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Exposure to heavy metal stress does not increase fluctuating asymmetry in populations of isopod and hardwood trees

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ABSTRACT

Fluctuating asymmetry (FA) refers to random, small and non-directional deviations from ideal bilateral symmetry is proposed as a bio-indicator of abiotic stress in both animals and plant. We investigated the effect of heavy metal stress on FA levels of morphological traits in a terrestrial isopod (*Philoscia muscorum*) as well as in the leaves of two hardwood tree species: Gray birch (*Betula populifolia*) and eastern cottonwood (*Populus deltoides*), in an urban brownfield in New Jersey. FA levels measured for five traits (length of two segments of antennae and three segments of the seventh legs) were compared in male and female populations of *P. muscorum* sampled from three low and three high soil metal load sites within the brownfield. FA levels measured for leaf width (perpendicular distance from a midpoint on midrib to the widest point of the lamina in right and left half in a leaf) were compared for both gray birch and eastern cottonwood leaves collected from the same low and high soil metal load sites. Contrary to the hypothesis that FA increases with higher heavy metal stress in isopods and trees, our results revealed that true asymmetry in gray birch and for some isopod traits (2nd antenna article, 3rd antenna article and merus of males and females, 3rd antenna article of males, and prodopus in females) did not differ between low and high metal contaminated sites. Furthermore, FAs measured in eastern cottonwood leaves and other isopod traits (carpus and prodopus in males) were found to be even lower at high metal contaminated sites than the low metal load sites. The overall effect of metal stress was shown as reduction in growth (measured as body size for a given head width in an individual) of isopods at high metal load sites as compared to the low metal load sites. Various hypotheses including induction of detoxification mechanisms in response to metal stress, selection against individuals with presumably lower fitness (high FA), difference in sensitivity of traits to stress, and plasticity are discussed to explain the observed lack of a significant association between FA and heavy metal stress in isopods and trees.

1. Introduction

Soil contamination with metals is a widespread environmental problem in urban and post-industrial regions throughout the world (Alloway 1995; Mireles et al., 2012; Yaylalı-Abanuz 2011). Metals such as zinc, lead, copper, arsenic, are often present in urban soils at concentrations greater than background and regulatory levels (Ge et al., 2000). Industrial activities, energy production, construction activities, traffic and vehicular emissions, and municipal wastes are main sources of heavy metals in urban soils (Wei and Yang 2010; Wong et al., 2006). Most of these metals persist in the soils for long time as they are not biodegradable. Therefore they have the potential to alter the physical, chemical and biological properties of the soils (Friedlova 2010; Pouyat et al., 2010) ultimately having adverse effects on urban ecosystems. Considering the growth of urban landscapes, their complexity and input of metals from a variety of sources; monitoring the impacts of metal induced stress on urban ecological health is an increasing challenge for biologists and environmental scientists (Li et al., 2013).

To assess the effect of metal induced stress on ecosystem health, previous studies have focused on quantifying metal concentrations in soils or in tissues of various plants and animals (Ge et al., 2000; Gallagher et al., 2008a; Godet et al., 2011; Manta et al., 2002; Sawidis et al., 2011; Tomašević et al., 2004), and on examining changes in species richness or diversity of different taxa of plants and animals (Fountain and Hopkin 2004; Murray et al., 2000). Most of the methods employed in these studies are often quite expensive, invasive, and time consuming. Over recent years, examining fluctuating asymmetry (FA) in populations of plants and animals has been proposed as an attractive, inexpensive, yet efficient, non-invasive

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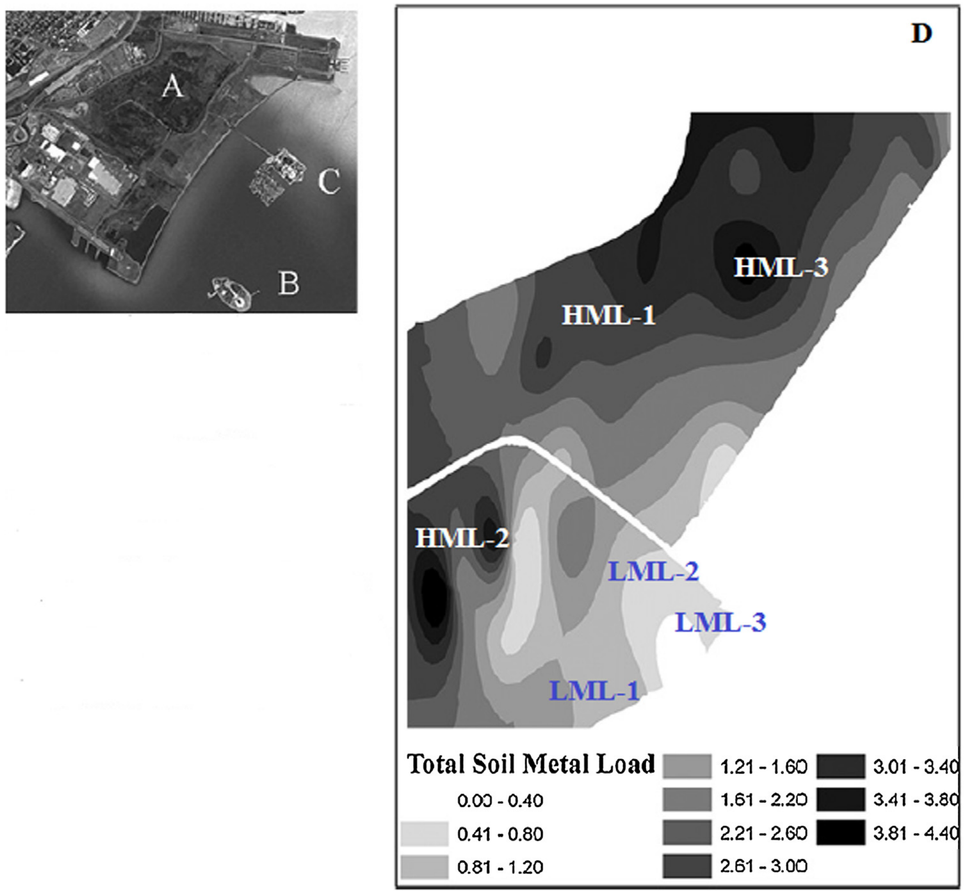

