Revista Brasileira de Entomologia 68(2):e20230045, 2024

SOCIEDADE BRASILEIRA DE ENTOMOLOGIA FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution

Limitations of allometry, morphometry, and fluctuating asymmetry in detecting environmental stress caused by lead soil contamination in aphids under field conditions

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ARTICLE INFO

Article history:
Received 30 June 2023
Accepted 02 April 2024
Available online 13 May 2024
Associate Editor: Lucas Kaminski

Keywords:
body size
Brevicoryne brassicae
heavy metal
kale
soil contamination

ABSTRACT

One of the tools used to investigate the influence of environmental contaminants and other stresses on the development of organisms is the analysis of morphometric traits used to detect changes in growth and size patterns. To evaluate the effects of the heavy metal lead (Pb) present in experimentally contaminated soil on the morphometric traits of the aphid *Brevicoryne brassicae* (L.) reared on cultivated *Brassica oleracea* L. (var. *acephala*), we analyzed three different metrics: morphometry, allometry, and fluctuating asymmetry (FA). Additionally, we aimed to assess the effectiveness of these analyses in detecting environmental stress. We cultivated kale plants in soil contaminated with the maximum allowable limit of Pb for local soils in Brazil, and the colonization of plants by aphids occurred naturally under field conditions. After collection, we photographed and measured the antennae, tibiae, and total body length of aphids from the control and contaminated treatments. We observed no significant differences in the allometry and morphometric analysis, and the presence of the contaminant did not result in any observed FA. The study demonstrated that the morphometric trait analyses employed were not effective in detecting environmental stress resulting from field exposure of aphids to Pb in soil, since these insects suffered a reduction in their population density in the presence of Pb, as indicated in our previous findings. This result diverges from those found in studies conducted under laboratory conditions. We discussed mechanisms of metal accumulation, detoxification in aphids, and population aspects in natural conditions.

Introduction

Changes in land use, combined with the overuse of agrochemicals, are one of the main causes of the biodiversity decline in various landscapes (Robinson and Sutherland, 2002; Benton et al., 2003; Bianchi et al., 2006). These intensive practices can cause chemical degradation of the soil due to the accumulation of toxic elements and/or compounds, such as heavy metals, at undesirable levels. Heavy metals such as lead (Pb), cadmium (Cd), and zinc (Zn), are often associated with agricultural, industrial, and mining activities. These metals possess the ability to move along the food chains, and their toxicity is mainly linked to the fact that they are not degradable, accumulating in organisms and in the environment (Ali et al., 2013; Butt et al., 2018). In the case of Zn and other metals, such as iron (Fe) and copper (Cu), there is an essential participation of these elements, in small quantities, in the biological processes of organisms (Cempel and Nikel, 2006; Göhre and Paszkowski, 2006). However, when they reach high concentrations, these elements become equally toxic as other heavy metals (Chaffai and Koyama, 2011). Consequently, these elements can be incorporated by plants and transferred along the trophic chain (Gimeno-García et al., 1996; Ramalho et al., 2000).

The long-standing persistence of these toxic metals in soil, as well as their accumulation in plant tissues at sub-phytotoxic levels, may pose a serious risk to the upper strata of food chains (Zhuang et al., 2009; Gall et al., 2015). It was demonstrated that plants grown on substrates with heavy metals transfer these elements to herbivorous insects, which in turn transfer them to their natural enemies (Zhuang et al., 2009; Dar et al., 2015, 2017; Zhou et al., 2016; Naikoo et al., 2019; Shi et al., 2020). Indeed, insects are potential indicators of heavy metal contamination, mainly due to their responses to the impacts of this disturbance, which include reduced survival, growth, changes in development and emergence patterns, and changes and reductions at the population or community level, and therefore can be used for biomonitoring (Sildanchandra and Crane, 2000; Azam et al., 2015; Morales-Silva et al., 2022). The mechanism of metal accumulation in these insects can vary significantly, depending on the plant and insect

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https://doi.org/10.1590/1806-9665-RBENT-2023-0045

species, as well as the metals involved (Dar et al., 2017; Woźniak et al., 2017).

