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Maternal transfer of trace elements in the Atlantic horseshoe crab (*Limulus polyphemus*)

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Abstract The maternal transfer of trace elements is a process by which offspring may accumulate trace elements from their maternal parent. Although maternal transfer has been assessed in many vertebrates, there is little understanding of this process in invertebrate species. This study investigated the maternal transfer of 13 trace elements (Ag, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, and Zn) in Atlantic horseshoe crab (*Limulus polyphemus*) eggs and compared concentrations to those in adult leg and gill tissue. For the majority of individuals, all trace elements were transferred, with the exception of Cr, from the female to the eggs. The greatest concentrations on average transferred to egg tissue were Zn (140 µg/g), Cu (47.8 µg/g), and Fe (38.6 µg/g) for essential elements and As (10.9 µg/g) and Ag (1.23 µg/g) for nonessential elements. For elements that were maternally transferred, correlation analyses were run to assess if the concentration in the eggs were similar to that of adult tissue that is completely internalized (leg) or a boundary to the external environment (gill). Positive correlations between egg and leg tissue were found for As, Hg, Se, Mn, Pb, and Ni. Mercury, Mn, Ni, and Se were the only elements correlated between egg and gill tissue. Although,

many trace elements were in low concentration in the eggs, we speculate that the higher transfer of essential elements is related to their potential benefit during early development versus nonessential trace elements, which are known to be toxic. We conclude that maternal transfer as a source of trace elements to horseshoe crabs should not be overlooked and warrants further investigation.

Keywords Maternal transfer · Atlantic horseshoe crab · *Limulus polyphemus* · Trace elements · Tissue distribution

Introduction

The accumulation of trace elements in aquatic organisms is mostly dependent on the diet and aqueous exposure (Wang and Fisher 1999; Marsden et al. 2003). However, organisms may also accumulate trace elements prior to birth or hatching through maternal transfer. Maternal transfer is the process by which the offspring receive a portion of the parent's accumulated trace elements during oogenesis. While the maternal transfer of trace elements has been documented for a wide range of oviparous organisms, including birds (Kubota et al. 2002; Bryan et al. 2003), amphibians (Hopkins et al. 2006; Bergeron et al. 2010), reptiles (Guirlet et al. 2008; Metts et al. 2013), and fish (Bang et al. 2008; Kelly et al. 2011), few studies have focused on marine invertebrates, and in particular arthropods (see Conley et al. 2009; Saxton et al. 2013; Lavradas et al. 2014).

Some trace elements have beneficial effects (typically the essential ones) while others can have toxic effects (typically the nonessential ones). Therefore, regulatory mechanisms

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might exist in terms of maternal transfer in order to increase the benefit to the offspring. There may be two consequences of maternal transfer. One is to protect offspring during oogenesis whereby only essential trace elements (e.g., Cu, Fe, and Zn) are maternally transferred while nonessential trace elements remain in maternal tissue. For example, Saxton et al. (2013) suggests such a mechanism for Hg in arthropods and this also has been shown in freshwater fish where neither inorganic nor organic Hg were maternally transferred in high concentration (Hammerschmidt et al. 1999; Johnston et al. 2001). Evidence has also been found in various taxa that maternal transfer of essential trace elements could benefit offspring physiological function and development (Hopkins et al. 2006; Guirlet et al. 2008; Lavradas et al. 2014). A second consequence is that maternal transfer may act as a detoxification pathway to decrease the female's body burden (Saxton et al. 2013) and this has been shown in birds for Hg, Pb, and Cd (Burger and Gochfeld 1991; Hughes et al. 1997; Agusa et al. 2005). However, it is also possible that there is no regulatory mechanism and transfer may be passive which could either be beneficial or detrimental.

The Atlantic horseshoe crab (*Limulus polyphemus*) is a coastal arthropod found along the Atlantic coast of North America from Maine to Florida, with a population in the Mid-Atlantic that includes the New York region (King et al. 2005). Horseshoe crab eggs, embryos and larvae provide an important food source for a variety of organisms such as migratory shorebirds, crabs, loggerhead sea turtles, and fishes (Berkson and Shuster 1999; Walls et al. 2002). Additionally, adults are commercially harvested for use as bait in eel and conch fisheries and for the production of *Limulus* amoebocyte lysate (LAL) from essential proteins in their blood by the biomedical industry (Novitsky 1984; Mikkelsen 1988; Berkson and Shuster 1999; ASMFC 2013). Over the last 30 years, there has been a decline in horseshoe crab populations; much of this decline is due to overharvesting which led the Atlantic States Marine Fisheries Commission (ASMFC) to enact fishing regulations on all fisheries (ASMFC 1998, 2013). Stock assessments have found that many populations have stabilized as a result of stricter harvest regulations, except in the New England and New York region, suggesting that the observed population declines could be a result from regional factors such as pollution (ASMFC 2013).

Waters of Long Island Sound and the New York Bight which surrounds Long Island, NY, often contain elevated concentrations of trace elements, including, but not limited to As, Cd, Cr, Cu, Pb, and Zn (O'Connor and Ehler 1991; Cochran et al. 1998). Therefore, it is reasonable to expect that this population is exposed to pollutants through ingesting contaminated prey or by absorption across the gills through aqueous exposure. Because horseshoe crabs

are a vital economic, biomedical, and ecological resource, understanding the source of trace element accumulation is critical.

Although the maternal transfer of trace elements has never been investigated in horseshoe crabs, several studies have described the levels of trace elements in their adult tissues and eggs (Burger 1997; Burger et al. 2002; Burger and Tsipoura 2014). In the assessment by Burger et al. (2002), As, Cd, Cr, Pb, Mn, Hg, and Se, were found at differing concentrations among their tissues and sampling locations between Maine and Florida. Specifically, New York locations had some of the highest concentrations of Cd, Mn, and Pb in leg and egg tissues. Moreover, several laboratory studies have described elevated rates of developmental defects and mortality in embryos and larvae of horseshoe crabs' exposed to trace metals (Botton et al. 1998; Itow et al. 1998a, b; Botton 2000). For example, Itow et al. (1998a) observed high mortality and developmental abnormalities in embryos (i.e. abnormal eyes and defective limbs) when there was continuous exposure to organic Sn and Hg. Thus, determining the level of these trace elements within eggs prior to exposure within the sediment where they develop is an important aspect to understanding the amount of accumulation they can tolerate from the environment.