Fig. 1. The study site (A) in Liberty State Park on the west bank of Upper New York Bay (centered at 40°42′14"N; 74°03′14"W), (B) The Statue of Liberty, (C) Ellis Island, and (D) location and Total soil metal load (TML) of all the six sites (3 low meal load [blue] and 3 high metal load sites [white]) within Liberty State Park (from Gallagher et al., 2011).

and reliable method for monitoring heavy metal stress (Beasley et al., 2013; Lazić et al., 2013). FA refers to the small, non-directional deviations from ideal bilateral symmetry of morphological traits in organisms (Valen 1962). High levels of FA in a population is considered as an indication of increased developmental instability (DI); the inability of an individual to buffer development against environmental or genetic disturbances to produce a symmetric form (Lerner 1954; Waddington 1942). The underlying premise is that under increased levels of stress, organisms struggle to maintain necessary levels of developmental precision resulting in a more reliable relationship between FA and DI (Sommer 1996). Numerous studies have shown that abiotic stress such as increased heavy metal contamination, chemical pollution, increased human disturbances (e.g., habitat fragmentation), and changes in microclimate (Chang et al., 2007; Helle et al., 2011; Kozlov et al., 1996; Lazić et al., 2013; Mal et al., 2002; Vilisics et al., 2005) can disrupt developmental processes that in turn may result in increase of FA in both plants and animals.

The objective of this study is to quantify effects of heavy metal soil contamination on FA in populations at two distinct trophic levels, that of a terrestrial isopod: *Philoscia muscorum* (common striped woodlice) and two hardwood trees species: *Betula populifolia* (gray birch) and *Populus deltoides* (eastern cottonwood). In metal contaminated environments, plants take up metals from the soils and store it in various tissues including leaves (Castiglione et al., 2009; Gallagher et al., 2008b). Eventually, metals can be transferred to invertebrates directly through feeding on contaminated leaf litter (e.g. isopods) or indirectly via different tropic interactions resulting in accumulation of heavy metals in their body (Jelaska et al., 2007; Peterson et al., 2003). Studies have shown that metal accumulation at higher concentrations than required can negatively impact growth, reproduction, physiology, or body symmetry (Ambo-Rappe et al., 2011; Arena et al., 2013; Di Baccio et al., 2003; Godet et al., 2011; Mal et al., 2002; Todeschini et al., 2011) in both plants and invertebrates. Since metal stress can have detrimental effects on different components of an ecosystem including producers and decomposers, in this study we expect to find a positive relationship between FA and metal stress in case of both hardwood trees (primary producers) and isopods (decomposers). In this study, we examined FA: (a) in five different traits (length of two segments of antennae and three segments of seventh leg) in males and female isopod populations, and (b) for leaf width in gray birch and eastern cottonwood collected from low and high soil metal load sites at post-industrial landscapes preserved in Liberty State Park (LSP), Jersey City, New Jersey (USA). In addition to FA, we also compared the growth rate (ratio of body size to head width) of isopods between low and high metal load sites. We tested the hypothesis that symmetry of: (a) morphological traits in isopods and (b) leaves of gray birch and eastern cottonwood increases with heavy metal pollution.

2. Material and methods

2.1. Study site

The study was conducted in Liberty State Park (LSP), Jersey City, New Jersey on the west bank of Upper New York Bay (centered at 40° 42′ 14" N and 74° 03′ 14" W). The area originally was an intertidal mudflat before it was filled in the 1800's with debris from construction projects and refuse from New York City region. Developed by Central Rail Road of New Jersey (CRRNJ), the area was used as a rail yard and for the transport and storage of coal and other goods. After CRRNJ discontinued its operations in 1967, the State of New Jersey purchased this area to develop it as a state park. While most of the area was capped with clean soil, the central area of the park, approximately 41 ha was fenced off and left isolated and undisturbed that serves as our study site. Over the last five decades, the site has been reclaimed by spontaneous vegetation succession which includes dominant hardwood trees (*Betula populifolia, Populus deltoides*), shrubs (*Rhus typhina, R. glabra,* and *R. copallinum*) and many native and non-native forbs and grasses (*Solidago spp*., Eupatorium spp., *Artemisia vulgaris, etc.*) (Gallagher et al., 2011). Due to its industrial history, soils at the site are unevenly polluted with metal contaminants including Arsenic (As), Chromium (Cr), Copper (Cu), Lead (Pb), Vanadium (V), and Zinc (Zn) (Gallagher et al., 2008a). Based on the concentrations of these metals in soil, Gallagher et al. (2008a) developed a total metal load index (TML) index. The TML represented a rank order transformation of the normalized soil metal concentration and has a range from 0 (low) to 5 (high). The scale is used as a relative index to compare soil contamination levels between different sites (see Fig. 1 in Gallagher et al., 2008b). Previous work at LSP has shown that plant productivity and growth are negatively impacted by the heavy metal stress at sites with a threshold TML level of 3.0 and above (Dahle et al., 2014 Gallagher et al., 2008a; Renninger et al., 2013). It has been shown that the metal loads also had an influence on the assemblage and trajectory of the vegetation, with hardwood trees (gray birch and eastern cottonwood) being more dominant at sites with higher soil metal load (Gallagher et al., 2008b; Gallagher et al., 2011) (see Fig. 1 in Gallagher et al., 2008b). For the purpose of this study, we selected three low metal load (LML) sites (with TML <3.0, LML-1: site 48, LML-2: site 41, LML-3: site 47) and three high metal load (HML) sites (TML> 3.0, HML-1: site 10, HML-2: site 14/16, HML-3: site 25) (Fig. 1). The soil metal load levels and concentrations of heavy metals at 3 LML and 3 HML sites are shown in Fig. 2.