Herbivorous insects, such as aphids, respond directly to changes in the host plant, which may affect their development (Görür et al., 2007). Studies have demonstrated that aphids can accumulate metals at lower levels than in plants (biodilution) or even higher levels (biomagnification) in some cases. For example, Cd and Zn were biomagnified in the aphid *Lipaphis erysimi* (Kalt.) in mustard (*Brassica juncea* L.) while Pb was reduced (Dar et al., 2017). Despite that, in the aphid *Acyrthosiphon pisum* (Harris) in pea (*Pisum sativum* L.), Pb was biomagnified (Woźniak et al., 2017). Experimental exposure of aphids to contaminants also indicated the occurrence of adverse effects on the biology of these insects, even in treatments with low concentrations. Woźniak et al. (2019), studied the effects of Pb on the demographic parameters of the aphid *A. pisum* population in pea plants treated with hormonal (0.075 mM $Pb(NO_3)_2$) and sublethal (0.5 mM $Pb(NO_3)_2$) doses. Both Pb treatments were observed to result in a decrease in fecundity compared to the control. The treatment with the higher concentration also exhibited a decrease in the longevity, reproduction period, fertility, net reproduction rate, and even induced behavioral changes, significantly prolonging the time required for aphids to reach the phloem for feeding. Similarly, studies have shown that life history characteristics and population dynamics of aphids, including reproduction and life expectancy, are adversely affected by other contaminants at low or high concentrations, such as petroleum derivatives and insecticides (Ullah et al., 2019a, 2019b, 2020). This effect is not yet clear in metal contamination.

Regarding the effects of heavy metal contamination on the body size of organisms, there are fewer studies. Size is an important indicator of fitness in insects (Beukeboom, 2018) and may suffer changes with other morphometric traits due to genetic and environmental factors present during development, influencing morphometry, allometry and fluctuating asymmetry (FA) (Klingenberg and Nijhout, 1999; Shingleton et al., 2007). Populations that develop in suboptimal conditions may present phenotypic responses to this stress, which allows the identification of this type of disorder through analyses of body variations, such as morphometry, allometric patterns, and developmental stability (Lazić et al., 2015). Morphometry is used to perform quantitative analyses of shape by evaluating size and body parts (Adams et al., 2004), while allometry is the relationship between the size of an organ and the total body size or between two organs (Stern and Emlen, 1999). Changes that result in bilateral features being larger or smaller than their normal size can be estimated using FA (van Valen, 1962; Leary and Allendorf, 1989). Variations in these metrics can be considered a reflection of environmental disturbances.

FA can serve as an indicator of organisms that are experiencing some form of stress, such as those that have developed in habitats affected by heavy metals or other toxic substances (Clarke, 1993). Görür (2006) evaluated how Pb and Cu affect stability in the development of aphids in closed cultivation of cabbage and radish, finding higher levels of FA in individuals exposed to heavy metals via plant irrigation. However, the FA response can change to different organisms and metals. FA was not a good indicator of environmental stress in ants *Lasius flavus* (Fabricius) collected along a heavy metal gradient. Despite eye reduction, in relation to morphometry, there was no significant difference between the right and left sides (Grześ et al., 2015). In addition, studies conducted in laboratory conditions can detect more significant effects on the FA of the insects than the studies conducted in the field (Beasley et al., 2013). There is a lack of knowledge about the effects of heavy metals on other morphometric variables and which methods can be used to detect morphological variations and environmental stress more clearly.

We previously demonstrated a drastic reduction in aphid population density on kale plants (*Brassica oleracea* L. var. *acephala*) grown in field conditions with soil contaminated by Pb concentrations permitted in Brazil. Additionally, we observed a decrease in the population of other insects in this system, including lepidopterans, parasitoids, and predators, resulting in the simplification of the community and food web (Morales-Silva et al., 2022, 2023). Aphids stand out as a model for detecting environmental influences on the morphology of individuals and on population structure due to their reproductive mode (parthenogenesis), which results in large numbers of offspring living in aggregated colonies and being simultaneously exposed to possible stresses and temporal instabilities (Souto et al., 2012). Thus, this work sought to evaluate the influence of Pb in the soil on the morphometric traits of the aphid species *Brevicoryne brassicae* L. individuals, which developed in open cultivation of kale, under field conditions, and the effectiveness of these analyzes in detecting environmental stress suffered by aphids, previously detected by the reduction in their population density. We investigated whether the presence of the contaminant affects body size and appendages, resulting in changes in allometric patterns and generating asymmetric fluctuations in the aphids. We hypothesized that aphids will experience stress in their morphometric traits due to the presence of Pb, resulting in smaller, asymmetrical individuals with alterations in their developmental patterns.