The objectives of the present study were first to determine the degree of maternal transfer of seven essential elements (Co, Cu, Fe, Mn, Ni, Se, and Zn) and six non-essential elements (Ag, As, Cd, Cr, Hg, and Pb) in Atlantic horseshoe crab eggs and second to determine if the levels in eggs are correlated with those in the legs and gills of their maternal parents.

The egg tissue assessed in this study is derived from "pre-spawned" eggs that fill the space in the female's carapace. We predicted that essential trace element concentrations will be maternally inherited in greater concentrations than nonessential trace elements based on two possible, but not mutually exclusive, ideas. First, because essential elements are important for egg development and physiological function (Rainbow 1993; Nichol et al. 2002; also see references above), it is likely that regulatory mechanisms have evolved to foster their efficient maternal transfer to eggs. Second, essential elements may simply be in higher concentrations in female tissues than nonessential elements. Similarly, we expect the transfer of nonessential trace elements will be lower because in comparison to essential trace elements, they could be lower in adult concentrations and furthermore, exposure can result in lethal and sublethal effects at low concentration (Rainbow 1985). For maternal tissue we chose two different tissue types; completely internalized adult tissue (leg) and tissue that boundaries the environment (gill). While establishing a specific uptake mechanism for each element is beyond the scope of this

study, uncorrelated levels between egg tissue and adult tissue would provide evidence that gametic tissue, which ultimately forms and houses the eggs leading to maternal transfer, responds differently than somatic tissue accumulation.

With the exception of Co, Fe, and Mn, all of the investigated trace elements are listed as priority pollutants under the Clean Water Act by the United States Environmental Protection Agency and are of particular environmental concern. The majority of these elements are found at elevated concentrations in heavily populated and industrialized coastal areas due to anthropogenic activities including coal-fired power plants, industrial activities, wastewater treatment plants, boating activities, and agricultural and urban runoff (Querol et al. 1995; He et al. 2005; Duruibe et al. 2007; Singh and Turner 2009; Maranhão et al. 2015).

Materials and methods

Study sites and sample collection

Forty adult female horseshoe crabs were collected during evening spring high tides from four different beaches on Long Island, NY ($N = 10$ per field site) during May to June 2015. Collections were made at two locations on the north shore of Long Island [Beekman Beach, Oyster Bay ($40^{\circ}52'34.3''N$, $73^{\circ}32'26.3''W$) and Sands Point, Manhasset Bay ($40^{\circ}50'01.9''N$, $73^{\circ}43'37.2''W$)], and two locations on the south shore of Long Island [Plum Beach, Jamaica Bay, Brooklyn ($40^{\circ}34'53.3''N$, $73^{\circ}54'52.0''W$) and Pikes Beach, Westhampton Beach ($40^{\circ}46'55.0''N$, $72^{\circ}42'19.3''W$)]. Site differences were not assessed as this was outside the scope of our current study on maternal transfer. Furthermore, the longevity and migratory behavior of horseshoe crabs indicates individuals are not likely to be representative of the habitat from which they were captured (Smith et al. 2010; Scalfani, unpublished data). Utilizing multiple sites limited any potential negative impact the collection of adult females might have at any one site during the spawning season. All appropriate federal and state permits were acquired prior to sampling.

Adults were immediately placed on ice, and transported to the laboratory at Hofstra University where they were frozen until dissection. During dissection, prosomal width (cm) and weight (g) values were recorded and eggs, leg muscle, and gill tissue were removed, dried at $60^{\circ}C$ for 48 h and homogenized into a fine powder using a pestle and mortar. The prosomal widths ranged from 21.9 to 29.8 cm (mean \pm S.D. = 26.1 ± 1.7 cm) and the weights ranged from 1.4 to 3.7 kg (mean \pm S.D. = 2.4 ± 0.5 kg). Before drying, the gills were further dissected to remove *Limulus* leaches (*Bdelloura candida*) which are commonly found within

horseshoe crab book gills; thus element concentrations reported for gills only represent maternal tissue. To allow for comparisons with other studies, water content percentage in the tissue was calculated by comparing wet weight to dry weight. The water content for each tissue was 73 % (SD = 0.03; $N = 30$) for eggs, 81 % (SD = 0.03; $N = 11$) for leg and 85 % (SD = 0.05; $N = 6$) for gills.

Sample preparation and ICP-MS analysis

All samples were transferred to Adelphi University (Garden City, NY) for closed-vessel acid digestion and the digested samples were then sent to the Trace Element Analysis Core Laboratory at Dartmouth College (Hanover, NH) for ICP-MS analysis. To summarize the digestion procedure, 0.25 g of homogenized tissue was digested in 4 ml of acid (9:1 nitric acid:hydrochloric acid) in an Ethos-EZ microwave (Milestone Inc, Shelton, CT) using the following procedure: 20 min ramp time to $220^{\circ}C$, followed by 25 min hold time at $220^{\circ}C$ and 20 min cool down. The digested samples were then diluted with 26 ml of deionized water, resulting in a total digested sample volume of approximately 30 ml (dilution factor = ~ 120). All of the sample processing and microwave digestion was carried out using a trace element clean technique to avoid contamination of the samples.

To summarize the ICP-MS procedure, the samples were run by collision/reaction cell ICP-MS (8800 ICP-QQQ, Agilent, Santa Clara, CA) using He as a collision gas and H_2 and O_2 as reaction gases. The instrument was calibrated using NIST traceable standards and initial and continuing calibration checks and blanks were run every 10 samples. Analysis duplicates and spikes were performed at a frequency of one each per 20 samples. Internal standards were used to control for instrument drift and the instrument was recalibrated if necessary. Criteria for quality control samples adhered to EPA recommendations. All of the data is reported as $\mu g/g$ dry weight.

QA/QC

Blanks ($N = 8$), duplicate samples ($N = 9$), spiked samples ($N = 10$) and certified reference material (DORM-4 fish protein, National Research Council Canada; $N = 8$) were used for quality control. The blanks were BDL (below detection limit) for all elements. The recoveries for the spiked samples and DORM-4 ranged from 89 to 104 % for all elements, and the percentage difference between analysis duplicates ranged from 0.1 to 15 % for all elements.