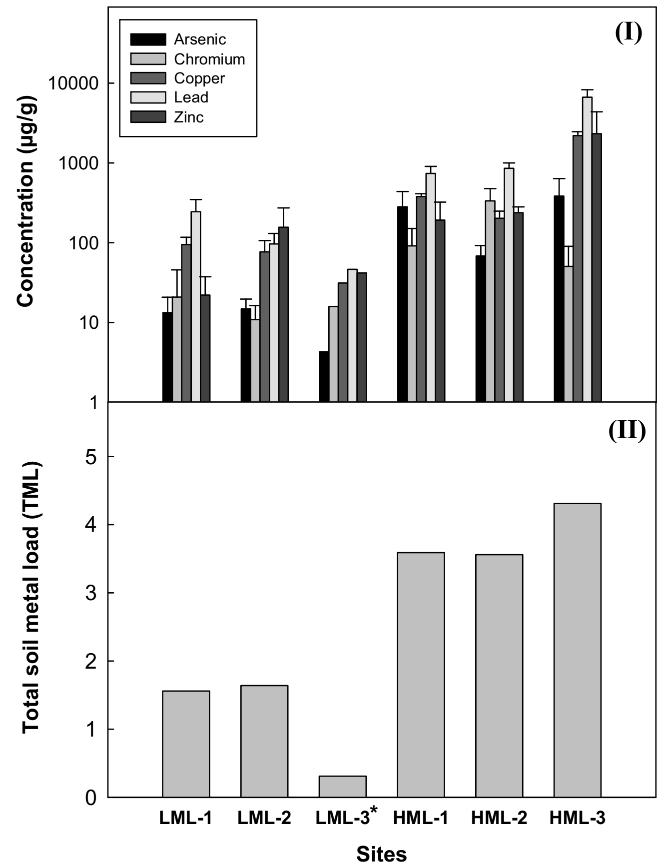

Fig. 2. (I) Soil metal concentrations (average value with standard deviation, x-axis is in log scale), and (II) total soil metal load for low metal load (LML) and high metal load sites (HML) study sites Soil metals µg/g (2005) * Sampled in 1995, one sample was analyzed from this site.

2.2. *Choice of organisms and data acquisition*

Terrestrial isopods (Isopoda: *Oniscidea* sp.) were selected for the FA analysis as they are one of the most abundant soil dwelling arthropods (David and Handa 2010). They have remarkable ability to accumulate large amounts of heavy metals in their body (in hepatopancreas) (Gál et al., 2008) and play an important role in the litter decomposition process. Based on pitfall sampling, we found that *Philoscia muscorum* was the most dominant of all the isopods species at our study sites. The species is common in the Mid-Atlantic region of the United States and primarily feeds on the decaying leaf litter (Hassall and Jennings 1975). Isopods were sampled using pitfall traps during June-July of 2013 and May of 2014. For FA analysis, 18–25 male and 19–26 female individuals were selected from each of the six study sites (for a total of 279 individuals, 141 male and 138 female individuals from six sites). Sampled isopods were individually stored in polypropylene vials with 3 ml of 70% ethanol in the laboratory. For FA analysis, five traits were measured: (1) length of two antennae segments (Fig. 3I): article 2 and article 3, and (2) length of three segments of seventh leg (pereiopod) merus, carpus and prodopus from the collected specimens (Fig. 3II). As reported by previous studies (Vilisics et al., 2005), these traits were selected as they are not difficult to measure and also the measurements are repeatable. In addition, to the two antenna and three pereipodal segments, head width of each individual was also measured to correct for the size dependence. To examine the impact of metal contamination of soils on the growth rate of isopods, maximum body length (length from middle point on the head to pleotelson) of randomly selected isopods from LML (N = 54) and HML (N = 62) sites was recorded. To compare the growth rate of isopods between LML and HML sites, growth rate of each individual was estimated as the ratio of body size to the head width (or body size for a given age, using head width as a proxy for age (Donker et al., 1993; Jones and Hopkin, 1998)). For all the *P. muscorum* individuals, images of the selected antennae and pereopodal traits (right and left sides) were taken with Infinity-1 digital camera connected with a NIKON – SMZ745T stereomicroscope (Nikon Europe, Düsseldorf, Germany). Selected traits were measured using Image J morphometrical software (http://imagej.nih.gov/ij/).

Leaves of gray birch *(Betula populifolia)* and eastern cottonwood (*Populus deltoides*), the dominant hardwood trees at the study sites, were selected for FA analysis. Previous work at these sites has shown that these two trees tend to accumulate heavy metals in plant tissues, specifically zinc in leaves (Gallagher et al., 2008b). In addition, long term growth rate of both gray birch and eastern cottonwood is negatively impacted by heavy metal stress at HML sites as compared to LML sites (Dahle et al., 2014; Renninger et al., 2013). For the FA analysis, we collected 92–105 leaves of gray birch and eastern cottonwood respectively from each of three LML and three HML sites at LSP in May 2014. At each site, leaves were collected randomly from six-seven trees (gray birch was absent at 2 sites: site 10, HML-1 and site 47, LML-3). In the laboratory, leaves were pressed in an herbarium press and then scanned at 800 dpi with a scanner (EPSON perfection V30, Seiko Epson Inc., Long Beach, CA, USA). For FA analysis, leaf width of the right (R) and left sides (L) was measured for each sampled leaf using Image J morphometrical software (http://imagej.nih.gov/ij/). Leaf width on either side

