BDL	0.17 ± 0.01	BDL

elements. In addition, fortified blanks (spiked blanks taken through the digestion procedure) had a recovery of 94 ± 7% for the volatile elements (Se and Zn) showing there was no loss of these elements from the sediment during the open vessel microwave digestion procedure. For water, the recovery of SRM 1640a ranged between 91 and 107%, the recovery of the spiked samples ranged between 89 and 106%, and the percentage difference between analysis duplicates averaged between 0.6 and 18% for all elements.

2.5. Statistical analysis

Trace element concentrations did not satisfy parametric assumptions for normality or homogenous variances and therefore nonparametric tests were used. A Kruskal Wallis test was used to compare egg, embryo and larvae concentrations. A significant effect for the Kruskal Wallis test was followed by pairwise comparisons of egg to embryo and embryo to larvae using a Dunn post hoc test with a Bonferroni correction for multiple comparisons. If no post hoc test was significant, the overall significant Kruskal Wallis suggests that concentrations progressively changed from egg to larvae. Embryo and larvae Co concentrations for WHB (embryo: $N = 7$; larvae: $N = 5$) and egg Ni concentrations for WJB ($N = 5$) were BDL (<0.01 μg/g for Co and <0.12 μg/g for Ni) for >50% of samples and therefore were removed from the analysis. Embryo and larval stages were BDL for <50% of samples for Co at WJB (embryo: $N = 1$; larvae: $N = 1$) and for JBY (embryo: $N = 1$; larvae: $N = 3$); therefore were replaced with 50% of the detection limit (Hopkins et al. 2006). Nickel for embryo and larvae stages for WJB were compared using a Mann Whitney-*U* Test. All statistical analyses were performed using SPSS version 22.0 (IBM Corp., 2013).

3. Results

3.1. Environmental differences

Overall, the concentration of essential trace elements in sediment varied between sites (Table 1). Iron showed the highest concentration of all elements that were detectable at each site (364–2849 μg/g), followed by Mn, Zn [although Zn was BDL (<4.00 μg/g) for WJB and WHB], and Co had the lowest detected sediment values of all elements across sites (0.14–1.05 μg/g). Nickel and Cu levels fell between Zn and Co for all sites, with Ni being higher than Cu at two sites (JBY and MBY) and lower at the other three. Selenium was BDL (<0.08 μg/g) in sediment at all investigated sites.

Zinc had the highest concentration of all investigated elements in pore water and overlying water when it was detected and the average concentration was consistently higher in pore water compared to overlying water (Table 1). Iron and Ni were the lowest of all elements for water values as they were BDL (Fe = <4.90 μg/L, Ni = <1.61 μg/L) for pore water and overlying water at all sites. Copper was BDL (<2.10 μg/L) for all overlying water at each site and all but two sites (JBY and MHB) for pore water. Cobalt consistently had the lowest of all measurable water values showing a comparable value for pore water and overlying water. Selenium always showed higher pore water values while Mn typically showed higher overlying water values (except for WHB where the values were similar).

3.2. Stage differences

The average concentration of essential trace elements in Atlantic horseshoe crab eggs, embryos and larvae is shown in Table 2. For each early life stage, Zn was found at the highest concentration, followed by Cu, Fe, Mn, Se, Ni, and Co was found at the lowest concentration. All element concentrations showed significant differences between horseshoe crab egg, embryo, and larval stages for both north and south shore sites, though the statistical pattern of differences was not similar across sites (Table 3). A Mann Whitney-*U* Test showed there was a significant difference between embryo and larvae at WJB for Ni ($U = 11.0$; $P = 0.002$; see Table 2 for life stage means).