Statistical analysis

Any trace element concentrations that did not satisfy parametric assumptions for normality or homogenous variances were log transformed. If the log transformed data did not meet parametric test assumptions, then nonparametric tests were used for analysis. Trace element concentrations for As, Hg, and Se in the egg, leg, and gill tissue were log transformed and compared among tissue type using parametric repeated-measures one way analysis of variance (ANOVA) test. All three trace elements for all tissues were found to not have sphericity and we therefore used the Greenhouse-Geisser statistic. If a significant effect was found using the ANOVA, pairwise comparisons were made using a Bonferroni post hoc test. Pearson correlation coefficients were used to describe relationships between eggs and maternal tissues (leg and gill) for each trace element that were maternally transferred. Leg and gill correlations were also conducted to evaluate the relationship between adult tissue completely contained within the body wall (i.e. leg appendage) and adult tissue that is in direct contact with the environment (i.e. gills). Pearson correlation significance tests for each element were Bonferroni corrected and tested at an alpha level of 0.025.

For all other trace elements the concentrations in leg, gill, and egg tissue were compared among tissue types using the nonparametric Friedman test. If an effect was found using the Friedman test, pairwise comparisons were made using a Dunn post hoc test. Spearman Rank-Order Correlations

were used to describe relationships among tissues for each trace element. Spearman Rank-Order Correlations significance test for each element were Bonferroni corrected and tested at an alpha level of 0.025.

The Cr concentration in eggs was below the detection limit for 93 % of samples ($N = 37$; $<0.12 \mu\text{g/g}$) and therefore were removed from the analysis. A Wilcoxon Signed-Rank test was used to compare leg and gill concentrations of Cr. Trace element concentrations for egg samples were BDL for Cd ($N = 4$; $<0.125 \mu\text{g/g}$), Hg ($N = 5$; $<0.042 \mu\text{g/g}$), and Pb ($N = 4$; $<0.012 \mu\text{g/g}$), and therefore were replaced with 50 % of the analytical detection limit as performed by Hopkins et al. (2006). Leg tissues were also BDL for Ag ($N = 1$; $<0.024 \mu\text{g/g}$), Cr ($N = 1$; $<0.12 \mu\text{g/g}$) and Ni ($N = 4$; $<0.12 \mu\text{g/g}$) samples and were replaced with 50 % of the detection limit for statistical analysis. All a priori analyses were tested at an alpha level of 0.05 and post hoc tests at 0.017. Statistical analysis was performed using SPSS version 22.0 (IBM Corp., Armonk, NY, 2013).

Results

Tissue trace element concentrations

The majority of adult leg and gill tissue collected had a higher concentration of essential and nonessential trace elements compared to egg tissue (Table 1). The essential elements found in the highest concentration for each

Table 1 Essential and nonessential trace element concentrations ($\mu\text{g/g}$ dry weight) in Atlantic horseshoe crab (*Limulus polyphemus*) egg, leg, and gill tissue

Trace element	Egg		Leg		Gill	
	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE	Range
Essential						
Co	0.16 ± 0.01	0.08–0.35	0.24 ± 0.01	0.12–0.41	0.40 ± 0.04	0.12–1.53
Cu	47.8 ± 1.79	14.4–68.4	86.9 ± 4.95	36.2–185	126 ± 7.70	42.2–246
Fe	38.6 ± 2.51	24.7–91.12	65.8 ± 6.75	29.6–235	2034 ± 289	161–9347
Mn	9.29 ± 1.29	2.70–40.7	5.07 ± 1.02	1.40–38.3	178 ± 40.3	8.88–1287
Ni	0.58 ± 0.18	0.13–6.94	0.25 ± 0.05	BDL–4.43	1.21 ± 0.16	0.34–4.43
Se	1.67 ± 0.12	0.69–4.63	4.10 ± 0.31	1.63–9.36	2.74 ± 0.09	1.87–4.97
Zn	140 ± 4.91	99.7–246	369 ± 3.89	321–427	69.3 ± 2.49	42.9–109
Nonessential						
Ag	1.23 ± 0.10	0.67–3.68	0.65 ± 0.16	BDL–5.54	1.77 ± 0.19	0.23–4.28
As	10.9 ± 0.65	4.03–22.2	52.3 ± 3.14	18.4–118	27.7 ± 1.47	14.5–53.2
Cd	0.04 ± 0.005	BDL–0.17	0.03 ± 0.004	0.01–0.11	0.41 ± 0.03	0.14–0.81
Cr	BDL	BDL	0.44 ± 0.05	BDL–1.64	2.23 ± 0.34	0.42–10.9
Hg	0.07 ± 0.004	BDL–0.16	0.30 ± 0.03	0.09–0.63	0.12 ± 0.01	0.04–0.31
Pb	0.05 ± 0.007	BDL–0.22	0.20 ± 0.04	0.04–1.61	14.4 ± 2.35	0.39–60.2

All BDL (below detection limit) values were excluded from the mean and standard error calculations. $N = 40$ for all elements and tissues except for Cd, Hg and Pb in egg tissue ($N = 36, 35, 36$, respectively) and Ni, Ag and Cr in leg tissue ($N = 36, 39, 39$, respectively)

Table 2 Nonparametric statistical comparisons and correlations for egg, leg, and gill tissue

Trace element	Friedman test X^2	Pairwise comparisons			Spearman rho		
		Leg versus egg	Gill versus egg	Leg versus gill	Leg versus egg	Gill versus egg	Leg versus gill
Essential							
Co	33.45 (0.001)*	0.133	<0.001*	<0.001*	0.180 (0.267)	0.164 (0.312)	0.451 (0.004)*
Cu	66.95 (0.001)*	<0.001*	<0.001*	<0.001*	0.252 (0.117)	0.111 (0.497)	0.577 (<0.001)*
Fe	71.25 (0.001)*	0.002*	<0.001*	<0.001*	0.256 (0.111)	0.086 (0.598)	0.531 (<0.001)*
Mn	68.45 (0.001)*	0.011*	<0.001*	<0.001*	0.493 (0.001)*	0.585 (<0.001)*	0.432 (0.005)*
Ni	57.05 (0.001)*	0.011*	<0.001*	<0.001*	0.364 (0.021)*	0.356 (0.024)*	0.795 (<0.001)*
Zn	80.00 (0.001)*	<0.001*	<0.001*	<0.001*	0.129 (0.429)	0.232 (0.149)	0.048 (0.769)
Nonessential							
Ag	44.15 (0.001)*	<0.001*	1.00	<0.001*	0.211 (0.191)	0.191 (0.237)	0.660 (<0.001)*
Cd	60.80 (0.001)*	1.00	<0.001*	<0.001*	0.182 (0.262)	0.069 (0.671)	0.512 (0.001)*
Cr	BDL	BDL	BDL	NA	BDL	BDL	0.364 (0.021)*
Pb	76.20 (0.001)*	<0.001*	<0.001*	<0.001*	0.586 (<0.001)*	0.305 (0.056)	0.682 (<0.001)*