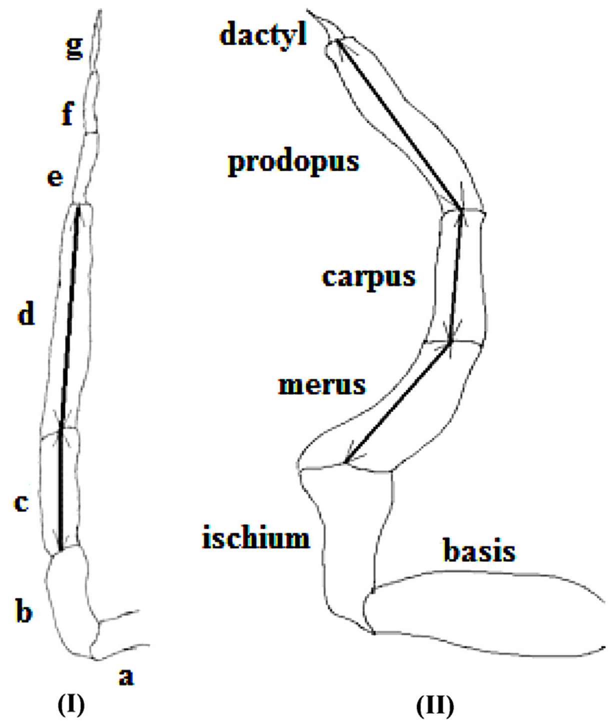

Fig. 3. (I) *Philoscia muscorum* antennae segments (a: basis; b: article 1; c: article 2; d: article 3; e: flagellum 1; f: flagellum 2; g: flagellum 3), (II) *P.m.* pereiopod segments, and (III) leaf-width in right and left half of an eastern cottonwood leaf. Lines in the antennal segments (c and d) and pereipodal segments and in the leaf indicate the measured lengths of selected segments.

(right or left) of a leaf is defined as the perpendicular distance from a midpoint on midrib (mid-point between the base and the tip) to the leaf margin on that side (right or left) (Fig. 3III).

2.3. Data analysis

For all the traits examined in isopods and trees, asymmetry index (A.I) was calculated as the right minus left side value of the trait. In order to assess if variation between sides is greater than differences due to measurement error (ME), separate two-way ANOVA (as recommended by Palmer and Strobeck, 1986) was performed for each trait for male and female populations from each of the six sites. This analysis also tests the presence of directional asymmetry (DA), non-directional asymmetry (refers to FA or antisymmetry), and differences due to size/shape within the individuals. In addition to two-way ANOVA, DA was also evaluated using one sample t-test against a mean of zero. Results of two-way ANOVA tests showed significantly higher variation for MS_{SI} (mean squares of the side X individuals) than MS_{ME} (mean squares of the measurement error) ($p < 0.05$) for all tests performed (isopods and leaves). In addition, no significant differences between sides were observed indicating absence of DA ($p > 0.05$). Results of the one-sample *t*-test performed for each trait, gender and site also revealed absence of DA ($p > 0.05$) in almost all the tests realized (for isopods and leaves) except for only 2 out of 60 tests (2nd antenna article and carpus in females from LML-1) and 2 out of 10 tests (eastern cottonwood from HML-2 and gray birch from LML-2) in isopods and tree species respectively.

With regards to antisymmetry, we found some of the FA indices (3 out of 60 ANOVA tests performed for each trait, site and gender) were influenced by antisymmetry. It is important to consider that if a trait shows presence of antisymmetry, some part of this between side variation might have a genetic component, therefore, these between side differences might not be necessarily attributable to developmental noise (Palmer and Strobeck, 1992). As a result, deviations of A.I values from normality were also checked with Kolmogorov–Smirnov test for each trait, gender and site. Normal distribution of right minus left (R-L) value differences ($p > 0.05$) were observed for all traits in both the data sets (isopods and tree leaves).

To test for the size dependence of FA in the data, regression analysis was performed between |R−L| (absolute value of right minus left trait value) and width of the head capsule (a measure of body size) for each trait, gender and site (method following Palmer 1996). Similarly, regression was tested between leaf width and |R−L| (absolute value of right side width minus left side width value). Since significant positive relationships were found in 18 out of the 60 regression tests performed for the isopod data and 7 out of 10 tests performed for leaves data, FA indexes were calculated with trait size correction as:

$FA = |R-L|/((R+L)/2)$ following Palmer and Strobeck (1986).

FA values measured for all the traits were analyzed in two different ways. Firstly, FA values estimated for each trait were compared separately for male and female isopods between all the six sites using one-way ANOVA with post-hoc Tukey test. Similar analysis was performed to compare FA levels for leaf width in gray birch and eastern cottonwood among all sites. For the second approach, trait specific FA values were grouped together from LML and HML sites for male and female isopod populations and gray birch and eastern cottonwood leaves. One-way-ANOVA with post-hoc Tukey tests was performed to compare the differences among male and female populations from LML and HML sites. To determine differences in growth rate (ratio of body size to head width) and head width of isopods between LML and HML sites (combined data) two tail t- tests were performed

Finally, to test the differences in FA in *P. muscorum* populations, a general linear model analysis (GLM) was performed between genders, (male and female) and total soil metal loads (HML and LML) with gender and total soil metal load as fixed factors and traits as random factors. Similar GLM analysis was performed for leaf width between tree species, and soil metal loads. Tests for normal-

Table 1
Results of the GLM (general linear model) analysis of fluctuating asymmetry among 5 traits (2nd article and 3 rd article of antennae and merus, carpus and prodopus of the 7th pairs of pereipods) observed for *P.muscorum* between genders (male and female) and between two habitats (low metal load and high metal load).

Source of Variation	Df	MS	F-test
Intercept	1	7.995	85.825*
Habitats (H, fixed)	1	0.016	1.103
Gender (G, fixed)	1	0.004	0.829
Traits (T, random)	4	0.093	18.464
G x H	1	0.001	0.350
G x T	4	0.005	0.364
H x T	4	0.014	0.984
H x G x T	4	0.014	2.922*
Error	1330	0.005	

*Significant differences: $p < 0.05$.

ity, two tail and one sample *t*-tests, analysis of variance (ANOVA) with Tukey tests, and GLM analysis were performed on the data sets using SPSS version 21.0 for Windows (SPSS, Chicago, IL).