Fig. 1 shows the concentration of each essential element in Atlantic horseshoe crab early life stages and allows for a visual comparison of element concentration across successive life stages. Cobalt showed no change between egg, embryo, and larvae for two of the five sites (OBY and WHB); however, for two sites (WJB and MBY) there was a statistically significant increase from egg stage to embryo, with no significant change from embryo to larvae, while for one site (JBY) there was no significant change from egg to embryo but there was a statistically significant decrease from embryo to larvae. For 4 of the sites, the Cu concentration increased with successive life stage (Fig. 1, Table 2). There was a statistically significant increase in Cu across stages in all but one site (WHB); while only one sites showed a significant increase between successive stages (WBY; embryo versus larvae), all other sites showed a significant overall Kruskal Wallis ($P < 0.05$) indicating that terminal stages were higher than early egg stages (Table 3). The average Fe concentration increased from egg to larvae at 3 of the sites (JBY, MBY, and OBY; Table 2). Iron showed a significant increase in concentration from egg to embryo at only one site (WJB), however, the overall Kruskal Wallis ($P < 0.05$) indicates there was a significant increase in concentration from egg to larvae stage at all other sites (Table 3). The accumulation of Mn showed no relationship with developmental stage for the majority of sites, with the embryonic stage having the highest concentration at all sites except MBY (Fig. 1, Table 2). All sites except one (OBY) showed a significant change in concentration for Mn, but the pattern of change differed between sites. Three sites (JBY, WJB, and MBY) showed a significant increase from egg to embryo, while two of these three sites (JBY and, WJB), and an additional site (WHB) showed a significant decrease in Mn concentration from embryo to larvae (Fig. 1, Table 3). No clear accumulation pattern was observed for Ni (Fig. 1, Table 2). Three of the sites showed a significant change across developmental stage but again the pattern of change varied. OBY showed a progressive decrease in Ni concentration from egg to larvae, MBY showed a significant increase from egg to embryo, and WHB showed a significant decrease from egg to embryo and a significant increase from embryo to larvae (Fig. 1). The average concentration of Se increased from egg to larvae at 3 sites (JBY, WJB, and WHB), whereas Se was the highest in the embryos at the other 2 sites (Table 2). Selenium concentration increased after egg stage in four of the five sites; three sites (JBY, WJB, and OBY) showed a significant increase from egg to embryo while only one (WHB) showed an overall significant Kruskal Wallis (Table 3). Lastly, the accumulation of Zn decreased from egg to embryo and then increased from embryo to larvae at 4 sites, and decreased with successive life at one site (JBY; Fig. 1, Table 2). Zinc showed a statistically significant change across stages (Table 3), but the pattern varied between sites; one site (JBY) showed a significant decrease from egg stage to larvae, while two other sites (OBY and WHB) showed a significant decrease between egg and embryo stages. Furthermore, three sites (WJB, MBY, and OBY) showed a significant increase from embryo to larvae stage.

4. Discussion

To our knowledge this is the first in-depth study assessing local habitat essential trace element concentrations and relating these values to accumulated levels in developing embryo and larval stages in marine habitats. Prior studies have assessed the toxicological effects of some of these elements (Cu and Zn) in horseshoe crabs (Botton et al., 1998; Itow et al., 1998a, b), but none of these studies have assessed the influence of sediment and water and the relationship between these environmental factors and trace element levels in early life stages. No element showed the same accumulation pattern in all 5 of the investigated sites. Overall, we found essential trace element concentrations to be different in embryo and larval stages from pre-spawned egg levels indicating there was uptake of some elements (Cu, Fe, and Se) from the

Table 2
Essential trace element concentrations (μg/g dry weight) in Atlantic horseshoe crab (*Limulus polyphemus*) eggs, embryos, and larvae. Values represent the mean ± 1 standard error and the range is provided in parentheses. All BDL (below detection limit) values were excluded from the mean and standard error calculations. BDL is <0.01 μg/g for Co and <0.12 μg/g for Ni. $N = 10$ for all elements and life stages. Egg data for all sites, with the exception of WJB, is from a previous study (Bakker et al., in review).