Chi square statistic with p value in parentheses provided for Friedman Test. Significant overall Friedman tests were followed by post hoc pairwise comparisons of each tissue combination and p values provided ($\alpha = 0.0175$). Rho values for Spearman Rank-Order Correlations provided for each tissue combination with p values in parentheses ($\alpha = 0.05$). Significant p values indicated by *. (BDL below detection limit for at least one tissue prevented statistical analysis, NA not applicable, analyzed with Wilcoxon Signed Rank Test, see Materials and Methods for all statistical details)

respective tissue were; egg: Zn (140 $\mu\text{g/g}$) and Cu (47.8 $\mu\text{g/g}$), leg: Zn (369 $\mu\text{g/g}$) and Cu (86.9 $\mu\text{g/g}$), and gill: Fe (2034 $\mu\text{g/g}$) and Mn (178 $\mu\text{g/g}$). The nonessential elements found in highest concentration were for egg: As (10.9 $\mu\text{g/g}$) and Ag (1.23 $\mu\text{g/g}$), leg: As (52.3 $\mu\text{g/g}$) and Ag (0.65 $\mu\text{g/g}$), and gill: As (27.7 $\mu\text{g/g}$) and Pb 14.4 $\mu\text{g/g}$. Chromium was the only element not observed in egg tissue.

The one-way repeated measure ANOVA for the essential trace elements comparing all tissues showed a significant overall effect for Se [$F(1.954, 76.206) = 120.122$, $p < 0.0001$]. Additionally, a post hoc Bonferroni test indicated significant differences whereby leg tissue was higher than egg ($p < 0.0001$), gill tissue was higher than egg ($p < 0.0001$), and leg tissue was higher than gill ($p < 0.0001$; see Table 1 for tissue means). A Friedman test showed a significant overall effect among all three tissues for all other essential trace elements (Table 2). Post hoc tests for Co, Cu, and Fe showed that concentrations were highest in the gill compared to leg and egg, and Cu and Fe were highest in the leg when compared to egg (Tables 1 and 2). The concentrations of Mn and Ni were highest in the gill compared to leg and egg, but egg was higher than leg. Lastly, the concentration of Zn was highest in the leg compared to gill and egg, and egg was higher than gill (Tables 1 and 2).

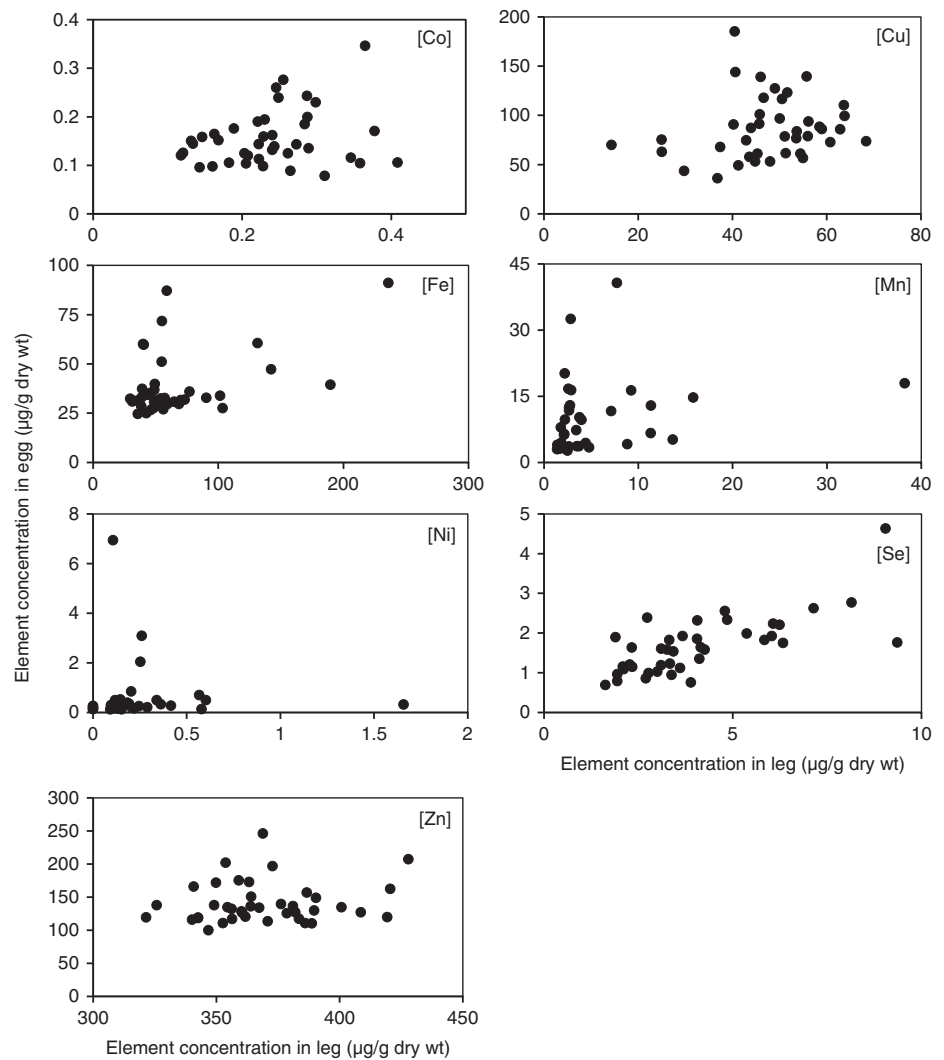
The one-way repeated measures ANOVA for the non-essential elements comparing all tissues showed a significant overall effect for As [$F(1.442, 56.237) = 280.247$, $p < 0.0001$] and Hg [Hg: $F(1.31, 52.13) = 133.299$, $p < 0.0001$]. Post hoc Bonferroni tests indicated a significant differences whereby leg tissue was higher than egg ($p <$