Material and methods

Study site and experimental procedure

We chose Pb as the study heavy metal. Pb contamination is associated with activities such as battery manufacturing, combustion of leaded gasoline and, mainly, herbicides and insecticides (Thangavel and Subbhuraam, 2004; Wuana and Okieimen, 2011). The Contaminated Areas Inventory carried out by the "*Fundação Nacional do Meio Ambiente* (FEAM)" (FEAM, 2015) identified Pb as the most frequent heavy metal in contaminated areas in the state of Minas Gerais, Brazil, where we conducted the study. We chose the plant species *B. oleracea* for two reasons: (1) Previous work demonstrated that plants in the Brassicaceae family can absorb heavy metals from soil (e.g. Chatterjee and Chatterjee, 2000; Xiao et al., 2017; Sahay et al., 2019). (2) The insects associated with this species are easy to identify, thus avoiding errors related to taxonomy.

In January 2019, we planted kale seedlings in uncontaminated (control) and contaminated soils, with 10 plants in each treatment, totaling 20 plants arranged randomly. We individualized and cultivated seedlings in pots with a capacity of 12 L containing a mixture of 2 kg of expanded clay for drainage and 8 kg of substrate containing vegetable soil (60%), sand (30%), organic fertilizer (8%) and limestone (2%). Each pot was connected to a PET bottle through a hose installed at the bottom of the pot, closing the water circuit and preventing local contamination. The irrigation of plants occurred two times per week. We conducted the experiment in an open greenhouse to enable insects to colonize the plants naturally, thereby simulating field conditions. Only the top portion of the greenhouse was covered with a transparent tarpaulin, as the closed-loop irrigation system could not withstand heavy rainfall. The greenhouse was located at the experimental farm of the Federal University of Lavras (UFLA), located in the municipality of Ijaci, Minas Gerais, Brazil.

We contaminated the soil three months before planting (October 2018), thus increasing the adherence of heavy metals to the soil, and used Pb in the form of nitrate ($Pb(NO_3)_2$) due to the ability to apply it through dilution in water. The lead nitrate concentration was 600 mg/kg of soil, which is equivalent to the Pb limit concentration for soils intended for plant cultivation (residential soil), according to "*Conselho Nacional do Meio Ambiente*" (CONAMA) (Brazilian National Environment Council)

Resolution 460/2013 (CONAMA, 2013). Values above this threshold are sources of potential direct or indirect risks to human health. We diluted the lead nitrate in 2 L of water and applied it directly to the soil already prepared in each pot. In the control treatment, we applied 2 L of water only. Further details about the experimental setup can be found in Morales-Silva et al. (2022).

Obtaining and measuring insects

In June 2019, after natural establishment of aphid colonies on plants, we collected the insects on host plants removing the aphids from the kale with a soft paintbrush. The aphids were stored immediately in 70% alcohol to preserve their body structures. We selected the aphid species *B. brassicae* for this study, as they were the most abundant in the experiment. We previously demonstrated that this population of *B. brassicae* experienced a drastic reduction in its population density in the presence of Pb in the soil, regardless of the concentrations (144, 360, or 600 mg of lead nitrate/kg of soil) (Morales-Silva et al., 2022). In each treatment, we selected six plants with a large number of aphids and we randomly selected 12 adult wingless females of *B. brassicae* from each plant for the measurement of morphometric traits, totaling 144 aphids measured. We used wingless females because of their limited mobility, ensuring that they fed in the same location where they were collected.

We placed each *B. brassicae* individual in a dorsal-ventral position on a microscope slide. We photographed with a Zeiss Axio Zoom v16 stereomicroscope with an Apo Z 1.5x/0.37 FWD 30 mm lens. The measurements we made in Zen 2.3 software at the Center of Studies of Subterranean Biology (CEBS) of the UFLA. As a result, we captured a photograph to measure the total body length (from the top of the head to the final portion of the last abdominal segment) (Fig. 1a). Additionally, we took another photograph to measure the flat appendages of the body, the antenna (antennomeres) (Fig. 1b), and the posterior tibia (from the intersection of the femur with the tibia to the tibia with the tarsus) (Fig. 1c). The appendages measurements occur three times on the right and left sides on different days to exclude measurement errors from the FA tests (Palmer and Strobeck, 1986; Graham et al., 2010; Souza et al., 2018; Oliveira et al., 2020).