	Co	Cu	Fe	Mn	Ni	Se	Zn
Jamaica Bay (JBY)							
Egg	0.16 ± 0.02 (0.08–0.24)	49.5 ± 2.76 (40.3–68.4)	39.0 ± 6.29 (25.1–91.1)	4.65 ± 0.98 (2.70–12.9)	0.70 ± 0.32 (0.13–3.10)	1.01 ± 0.09 (0.69–1.64)	137 ± 9.35 (113 − 202)
Embryo	0.20 ± 0.03 (BDL–0.42)	61.8 ± 3.25 (48.8–80.3)	44.6 ± 3.77 (30.8–70.4)	17.0 ± 4.67 (3.93–55.4)	0.22 ± 0.02 (0.16–0.36)	2.74 ± 0.54 (1.02–5.71)	118 ± 2.06 (107–127)
Larvae	0.12 ± 0.01 (BDL–0.15)	64.6 ± 2.31 (52.8–76.3)	48.2 ± 2.59 (37.7–63.4)	4.34 ± 0.29 (3.18–5.98)	0.31 ± 0.10 (0.03–1.06)	3.53 ± 0.82 (1.32–9.82)	109 ± 3.28 (94.9–127)
West Jones Beach (WJB)							
Egg	0.12 ± 0.01 (0.05–0.17)	53.9 ± 4.17 (35.2–70.8)	40.5 ± 2.70 (32.6–58.5)	4.56 ± 0.49 (2.52–7.25)	BDL	1.27 ± 0.18 (0.73–2.45)	121 ± 5.49 (102–157)
Embryo	0.25 ± 0.04 (BDL–0.49)	50.6 ± 3.57 (36.8–65.9)	552 ± 197 (94.4–1870)	18.6 ± 6.60 (3.10–72.8)	0.37 ± 0.07 (0.17–0.80)	3.42 ± 0.40 (1.74–5.38)	105 ± 3.28 (90.7–124)
Larvae	0.15 ± 0.01 (BDL–0.18)	66.9 ± 3.63 (48.8–79.4)	127 ± 12.9 (55.2–217)	4.53 ± 0.50 (3.17–7.89)	0.17 ± 0.03 (0.08–0.37)	4.04 ± 1.01 (1.68–9.83)	124 ± 3.89 (108–154)
Manhasset Bay (MBY)							
Egg	0.14 ± 0.02 (0.10–0.28)	45.0 ± 3.91 (14.4–58.5)	36.1 ± 3.25 (24.7–59.8)	10.3 ± 1.75 (3.41–20.2)	0.27 ± 0.04 (0.13–0.50)	1.71 ± 0.16 (1.10–2.63)	134 ± 7.35 (99.7–173)
Embryo	0.23 ± 0.04 (0.15–0.36)	52.9 ± 2.64 (41.8–65.2)	75.3 ± 15.5 (37.2–154)	47.7 ± 6.86 (23.7–77.3)	0.63 ± 0.08 (0.35–1.08)	2.97 ± 0.93 (1.59–9.28)	116 ± 4.28 (99.3–134)
Larvae	0.36 ± 0.11 (0.14–1.14)	71.2 ± 2.44 (52.3–80.4)	194 ± 42.8 (76.1–442)	106 ± 31.4 (25.0–323)	0.88 ± 0.18 (0.32–1.91)	2.05 ± 0.17 (1.47–3.19)	139 ± 3.85 (119–155)
Oyster Bay (OBY)							
Egg	0.17 ± 0.02 (0.09–0.34)	51.5 ± 3.78 (27.9–63.7)	44.4 ± 5.99 (28.7–87.2)	16.9 ± 3.61 (5.20–40.7)	0.45 ± 0.06 (0.24–0.85)	1.58 ± 0.13 (0.96–2.39)	151 ± 13.5 (117–246)
Embryo	0.19 ± 0.02 (0.12–0.26)	64.3 ± 2.02 (56.8–71.0)	85.1 ± 16.8 (41.9–153)	26.6 ± 4.85 (7.69–40.2)	0.33 ± 0.09 (0.17–0.85)	2.66 ± 0.32 (1.62–4.09)	106 ± 2.34 (95.4–115)
Larvae	0.17 ± 0.01 (0.10–0.23)	69.9 ± 3.85 (54.6–88.2)	172 ± 13.2 (131–228)	22.3 ± 5.56 (10.0–50.8)	0.27 ± 0.02 (0.20–0.38)	2.05 ± 0.39 (0.78–3.75)	127 ± 2.88 (115–137)
Westhampton Bay (WHB)							
Egg	0.15 ± 0.02 (0.10–0.26)	45.1 ± 3.82 (25.1–63.8)	34.9 ± 4.13 (29.7–71.8	5.32 ± 1.04 (3.20–13.0)	0.91 ± 0.67 (0.14–6.94)	2.40 ± 0.27 (1.58–4.63)	141 ± 8.59 (110–197)
Embryo	BDL	51.7 ± 3.36 (36.3–73.1)	51.7 ± 14.4 (31.2–180	6.13 ± 0.88 (3.88–13.4)	0.09 ± 0.01 (0.04–0.16)	3.41 ± 0.38 (1.45–5.31)	103 ± 2.92 (87.0–116)
Larvae	BDL	58.4 ± 3.79 (42.6–75.8)	52.0 ± 3.76 (34.6–87.2	3.79 ± 0.24 (2.95–5.52)	0.28 ± 0.08 (0.08–0.91)	7.23 ± 1.87 (2.14–21.4)	107–2.33 (95.8–119)