0.0001), gill was higher than egg ($p < 0.0001$), and leg was higher than gill ($p < 0.0001$) for both elements (see Table 1 for tissue means). Similarly, the Friedman test showed a significant overall effect among all three tissues for Ag, Cd, and Pb (Table 2). Post hoc tests revealed that the Ag concentration in the gill was higher than in the leg and that egg was greater than leg; the concentrations of Pb was the highest in the gill compared to leg and egg, and leg was greater than egg; and the concentration of Cd was higher in the gill compared to egg (Tables 1 and 2). Chromium was the only element where more than 50 % of the samples were BDL for egg tissue and therefore only leg and gill were compared. Chromium levels in gills were greater than leg tissue (Wilcoxon signed-rank test; $Z = 5.39$, $p < 0.0001$).

Maternal transfer and tissue relationships

The relationship between the concentration of each essential trace element in the leg and egg, and gill and egg is shown in Figs. 1 and 2, respectively. A Pearson correlation coefficient was calculated for the relationship between leg versus egg and gill versus egg for Se. Selenium had a significant positive correlation for leg versus egg [$r(38) = 0.709$, $p < 0.001$] and gill versus egg [$r(38) = 0.413$, $p = 0.008$]. Additionally, the relationship between tissues for all other essential trace elements (Co, Cu, Fe, Mn, Ni, and Zn) were analyzed using a Spearman Rank-Order coefficient and Mn and Ni were the only two elements to be significantly and positively correlated between leg and egg, and gill and egg (Figs. 1 and 2, Table 2).

Fig. 1 Relationship between the concentration ($\mu\text{g/g}$ dry weight) of essential trace elements in egg and leg tissue in Atlantic horseshoe crab (*Limulus polyphemus*). $N=40$ for each element; BDL (below detection limit) values are included



The relationship between the concentration of each nonessential trace element in the leg and egg, and gill and egg is shown in Figs. 3 and 4, respectively. A Pearson correlation coefficient was calculated for the relationship between leg and egg, and gill and egg for Hg and As. A significant positive correlation was found for Hg in leg versus egg [Hg: $r(38) = 0.623$, $p < 0.001$; Fig. 3] and gill versus egg [Hg: $r(38) = 0.506$, $p < 0.001$; Fig. 4]. The concentrations of As was not significantly correlated for leg versus egg [As: $r(38) = 0.04$, $p = 0.81$; Fig. 3] and for gill versus egg [$r(38) = -0.067$, $p = 0.683$; Fig. 4]. Silver, Cd, Cr, and Pb were analyzed using a Spearman Rank-Order coefficient where Pb was the only element to be significantly and positively correlated between leg and egg (Figs. 3 and 4, Table 2); none of the investigated elements were correlated between gill and egg.

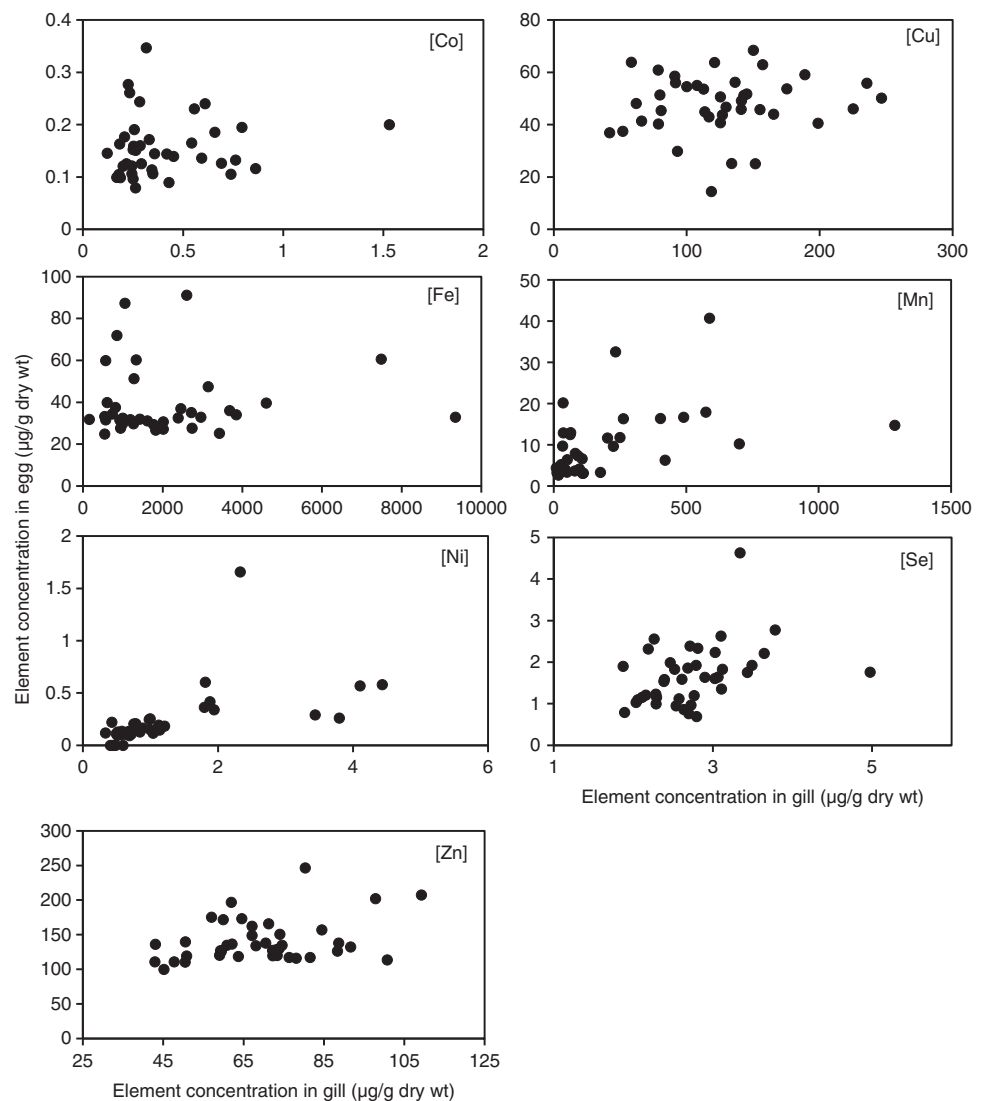
Lastly, the relationship between the concentration of essential trace elements in leg and gill are shown in Table 2. A Pearson correlation for Se found a significant and positive