3. Results

3.1. Fluctuating asymmetry in P. muscorum populations in relation to metal load

Overall there was no significant effect of metal load on the magnitude of FA when all traits are considered (Table 1), however as indicated by the significant three-way interaction revealed in the GLM analysis, FA for some traits varied in relationship to both metal load, and gender. Accordingly, when individual traits were compared for male and female populations, only few FA values differed between metal loads (Fig. 5). These included FA measurements for the pereiopodal segment, carpus of males from HML sites that were significantly lower than both males and females from LML sites (Fig. 5). FA levels measured for prodopus in males from LML sites was also significantly greater than males from HML sites (Fig. 5) ($p < 0.05$).

Comparing the individual traits in male populations across all six sites, the differences that were found for males were the FA values estimated for carpus in individuals from site LML-1 that were higher than individuals from sites HML-1, HML-2 and HML-3 respectively ($p < 0.05$, Fig. 4). In addition, FA values observed for prodopus were significantly higher in individuals from site LML-2 than individuals from sites HML-1, HML-2 and HML-3, respectively ($p < 0.05$, Fig. 4).

For females in the populations of *P. muscorum*, FA measured for the 2nd article of antenna, merus, carpus, and prodopus did not differ ($p > 0.05$, Fig. 4) between all of the six sites (3 LML and 3 HML sites). In case of the 3rd antenna article, FA values estimated in females from site LML-1 were significantly greater than individuals from site HML-2 ($p < 0.05$, Fig. 4).

3.2. Head width to body length ratio analysis in population of P. muscorum

The ratio of body size to head width was significantly higher in isopod populations collected from LML sites than from HML sites (Fig. 6a). The results showed that growth (measured as ratio of body size and head width in an individual) of isopods is significantly reduced at HML sites as compared to LML sites ($p < 0.05$, two-tail *t*-test). However, the head-width in the isopod populations from low metal load and high metal load sites were not significantly different from each other ($p > 0.05$ two tail *t*-test) (Fig. 6b).

Table 2
GLM (general linear model) analysis for fluctuating asymmetry in leaves of gray birch and eastern cottonwood from two habitats (low metal load and high metal load).

Source of Variation	Df	MS	F-test
Intercept	1	4.810	1500.937*
Habitats (H, fixed)	1	0.016	5.126*
Species (S, fixed)	1	0.012	3.729
H x S	1	0.000	0.033
Error	994	0.003	

* Significant differences: $p < 0.05$.

3.3. Fluctuating asymmetry in leaves of gray birch and eastern cottonwood

Results of the one way ANOVA analysis revealed no significant differences between FA values calculated in leaves of gray birch and eastern cottonwood between at the six sites (Fig. 7a) ($p > 0.05$). However, for the data combined on basis of soil metal load (HML and LML), FA was higher in eastern cottonwood leaves from HML than LML sites ($p < 0.05$, two-tail *t*-test) (Fig. 7b). No differences were found between gray birch leaves from LML and HML sites ($p > 0.05$, two-tail *t*-test) (Fig. 6b).

GLM analysis performed on FA measured in gray birch and eastern cottonwood leaves for lumped LML versus HML sites showed that differences in FA values were dependent on the total soil metal load ($p < 0.05$) but not on species ($p > 0.05$, Table 2).

4. Discussion

The magnitude of asymmetry and in particular fluctuating asymmetry (FA) has been proposed as a reliable indicator of abiotic stress in different organisms including plants (Jari and Mikhail, 2001; Kozlov et al., 1996; Mal et al., 2002) and invertebrates (Chang et al., 2007 Peters et al., 2001; Vilisics et al., 2005). Therefore, we had hypothesized that FA levels for antennae and pereiopod segments in isopods and for leaf width in trees increase with higher metal stress. In contrast, the results of this study showed that FA in isopods and trees either did not increase or even decreased with soil metal induced stress. That this is not simply due to the lack of overall pollution effects as indicated by the expected reduction of growth associated with higher metal levels. As expected, isopods in sites with higher metal loads are relatively smaller in size (indicated by lower body size to head width ratio) compared to sites with lower metal stress, this even though their ages as indicated by head width (Donker et al., 1993; Jones and Hopkin, 1998) were similar. Likewise, previous work at Liberty State Park has demonstrated that growth rates of the two tree species (gray birch and eastern cottonwood) are also impaired by soil metal contamination (Renninger et al., 2013; Dahle et al., 2014). Similar to our results, the lack of any relationship, or even negative relationship between FA and stress across different traits and species have also been reported by others researchers (Ambo-Rappe et al., 2008; Floate and Fox, 2000; Godet et al., 2012; Hódar 2002; Rabitsch 1997). For instance, Godet et al. (2012) observed that FA measured for antennae and periopod segments in male population of terrestrial isopod, *Porcellio scaber* were lower at heavy metal contaminated sites as compared to uncontaminated ones. In another study, Rabitsch (1997) also found no relation between FA of four morphological traits in the ant *Formica pratensis* and levels of heavy metals. Similarly, Ambo-Rappe et al. (2008) found no increase in FA of the seagrass *Halophila ovalis* sampled from a heavy metal polluted site and a control site. Overall, the inconsistency in literature concerning the relation of FA and stress is demonstrated by the fact that some studies have reported increased FA in response to stress (Jari and Mikhail, 2001 Kozlov