habitat and excretion or limited uptake of others (Co, Mn, Ni, and Zn). Each element has different chemical properties which interact with the sediment and water to influence their bioavailability and therefore accumulation. In addition, the excretion of essential trace elements in

Table 3
Nonparametric statistical comparisons and correlations for egg, embryo, and larvae. Kruskal Wallis statistic with *P* value in parentheses. Significant overall Kruskal Wallis tests were further tested with a with Dunn post hoc comparisons of each stage combination. Significant *P* values ($P < 0.05$) are indicated by *. BDL = below detection limit for at least one tissue prevented statistical analysis; NA = not applicable because analysed with Mann Whitney-*U* Test, see Methods for all statistical details. BDL for Co is <0.01 μg/g.

	Co	Cu	Fe	Mn	Ni	Se	Zn
Jamaica Bay (JBY)							
Kruskal Wallis	7.69 (0.02)*	10.6 (0.01)*	6.48 (0.03)*	15.0 (<0.01)*	0.62(0.73)	14.9 (<0.01)*	9.81 (<0.01)*
Egg versus embryo	1.00	0.06	0.27	<0.01*		0.01*	0.51
Embryo versus larvae	0.02*	1.00	1.00	0.01*		1.00	0.24
West Jones Beach (WJB)							
Kruskal Wallis	6.41 (0.04)*	8.24 (0.01)*	20.1 (<0.01)*	8.18(0.01)*	NA	15.3 (<0.01)*	9.61 (<0.01)*
Egg versus embryo	0.03*	1.00	<0.01*	0.04*		<0.01*	0.09
Embryo versus larvae	0.49	0.09	0.89	0.03*		1.00	<0.01*
Manhasset Bay (MBY)							
Kruskal Wallis	11.6 (<0.01)*	16.8 (<0.01)*	18.7(0.001)*	19.6 (<0.01)*	13.63 (<0.01)*	2.64 (0.268)	8.12 (0.01)*
Egg versus embryo	0.01*	0.99	0.09	<0.01*	<0.01*		0.12
Embryo versus larvae	1.00	0.02*	0.17	0.98	1.00		0.01*
Oyster Bay (OBY)							
Kruskal Wallis	0.85 (0.66)	11.3 (<0.01)*	16.4 (<0.01)*	2.61 (0.27)	7.29 (0.02)*	6.76 (0.03)*	15.1 (0.01)*
Egg versus embryo		0.09	0.19		0.09	0.02*	<0.01*
Embryo versus larvae		0.98	0.14		1.00	0.45	0.02*
Westhampton Bay (WHB)							
Kruskal Wallis	BDL	5.13(0.08)	13.8 (<0.01)*	9.79 (<0.01)*	14.64 (<0.01)*	9.45 (<0.01)*	16.5 (<0.01)*
Egg versus embryo			0.11	0.16	<0.01*	0.35	<0.01*
Embryo versus larvae			0.31	<0.01*	<0.01*	0.40	1.00