relationship between leg versus gill [$r(38) = 0.744$, $p < 0.001$]. Additionally, a Spearman Rank-Order found that Co, Cu, Fe, Mn and Ni were also positively correlated between the tissues. When comparing the concentration of As and Hg in leg and gill tissue there was a significant and positive correlation [As: $r(38) = 0.741$, $p < 0.001$; Hg: $r(38) = 0.658$, $p < 0.001$]. In addition, a Spearman Rank-Order correlation found that Ag, Cd, Cr, and Pb were correlated between the tissues (Table 2). There were no significant correlations between any tissues for Zn.

Discussion

To our knowledge this is the first thorough study investigating the maternal transfer of trace elements in the Atlantic horseshoe crab. While Burger et al. (2002) investigated the concentration of trace elements in egg and leg tissue in horseshoe crabs collected from Maine to Florida, maternal

Fig. 2 Relationship between the concentration ($\mu\text{g/g}$ dry weight) of essential trace elements in egg and gill tissue in Atlantic horseshoe crab (*Limulus polyphemus*). $N = 40$ for each element; BDL (below detection limit) values are included



transfer was not discussed. In the present study, both essential and nonessential trace elements were found to be maternally transferred to horseshoe crab eggs, with the exception of Cr.

Supporting our prediction, the majority of essential elements were maternally transferred in greater concentrations than the majority of nonessential elements, with Cu, Fe, and Zn accumulating to the highest concentrations in the eggs. There are two possible, non-mutually exclusive factors which could lead to this. First, essential elements could be required for embryonic development and physiological function (Botton 2000; Itow et al. 1998a, b; Srijaya et al. 2012) and therefore explains this difference. However As, a nonessential element, was also maternally transferred in high concentrations, though it was the only nonessential element to do so to this magnitude. Another possible factor is that the amount transferred is based in part on the concentrations within adult females. Ranking the elements

using the values in Table 1, we find that the top seven elements are ranked in this order from highest to lowest: Zn, Cu, Fe, As, Mn, Se, Ag for both egg and leg tissue suggesting there is an effect of adult female concentrations on the amount transferred to eggs. However, this does not explain all the elements. The rank of the egg concentrations of the remaining six elements from highest to lowest are Ni, Co, Hg, Pb, Cd, Cr and not related to their rank order for leg concentrations which is Cr, Hg, Ni, Co, Pb, Cd. The fact that the majority of elements that are found in highest concentration are also essential makes it difficult to distinguish between these two possibilities at this time.

Regarding somatic adult tissue, there were differences in concentrations as well. Leg muscle tissue had the highest concentration of Zn, Se, and As whereas gill tissue had the highest concentration of Cu, Fe, Mn, and Pb. While the reason for this is unclear, it is likely related to the direct exposure gills have to environmental levels of trace

Fig. 3 Relationship between the concentration ($\mu\text{g/g}$ dry weight) of nonessential trace elements in egg and leg tissue in Atlantic horseshoe crab (*Limulus polyphemus*). $N=40$ for each element; BDL (below detection limit) values are included