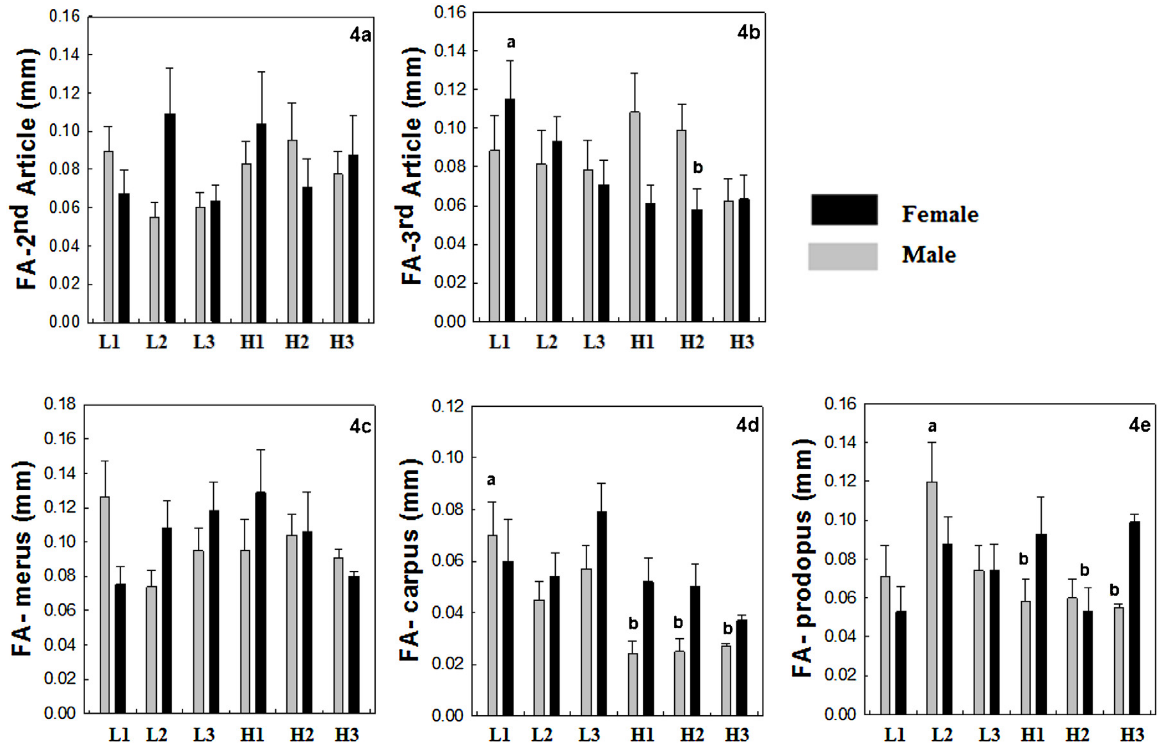

Fig. 4. Fluctuating asymmetry values (mean ± SE) observed for different traits: (a) 2nd and the (b) 3rd articles of antenna and merus (c), carpus (d) and propodus (e) of the 7th pereiopods in *P. muscorum* collected from the three low metal (L1, L2, and L3) and the three high metal load (H1, H2 and H3) sites in brownfields at Liberty State Park. Different letters indicate significant differences (P < 0.05) between sites tested by one-way ANOVA with post hoc Tukey for each trait among males and females respectively from all six sites.

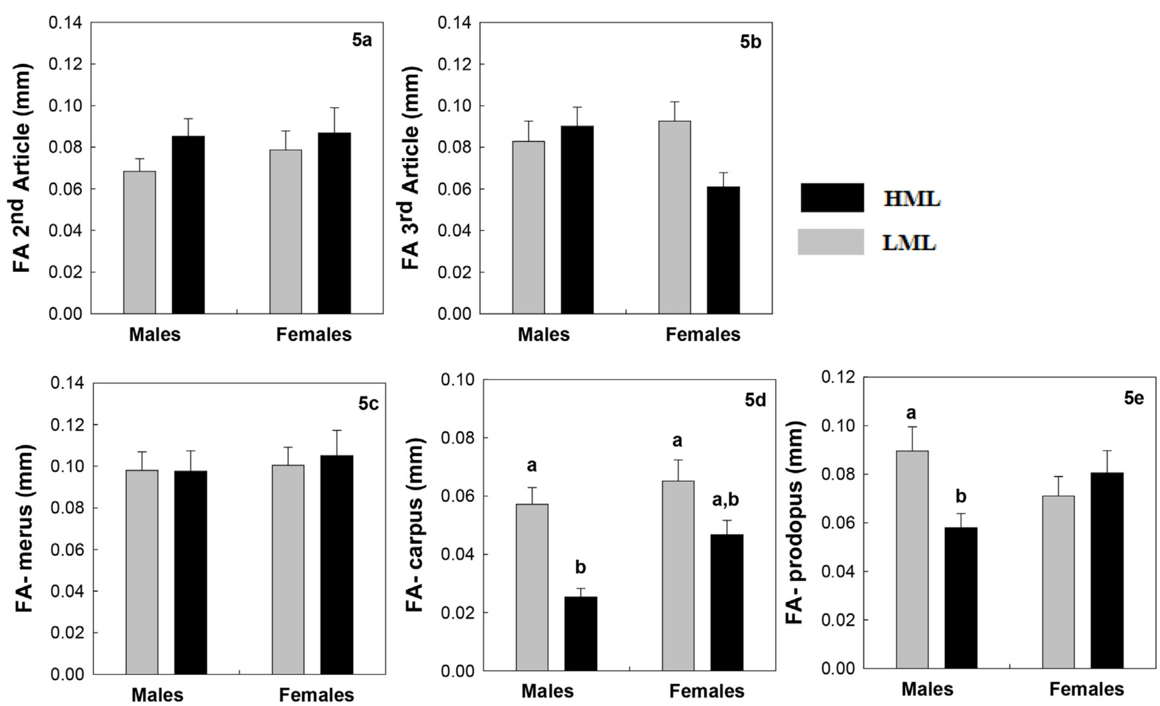

Fig. 5. Fluctuating asymmetry values (mean ±SE;) values observed for different traits: (a) 2nd and the (b) 3rd articles of antenna, and (c) merus, (d) carpus and (e) propodus of the 7th pereiopods in *P. muscorum* collected from brownfield sites at Liberty State Park and grouped by habitat type (low metal load and high metal load) and by gender (male and female). Different letters indicate significant differences (P < 0.05, one-way ANOVA, post-hoc Tukey tests) between different groups.

et al., 1996; Mal et al., 2002), whereas some including ours have failed to detect a clear link between FA and stress.