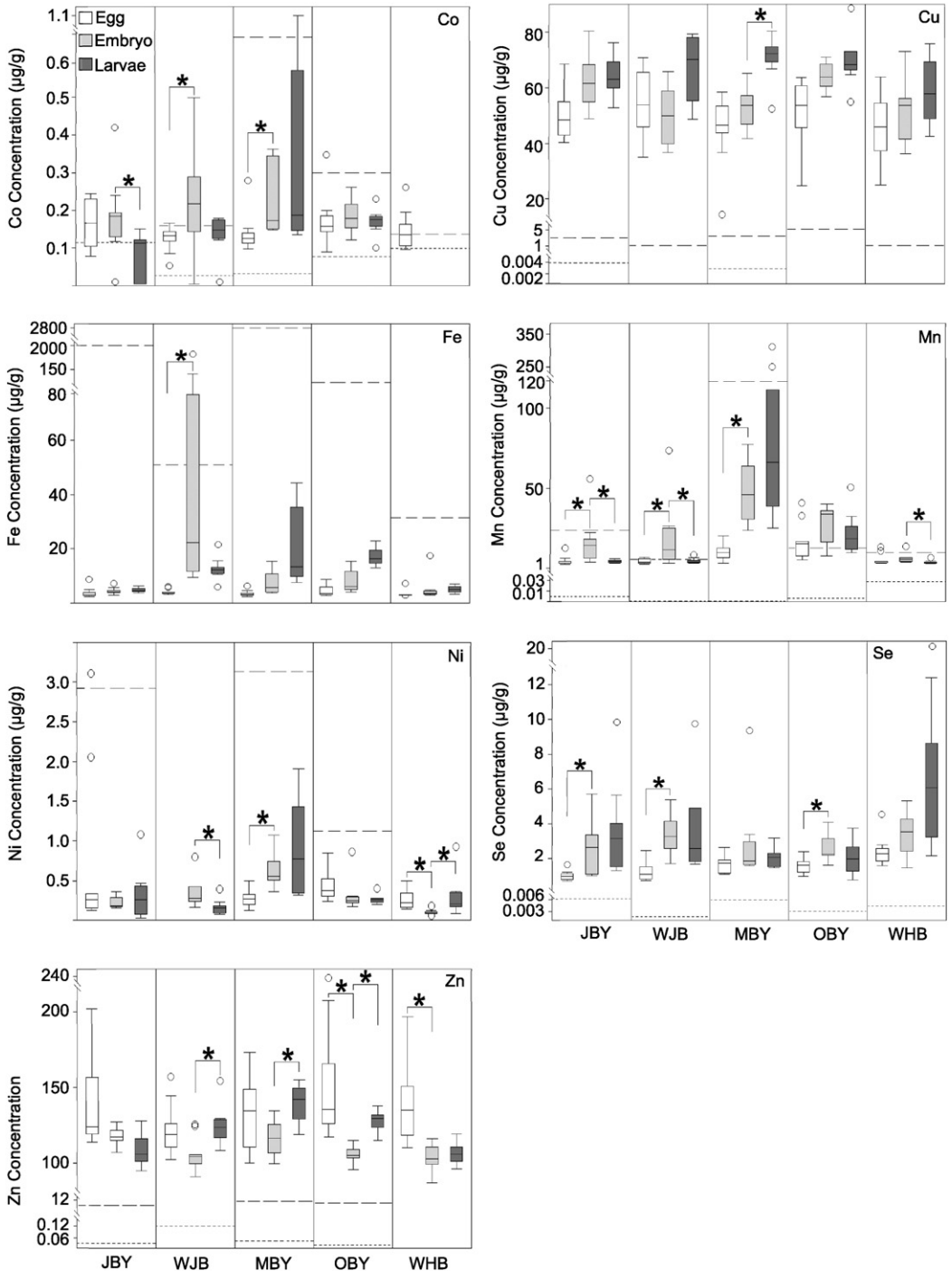

Fig. 1. Concentration (µg/g dry weight) of Co, Cu, Fe, Mn, Ni, Se and Zn in horseshoe crab (*Limulus polyphemus*) eggs (white), embryos (light grey), and larvae (dark grey). Box and whisker plot represents median (hash line within box), first and third quartiles. Whiskers indicate range between ± 1.58 IQR $n^{-1/2}$ (IQR = interquartile range; n = number of points in the distribution). Circles indicate data outside this range (outliers). Significant comparisons between stages within a site for each element are indicated; * = $P < 0.05$. Large dashed lines represent average sediment concentrations and small dashed lines represent average pore water concentrations (pore water values were converted from µg/L to µg/g for same unit comparison). Missing lines or boxes indicate BDL (below detection limit). Site abbreviations are as follows: JBY = Jamaica Bay; WJB = West Jones Beach; MBY = Manhasset Bay; OBY = Oyster Bay; and WHB = Westhampton Beach.

horseshoe crab larvae is influenced by physiological processes including growth dilution and ecdysis. Lastly, it is worth noting that the levels discovered in each life stage are not only due to the suspected predominant uptake route of that particular stage (i.e. aqueous exposure for embryos versus diet exposure for larvae), but the length of time each stage is exposed to trace elements while in the sediment. Combined, these processes could explain the observed accumulation patterns.

4.1. Local habitat factors

Within marine habitats, sediment acts as a primary sink for trace elements and interactions between various abiotic parameters in the pore water and overlying water, such as temperature, salinity, and pH, can alter element accumulation which directly affects benthic organisms (Tarique, et al., 2013). There have been previous studies in which

trace element concentrations found in tissues of benthic organisms, such as worms (*Nereis diversicolor*) and crustaceans (*Corophium volutator, Gammarus zaddachi, Palaemonetes varians, Crangon crangon, Carcinus maenas*) were representative of high element levels within the sediment (Amiard et al., 1987; Tarique, et al., 2013). However, for other benthic organisms this was not the case, as low levels in the sediment did not display the same outcome, as observed in the blue