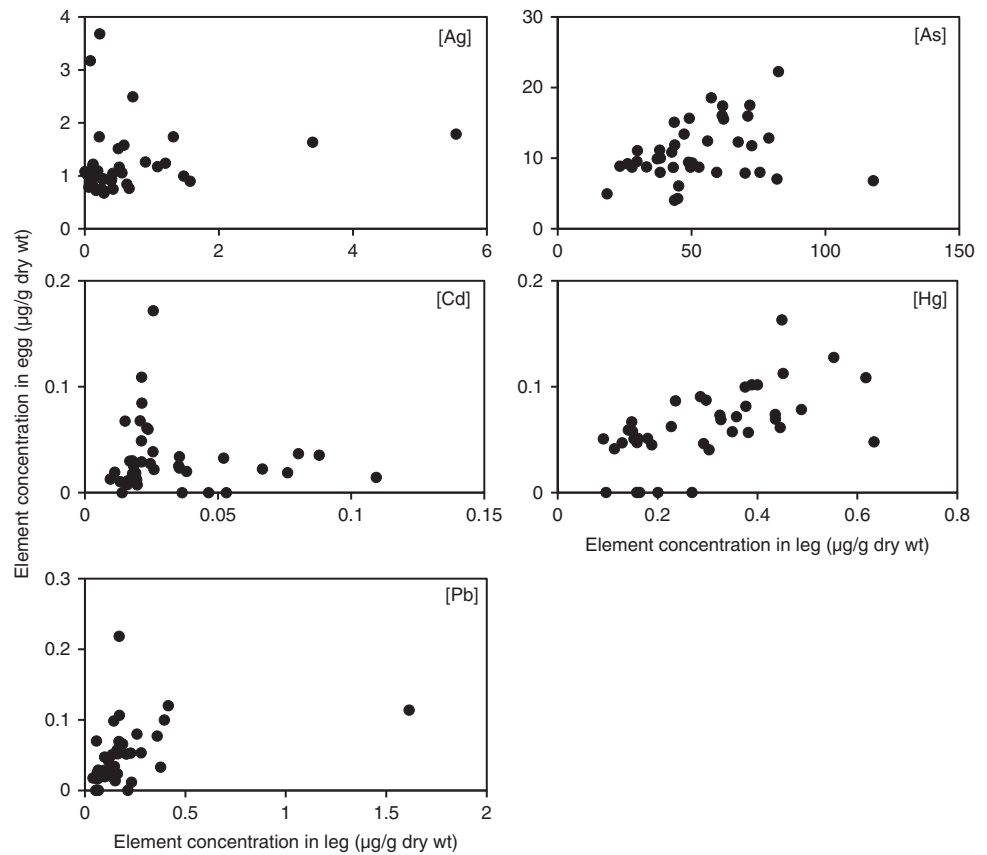
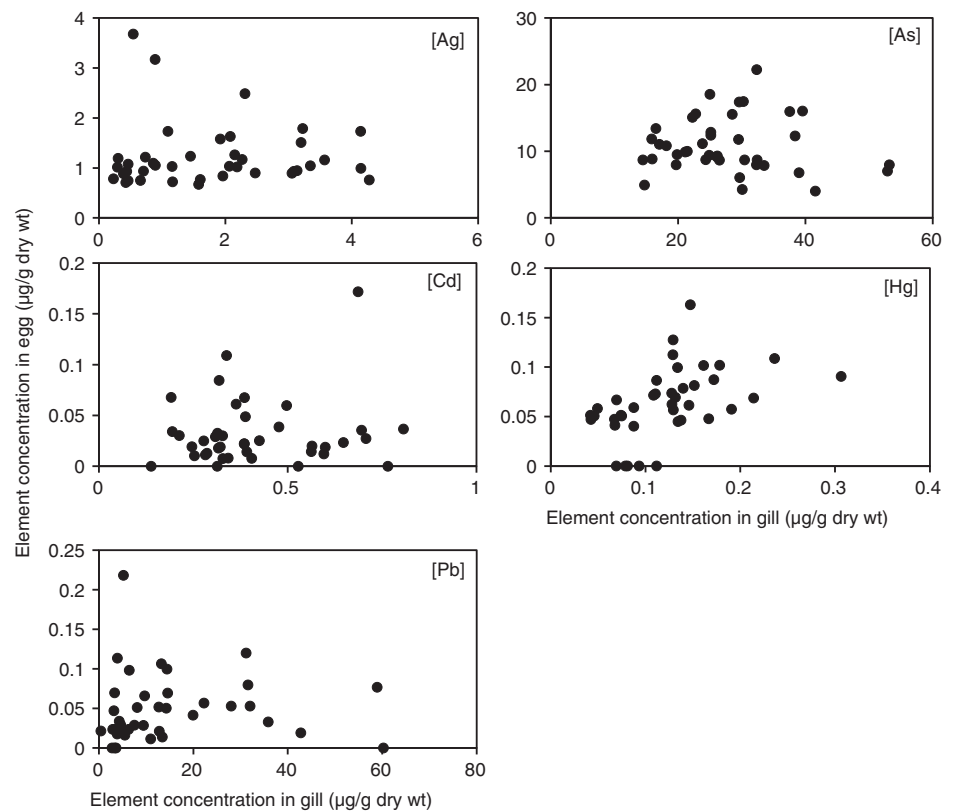


Fig. 4 Relationship between the concentration ($\mu\text{g/g}$ dry weight) of nonessential trace elements in egg and gill tissue in Atlantic horseshoe crab (*Limulus polyphemus*). $N=40$ for each element; BDL (below detection limit) values are included



elements as well as differences in tissue structure and function. Although it was not possible to address the specific mechanisms of uptake for all 13 elements across the three tissues examined, the relationship between the levels found in the different tissues indicate some interesting differences between the accumulation of trace elements in the eggs due to maternal transfer and that of somatic tissues. For example, with the exception of Zn, the concentration of all elements was related between the two adult somatic tissue types, leg and gill. This indicates that whatever the process is that underlies the uptake, it happens at consistent rates based on the presence of these elements surrounding these tissues within blood. However, only a subset of these elements (Hg, Mn, Ni, and Se) showed a similar uptake between both these adult tissues and egg tissue. All other elements lacked a significant correlation to both adult tissues indicating that their uptake in gametic tissue is different than the uptake that happens in somatic tissue. Thus, the process for maternal transfer for the elements that do not show a relationship between somatic tissue and egg tissue (Ag, As, Cd, Co, Cu, Fe, and Zn) is likely a mechanism related either to a specific physiologic process, or to a unique characteristic of egg tissue versus somatic tissue. Lead was the only nonessential element that only showed a related uptake between one somatic tissue (leg) and gametic egg tissue.

A subset of the 13 trace elements quantified here hold particular interest due to their known function. Two of these are Cu and Zn which were found in high concentrations in all tissues and were maternally transferred. Copper is required in hemocyanin, a group of metalloproteins that transport oxygen in horseshoe crabs and Zn is known to be involved in the horseshoe crab immune system (reviewed in Iwanaga and Lee 2005). The level of hemocyanin has been found to rapidly increase through horseshoe crab embryonic development (Sugita 1988; Rainbow 1993). In a previous experiment by Botton et al. (1998), horseshoe crab embryo and larvae were exposed to different concentrations of both Zn and Cu for 24, 48, and 72 h. In most trials, no mortality or developmental effects occurred between the embryo and larval stages, though some experienced structural abnormalities but only in higher concentrations than found naturally. While it is unclear if the maternal transfer that occurs is an active or passive mechanism, the high tolerance shown in Botton et al.'s study combined with the high levels we show to be maternally transferred might suggest this uptake is beneficial.

The non-significant correlations observed in this study for Zn and Cu between egg tissue and either adult somatic tissue could indicate a regulatory process specific to eggs influencing these elements transfer. If early accumulation of these essential elements has a developmental benefit, then maternal transfer could be a pathway by which females aid

their offspring. However, without data on the relationship between offspring survivorship and maternal transfer, such a conclusion can only be speculation at this time. It is also possible that this transfer could act as a purge of elevated adult female levels in an effort to release their body burden (Saxton et al. 2013).

Selenium is essential for all organisms that possess a nervous system because it is crucial for the activity of selenoenzymes, which protect the nervous systems from oxidative damage (Torres et al. 2014). In addition, it has long been perceived that Se may also protect organisms from Hg toxicity. Selenium is known to have an antagonistic relationship with Hg and studies investigating this relationship in fish have argued that if the Se:Hg molar ratio is greater than 1 then Se may offer some protection against the deleterious effects of Hg exposure (Kaneko and Ralston 2007; Peterson et al. 2009; Burger and Gochfeld 2013). The average Se:Hg molar ratio for horseshoe crab egg, leg and gill tissue in this study was 67.1, 34.9 and 57.3, respectively. These high Se:Hg molar ratios indicate that Se may have a protective role against Hg exposure, and therefore the uptake of Se in adult gametic as well as somatic tissue could be important physiologically.