4.1. Fluctuating asymmetry in isopods

The observed lower FA levels in the isopod population at highly contaminated sites may be related to synthesis of heat shock

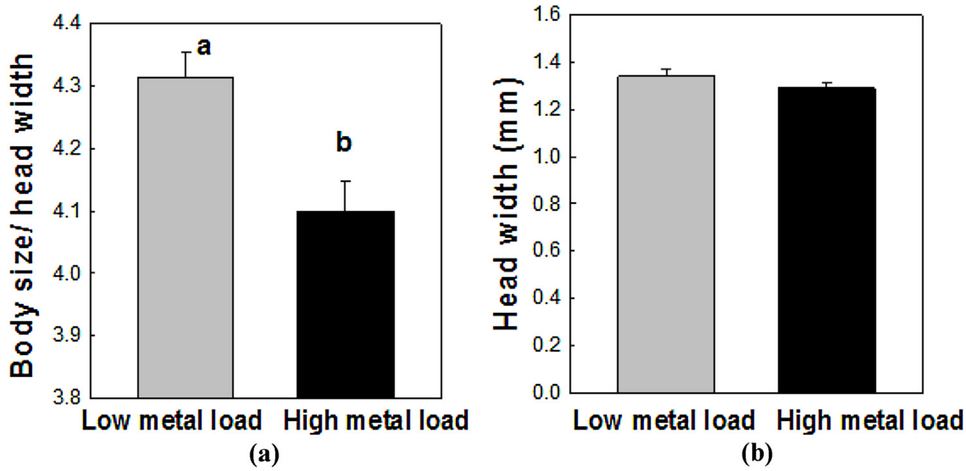

Fig. 6. (a) Head width to body length ratio in *P. muscorum* individuals collected from low metal load (N = 54) and high metal load sites (N = 62) (b) Head width of *P. muscorum* individuals collected from low metal load (N = 54) and high metal load sites (N = 62). Different letters indicate the significant differences (P < 0.05, two-tailed *t*-test) between isopod populations from low metal and high metal load sites.

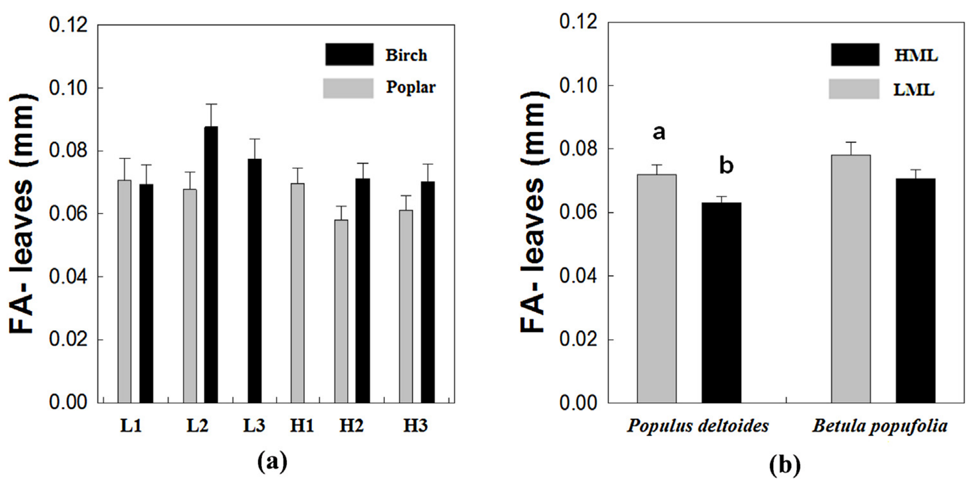

Fig. 7. (a) Fluctuating asymmetry values (mean ±SE;) for leaf width of *Populus deltoides* and *Betula populifolia* collected from three low metal load (L1, L2 and L3) and three high metal load (H1, H2 and H3) sites and grouped on the basis of habitat type (low metal load and high metal load) and according to tree species. 6(b) fluctuating asymmetry values (mean ±SE;) values observed for leaf width observed in *Populus deltoides* and *Betula populifolia* and grouped on the basis of habitat type (low metal load and high metal load) and according to tree species. Different letters indicate the significant differences (P < 0.05, two-tailed *t*-test) between *Populus deltoides* leaves sampled from low metal and high metal load sites.

protiens (HSPs) known to be stress-defense proteins, and the metal-binding proteins metallothioneins (MTs). These specific set of proteins play an important role in defense against metal toxicity and in maintaining cellular homeostasis (Ackerman and Iwama 2001; Beckmann et al., 1990; Viarengo et al., 1999). Previous studies have demonstrated metal induced expression of HSPs and MTs in several soil invertebrates (Arts et al., 1999; Morgan et al., 2004; Nadeau et al., 2001; Spurgeon et al., 2004; Sterenborg and Roelofs 2003). In terrestrial isopods, Mazzei et al. (2015) reported induction of Hsp-70 and MTs by metals in hepatopancreas, which is the primary organ for metal storage and detoxification in isopods. It is possible that activation of detoxification mechanisms based on the expression of HSPs or MTs protects isopod development from damage due to metal stress leading to a decrease in asymmetry. These protective mechanisms, however, are energetically expensive and may shift resources away from energy demanding process such as growth (Feder and Hofmann 1999; Krebs and Feder 1997; Schill and KÖhler 2004; Sibly and Calow 1989). This could explain why *P.muscorum* in the heavy metal load site tended to be smaller in size compared to sites with lower metal stress even though their ages as indicated by head width were similar.