mussel *Mytilus edulis* and multiple fish taxa (Amiard et al., 1987; Heiny and Tate, 1997). Diet also plays an important role in bioaccumulation, but the diet of horseshoe crab larvae is not well known. Based on stable isotope analysis it is assumed that second instar larvae feed on sedimentary organic matter and other organisms that may derive their nutrition from phytoplankton and macroalgal sources (Gaines et al., 2002).

A key finding of the present study is that all of the investigated essential trace elements investigated accumulated at some point among the developmental stages and investigated habitats. Two elements of particular interest were Zn and Cu; both have important metabolic and enzymatic functions during development (Botton et al., 1998) and the subsequent increase from egg to embryo to larvae seen at the majority of sites for Cu is logical as they have the respiratory pigment hemocyanin. However, this was not seen for Zn which has a similar physiological function to Cu (i.e. both are used for the synthesis of metallothioneins and metalloproteins; Kagi, 1991). For the majority of sites, Zn generally showed a decrease from the egg to embryo stage and then increased from embryo to larval stages. While this could represent the importance of accumulating Zn during the embryo stage then excreting it to lower levels at the larval stage, no evidence exists to support or contradict this tendency. Despite any physiological importance, the factors that influence the accumulation of any particular element could also be explained through different abiotic factors. For example, accumulation of Cu in the beach flea *Transorchestia chiliensis* was related to air or sea temperature (Marsden et al., 2003), and Zn is known to become more available for uptake in ionic form when there is an increase in the concentration of metal binding organic ligands or a decrease of salinity (Marsden and Rainbow, 2004). This could help explain the observed accumulation pattern in OBY, which has a freshwater creek that flows into the bay close to spawning sites, which would cause the lower salinity levels we reported in this study. However, the increase in larval concentrations of Zn also seen at WJB and MBY, which both had similarly higher salinity levels, suggests there is more factors involved with this uptake mechanism.