The remaining essential trace elements (Co, Fe, Mn, and Ni) were found to be transferred in varying concentrations, though still transferred in higher amounts than many of the nonessential elements. Less is known about these four elements, and thus explaining their transfer and uptake is difficult. Cobalt can be absorbed through Ca^{2+} channels as has been shown in the gills of rainbow trout (Richards and Playle 1998) and previous studies have shown both positive and negative effects with this element (reviewed in Kim et al. 2006). However, we are not aware of any current studies on the essential role it has in marine organisms. In many invertebrates, Fe is a catalyst for oxidative metabolism (Nichol et al. 2002) and is used in blood-born respiratory pigments such as an iron-bearing hemoglobin, chlorocruorin, or hemerythrin (Rainbow 1993). However, horseshoe crab blood contains copper-bearing hemocyanin so the possible mechanism of Fe uptake and use in this study is also unclear. The benefits of Mn are unclear, but it is a known cofactor for various body mechanisms in humans (Drown et al. 1986). Finally, Ni has been suggested to have a homeostatic regulation role in rainbow trout (Chowdhury et al. 2008), and a review by Muysen et al. (2004) explored the element's use in many different taxa yet no study has explored its biological function for invertebrates. Overall these four elements are considered essential and transfer might have beneficial effects on offspring development, however we can only speculate on the mechanism of their uptake. The levels of Co and Fe in gametic tissue were not correlated to the levels seen in either adult tissue (whose levels were correlated to each

other) indicating some regulatory process or structural trait specific to eggs that influences the maternal transfer of these elements. In comparison, the levels of Mn and Ni were both correlated among all tissues indicating the uptake processes among gametic and somatic tissue for these elements are likely the same.

Levels of As in leg and gill tissues were observed to be the highest of all nonessential trace elements, but only the levels found in leg tissue were correlated to levels found in egg tissue. Interestingly, although levels were correlated between egg tissue and leg tissue, egg values were fivefold lower than leg tissue. We speculate that a regulatory mechanism may exist that decreases the amount of As transferred to the eggs thus increasing offspring survival and development. Future studies should investigate whether it is inorganic or organic As that is maternally transferred. Studies using phytoplankton and fish have shown that inorganic arsenate [As(V)], the dominant As species in seawater (Neff 1997), shares the same uptake pathway as phosphate, an essential element, into cells (Sanders and Windom 1980; Beene et al. 2011). Once inside the cell, As (V) can be converted into arsenobetaine ($C_5H_{11}AsO_2$). In addition, previous studies have shown that marine invertebrates and fish cannot readily bioconcentrate inorganic As from seawater, but can bioaccumulate organoarsenic species, particularly arsenobetaine from their diet (Neff 1997).

While many nonessential elements were detected, with the exception of Cr, it is possible that concentrations in the female were not high enough to reach a threshold for transfer to occur into the eggs. Cadmium and Pb concentrations were also found in all tissues but with relative lower levels in the leg and egg. In freshwater fish, evidence has shown that Cd and Pb enter through the gills via the same uptake pathway as Ca^{2+} , an essential element (Franklin et al. 2005; Rogers and Wood 2004), which may explain significantly higher concentrations of Cd and Pb in the gills. It is unclear why levels of Cd were much lower in leg and egg compared to gills. We can only speculate that Cd is not readily redistributed from the gills to internal tissue thus resulting in these lower levels within internal tissue. However, it is worth noting that the Pb concentrations were significantly higher in leg than egg which may indicate a form of protection for the eggs.

Lastly, Cr was the only element to not be detected in any of the egg tissue, but was in the leg and gill tissue. The reason for this lack of maternal transfer is not apparent to us as Cr was not speciated into Cr(VI) and Cr(III) in this study. Both species of Cr are known to accumulate in marine invertebrates, as observed in mussels (Wang et al. 1997) and Cr(III), unlike Cr(VI), does not readily cross biological membranes (Oshida et al. 1981). It is also possible that concentrations in the female were not high enough to reach a threshold for transfer to occur into the eggs.

While the mechanistic processes of maternal transfer are unknown for invertebrates, including horseshoe crabs, evidence from what is known for vertebrate maternal transfer might provide some insight. For example, sharks nourish their offspring from primary energy stores that come from their lipid-dense liver, which is known to accumulate contaminants (Wourms and Demski 1993). This process was further demonstrated by Lyons and Lowe (2013), where Hg and organochlorine contaminants were transferred from the liver and not muscle tissue in the common thresher shark (*Alopias vulpinus*). If the hepatopancreas (a homologous organ to the vertebrate liver) of horseshoe crabs functions similarly, then this could be the starting point for assessing the process of this maternal transfer. As such, a study on the Japanese horseshoe crab (*Tachypleus tridentatus*) indicated the potential for maternal transfer of trace elements (Cd, Co, Cd, Fe, Hg, Ni Mn, Pb, and Zn) from the hepatopancreas during vitellogenesis (Kannan et al. 1995). However, a low sample size ($N = 1$) hindered conclusive evidence for such a process to be analyzed.

Though not addressed in this study, one final related factor that has important consequences for the interaction of trace element contamination and horseshoe crab population dynamics is bioaccumulation from the surrounding sediment, pore water, and overlying water during embryo development. Horseshoe crab eggs develop for approximately 4 weeks in sediments before hatching into larvae, where they then remain for varying amounts of time before entering the ocean (Loveland et al. 1996). Accumulation from the surrounding environment during these post spawning stages has been shown in turtle eggs (Marco et al. 2004) and is likely as important to trace element accumulation as maternal transfer dynamics. An increase in trace element accumulation due to the surrounding environment could have negative consequences if it increased levels that were already high due to maternal transfer processes. While some studies have simulated such a process by exposing horseshoe crab early life-stages to different levels of trace elements (see Botton et al. 1998; Itow et al. 1998a, b; Botton 2000), sediment in these studies was removed to control for elements binding to sediment particles. As such, more work needs to be done on the natural incubation period and dynamics of embryos in their natural environments. Such data could then be combined with the current study on maternal transfer processes to fully understand the dynamics of trace elements on horseshoe crab populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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