Exposure to abiotic stress can act also as a form of selection against developmentally unstable phenotypes if stress induced asymmetry is negatively correlated with natural and/or sexual components of fitness (Møller 1997). Higher FA in antennae and periopods might have serious fitness costs for individuals and therefore natural selection may remove asymmetric phenotypes from isopod population at highly contaminated sites. In addition, selection pressures by mate choice may also mask a relationship between FA and metal stress if asymmetry is associated with reduced mating success in isopods. These hypotheses are better understood when considering the function of antennae and periopods in isopods.

Antennae are involved in important sensory functions such as hygroreception and chemoreception (Haug and Altner 1984; Warburg 1993; Zimmer and Topp, 1996). It has been suggested for some isopods that the olfactory or chemoreceptors present on the antennae are responsible for their avoidance behavior towards contaminated food or soil (Weißenburg and Zimmer 2003; Zidar et al., 2005). Additionally, in some species antennae are used by courting males to locate a receptive female (Johnson 1985) and to assess female quality (Mead 1973). Furthermore, males also use antennae in aggressive behavior against other contesting males during mating (Lefebvre et al., 2000). Pereiopods are the walking legs in isopods (Webb and Sillem 1906) and in addition to locomotion, pereiopods, especially the 7th pair, are also used by males to grasp females during the mating process (Forest 1999). Assuming that presence of asymmetry in these traits might: (i) be associated with loss or impairment of functions that are critical for survival (a fitness component), and/or (ii) influence the process of mate choice in favor of symmetric individuals. It is therefore plausible that selection might explain the absence of a relationship between FA and metal stress at highly polluted sites. A number of studies have determined negative impacts of asymmetry on fitness components in different invertebrates (Allen and Simmons 1996; Møller and Zamora-muñoz, 1997; Naugler and Leech 1994; Thornhill 1992; Ueno 1994), however, these relationships have not been tested in the case of terrestrial isopods.

4.2. Fluctuating asymmetry in trees

We also hypothesized that FA in leaves of gray birch and eastern cottonwood increase with metal stress. However, despite the evidence for metal induced growth stress of gray birch and eastern cottonwood at highly contaminated sites (Dahle et al., 2014; Renninger et al., 2013), results of the FA analysis in this study do not support our hypothesis (Fig. 7a). One plausible explanation of our results could be activation of stress protection enzymes such as phytochelatin synthase upon the exposure to metal contamination. These enzymes catalyze the synthesis of metal binding proteins termed phytochelatins which play a key role in detoxification in plants. In fact, phytochelatin synthesis has been shown to be involved in maintenance of Zn homeostasis in eastern cottonwood (Adams et al., 2011). It is also known that Zn was the only metal to translocate to the leaves of these species at or above concentrations found in the soil. The other metals were sequestered within the root or excluded at the root soil interface (Gallagher et al., 2008b). It is possible that activation of phytochelatin synthase functions to protect plant development from high soil metal load, however the energy cost for such protection could impair long term growth (Dahle et al., 2014).

Some authors have argued that different traits vary in their sensitivity to stress and therefore examining FA levels of a sensitive trait can increase the likelihood of detecting FA and stress relations. For example, Ivanov et al. (2015) suggested that leaf width is not the most sensitive trait for FA analysis in birch due to metal contamination as compared to other traits such as distance between the bases of the first and second lateral veins. Other researchers also recommend examining FA for multiple traits as relations between FA and stress are usually weak, hence, studying multiple traits can maximize the odds of detecting such a relationship (Leung et al., 2000). On the other hand, some comparative studies have reported positive correlations between metal stress and FA measured for leaf width in plants (Franiel 2008; Mal et al., 2002). Therefore, it is difficult to argue whether selection of a single and/or a relatively insensitive trait (leaf width) for FA analysis in trees was one of the reasons why we did not observe an increase in FA with metal stress.

Plasticity could be another factor that might facilitate resistance to metal stress in trees at high metal load sites. Unlike most animals, sessile organisms such as plants which demonstrate indeterminate and modular growth (Fenster and Galloway 1997; Schmid 1992) can often respond to stressful environmental conditions through plasticity involving changes in their growth patterns (Bradshaw 1965; Schmid 1992). Trees have been shown to develop resistance in form of phenotypic plasticity to cope up with metal stress, for example, Watmough and Hutchinson (1997) showed that callus cell lines derived from mature maple trees growing in metal contaminated soils possessed the ability to tolerate elevated levels of metals. Studies have also reported evidence of phenotypic plasticity in plants in response to other factors such as herbivory, light gradient, salt, or water stress (Abbruzzese et al., 2009; Chazdon and Kaufmann 1993; Goulet and Bellefleur 1986; Pedrol et al., 2000; Rautio et al., 2002). Since gray birch and eastern cottonwood trees have been growing on high metal load sites for many years, it is possible that ability to acclimatize or plasticity might be a contributing factor to metal tolerance in tree populations resulting in absence of a relation between FA and metal stress.

5. Conclusions

The results of this study did not support our hypothesis that FA increases in isopods and trees with higher heavy metal stress even though a growth reduction was shown at the site. Our results support the growing body of literature demonstrating the failure to detect a direct link between stress and FA. This need not imply that FA should no longer be used as a marker for environmental stress as argued by some skeptics (Bjorksten et al., 2000), however, it does point to the need for identifying the reasons for these observed inconsistencies and for a better understanding of the underlying developmental mechanisms related to the origin of FA. Natural processes such as selection have been suggested in studies as a possible mechanism that can mask the direct relationship between FA and stress (Bergstrom and Reimchen 2003; Hendrickx et al., 2003). Experiments under controlled settings using multiple generations of isopods will be helpful to verify the negative effects of increased FA on fitness. In addition, one could ask whether traits that contribute less to fitness and therefore are less selected on, are better candidates to detect FA. Results of these studies may provide an insight into understanding the influence of selection on this relationship between FA and stress. Given the growing interest of researchers in studying FA as an indicator of stress, examining the role of detoxification mechanisms (e.g. induction of MTs, HSPs, or phylochelatins) in conjunction with FA measurements might also help to better explain FA and stress relations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2016.12.037.

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