Sediment naturally has a high concentration of Fe and Mn due to these elements being present in the earth's crust and is therefore not necessarily from high anthropogenic activities. Nevertheless, the uptake of Mn and Fe may also be related to environmental exposure time and conditions present in the habitat. For example, shallow sandy estuarine areas may intermittently encounter conditions from substantial coverage of floating macroalgae in overlying water (Viaroli et al., 1996; Krause-Jenson et al., 1999); subsequently, the sediment becomes anoxic causing the reduction of Mn and Fe oxides and ultimately the loss of Mn^{2+} and Fe^{2+} from the pore water to the overlying water (Kristiansen et al., 2002). This could explain why the Mn concentration was 2.8 to 156-times higher in the overlying water than the pore water at four of the investigated sites. These elemental forms of Mn^{2+} and Fe^{2+} are more soluble and thus become available for uptake by organisms who spend considerable amount of time in benthic zones. This effect was described for the Norway lobster, *Nephrops norvegicuus*, where an increase in concentration of Mn in tissues was found after a hypoxic event (Baden et al., 1995). A similar situation could be occurring on our north shore sites where the Long Island Sound experiences periodic hypoxic events due to algal blooms between July and September (O′ Donnell et al., 2008). Specifically MBY and OBY were found to have higher levels of Fe and Mn in the sediment, embryo, and larvae. However, oxygen parameters were not measured in this study, making any speculation about the bioavailability of Fe and Mn difficult.

Although different abiotic factors can increase the ability for uptake of trace elements, to our knowledge the mechanisms involved have not been well documented for Co, Ni and Se in aquatic organisms. While Co has been shown to accumulate in aquatic organisms via an aqueous exposure route, the conditions that influence this uptake have not been identified. A laboratory study by Rainbow and White (1990) observed the uptake of Co from solution for multiple adult crustacean species. Additionally, Co can be absorbed through the Ca^{2+} channels in gills, as has been observed for rainbow trout [*Oncorhynchus mykiss* (Richards and Playle, 1998)]. However, this may only apply to larval stages that have fully developed gills and it is not known whether embryos have calcium channels within their membranes. We only observed a statistically significant increase from egg to embryo stage at WJB and MBY and future studies are needed to identify the accumulation pathway. Nickel was also observed to increase from egg to embryo, but only at MBY. Patterns for Ni uptake may be related to similar factors for other elements like Co, Fe, and Mn because of similarities in their ionic radius and electronegativity (Hutchinson et al., 1981), however this is speculation because of the paucity of data available on this topic.

Selenium was BDL in the sediment but was detected in pore water and overlying water at all investigated sites. This observation (as well as Zn being BDL at two sites) is attributed to the sediment composition. At all investigated sites >97% of the sediment was comprised of sand and granule sized particles and had a low organic carbon content (<1%). In addition, the sediment was only covered by water during high tide and due to the high permeability of sand the surface sediment rapidly lost any subsurface water when exposed to the atmosphere. The combination of these factors resulted in a beach sediment with very low Se, and at two sites, Zn concentrations.

Uptake of Se from water has been shown in shrimp (Fowler and Benayoun, 1976) and in our current study uptake from egg to larvae occurred in three (JBY, WJB, and WHB) out of the five sites. Taken together, it appears that Se is more readily accumulated via an aqueous exposure, but there is no current data on what drives this mechanism. Though Se was not speciated, Se can be accumulated through sulfur channels (Cappon and Smith, 1982) which most likely occurred for the embryo stages. Nonetheless, the ready uptake of Se could be related to its physiologic function as it is known to be involved in the making of proteins such as selenoenzymes (Torres et al. 2014).