Among the appendages selected for measurement, the antenna constitute a sensory structure that has an orientation function, and the tibia are an important locomotion trait that allows greater mobility on the host plant and escape from predators (Ruiz-Montoya et al., 2005; Maia et al., 2017). Both structures are comparable to those of other groups of insects and are widely used in morphometric studies on insects, constituting a consensus among authors (e.g. Görür, 2006; Wadhwa et al., 2017).

Lead detection in leaf samples

After the field experiment, we took the plants to the laboratory for drying in an oven and subsequent grinding in a knife mill. We sent dry and ground leave samples from each plant to the company "SG *Soluções Científicas*" (São Carlos, SP, Brazil) for the determination of Pb using Inductive Coupled Plasma Optical Emission Spectrometer (ICP OES). The sample digestion occurred in a system assisted by an equipped digester block with perfluoroalkoxy (PFA) tubes (Savillex, MN,

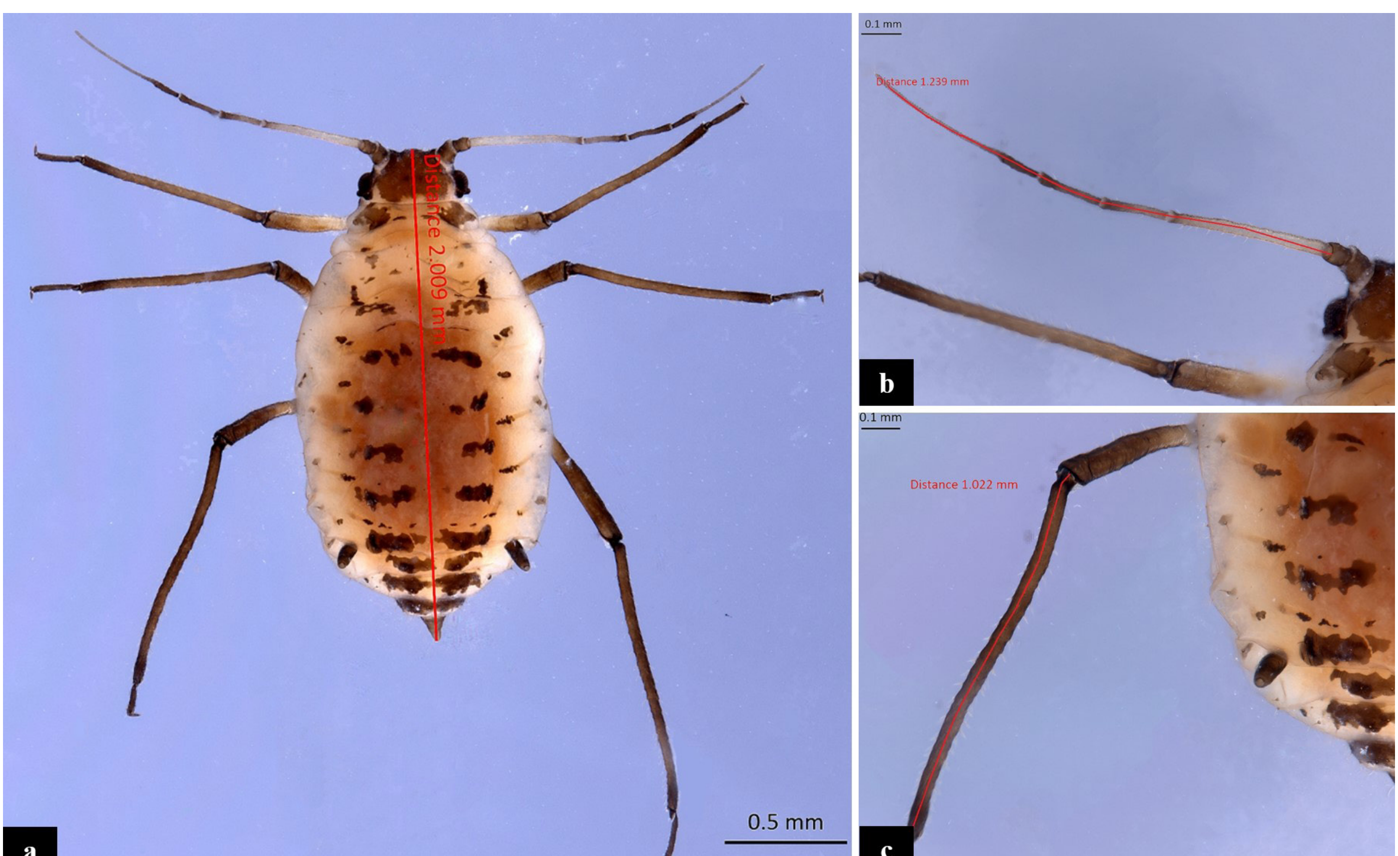

Figure 1 *Brevicoryne brassicae* placed in a dorsal-ventral position for structure measurement. (a): Total body length (b): antenomer length (c): length of the posterior tibia. Source: the authors.