4.2. Physiological factors

Despite the fact that larvae have a longer exposure time than embryos in the sediment which would influence trace element levels within each stage, other physiological processes associated with each stage may also contribute to control element concentrations and have a role in an element's efflux. Once taken up into the embryos and larvae, the internal trace element concentration will be determined by several factors, including tissue distribution, storage and sequestration, biotransformation and the rate of elimination. As these processes change through an organism's life stages, the degree to which they affect element levels within organisms may also change. For example, Cu, Fe, and Zn were accumulated which could be important for embryonic development (Botton, 2000; Itow et al., 1998a, b; Srijaya et al., 2012). However, these same processes could become impaired if trace element levels are high (Burger, 1997), so concentrations may be actively regulated to protect against these toxic effects as shown for Cu and Zn in crustaceans (Rainbow and White, 1989, 1990). Copper and Zn are known to be important metabolically and for the function of many enzymes so their active regulation would be expected (Botton et al., 1998). Furthermore, a field study on the mangrove horseshoe crab (*Carcinosscorpius rotundicauda*), observed a similar pattern as observed in the present study for Zn as there was a decrease between 25 and 35 days of development but increase on day 40 (Srijaya et al., 2012). In the present study, the similar concentration patterns for both Cu and Zn across developmental stages for all sites despite varying local

conditions at each individual site supports active regulation, however further studies on horseshoe crab uptake mechanisms are required.

Similar to uptake mechanisms, excretory mechanisms for each investigated element could also be related to life history strategies. As a horseshoe crab embryo matures, it regulates and absorbs water, ions (Na^+ and Cl^-) and other elements through a non-chitinous membrane while increasing water content and metabolizing energy stores (Laughlin, 1981). Thus, elements stored in the perivitelline fluid (extra-embryonic fluid found within the chorion that suspends the embryo) could be lost during hatching. Membranes surrounding embryos will be more permeable than a chitinous exoskeleton to essential trace elements and therefore reduced uptake could occur between these two stages resulting in growth dilution. While no published studies are presently available to support this hypothesis, such a process could explain our observed statistically significant decreases in Co, Mn, and Ni concentrations between embryo and larval stages. Concentration loss was not observed for all sites though, and this could be due to individuals who have undergone varying degrees of ecdysis at the time of sampling. For example, studies using mollusks and crustaceans have observed decreases in Cu, Zn, and Se levels following ecdysis, after which the levels increase again indicating the exoskeleton is a repository for these trace elements (Fowler and Benayoun, 1976; Engel et al., 2001). Therefore, the rate at which larvae are undergoing ecdysis between sampling periods and sites could be a source of variation, masking the interaction of local habitat levels and element uptake and excretion.

In addition to the local habitat factors and physiological factors already discussed, other conditions exist that could influence uptake of essential elements from the environment. Many nonessential elements may compete with essential elements and may share pathways for uptake. For example, Cu is known to interact and compete with Pb, while Zn is known to interact and compete with Cd, due to the similar chemical characteristics (Klaassen et al., 1999). In addition, many of the nonessential elements can have negative effects at lower doses (Botton, 2000; Itow et al., 1998a, b). Therefore, it is important to investigate what elements are present in these habitats, their route of uptake, and any interactions that may exist between essential and nonessential elements.

5. Conclusion

The data in the present study indicates a general accumulation of essential elements from the surrounding environment. Although some essential elements are required as a micronutrient, high concentrations can have toxic effects. Many studies have shown that only high doses of Cu and Zn cause developmental abnormalities and increased mortality during horseshoe crab embryo and larval stages. In the current study, Cu and Zn concentration levels were lower than the levels required to have such observed effects. Additionally, based on the uptake levels we present here, the other essential elements (Co, Fe, Ni, Mn, and Se) which have not been similarly assessed deserve further attention because some habitats may be of more concern than others. For example, from egg to embryo stage we saw a 5 fold increase in Mn at MHB, while similar dramatic increases were seen at WJB but for Co (a 2 fold increase) and Fe (a 13 fold increase). The biological significance of these uptake levels requires controlled laboratory studies to determine which habitats are the most suitable and which ones are most likely to pose a threat to developing horseshoe crabs.

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