USA). An analytical balance (model AY220, Shimadzu, Kyoto, Japan), was used to weigh approximately 1.00 g of leaf from each sample directly in the PFA tubes. After weighing the samples, they added an acidic mixture containing: 5.0 mL of HNO_3, 5.0 mL of deionized water and, 2.0 mL of H_2O_2. They performed two analytical blanks with the samples. Pb determinations in the properly digested samples were performed using an ICP OES, model Thermo ICP OES iCAP 7000 (Thermo Fischer Scientific, Madison, WI, USA). Details on the heating program, parameters and, operating conditions of the equipment are available in Tables S1 and S2 of the Supplementary material. The method detection limit was 0.001 (mg.kg^{-1}) and, the limit of quantification is 0.005 (mg.kg^{-1}).

Data analysis

To compare the amount of Pb in leaf samples from the control and contaminated treatments, we applied a Mann-Whitney test, since data did not present normal distribution and homogeneity of variances. We consider results below the Pb detection limit as zero.

We performed a correlation analysis between the right and left sides of the antennae and tibiae using the *chart.correlation* function from the *Performance Analytics* package (Peterson and Carl, 2020) to reduce and simplify the analysis of morphometry and allometry, in which the right side of the body was used to evaluate the contaminant influences. To test whether there were differences in the body measurements between the treatment with Pb and control, we used linear mixed models with the body measurements as the response variable (normally distributed) and the treatment as the explanatory variable. We included the plants as random variables to avoid errors due to repeated sampling. For this analysis, the *lmer* function from the *lme4* package was employed (Bates et al., 2015).

We investigated the allometric patterns of the tibia length and the antenna in relation to the total length of the body using the *major axis* (MA) method of the *lmodel2* package (Legendre, 2018). The α and b parameters are the allometric coefficient (the slope of the relationship) and intercept of the model, respectively. α indicates how the size of a structure varies with the size of another structure and/or the total individual size, and b indicates the difference in the proportional size of the evaluated structure (Shingleton et al., 2007). Allometry is detected when α is different from 1 (i.e., isometry); negative allometry (hypoallometry) occurs when $\alpha < 1$, demonstrating that organs grew at a slower rate than the body, while positive allometry (hyperallometry) occurs when $\alpha > 1$, demonstrating that organs grew at a faster rate than the body (Shingleton et al., 2008).

Before testing the occurrence of FA, we observed whether there was a bias in the measurements made in larger individuals. We verified the relationship between the size of the measured organs and the FA value (Palmer and Strobeck, 1986). Hence, we calculated FA according to the equation: $FA = (R - L)$, (Palmer and Strobeck, 1986), where R is the size measured on the right side and L is the size measured on the left side, for both the antennae and the tibiae (Grześ et al., 2015; Souza et al., 2018; Oliveira et al., 2020). We applied linear mixed models (LMMs) using the function *lmer* from the *lme4* package, considering the treatments as the explanatory variables and individual identity (measurement error) and plant identity as the random variables (Palmer, 1994). We performed all the statistical analyses with R version 4.0.2 (R Core Team, 2020).

Results

A total of nine of the ten leaf samples from the control treatment had Pb content below the detection limit of the method and one had a value of 0.17 mg.kg^{-1}, resulting in a mean of 0.02 (± 0.02) mg.kg^{-1} of Pb for the control treatment. The contaminated treatment samples showed values ranging from 0.23 to 0.60, with a mean of 0.35 (± 0.06) mg.kg^{-1}, which was significantly higher than the control treatment ($p < 0.001$). The quantity of Pb in each sample is available in Table S3 of the Supplementary material. The data on Pb quantification in kale were previously published in Morales-Silva et al. (2022). We encourage readers to consult this manuscript for further insight into Pb bioaccumulation in kale.

The *B. brassicae* individuals from the control, i.e., in the absence of the contaminant, had a mean total body length of 1.913 ± 0.193 mm, right antenna length of 1.137 ± 0.122 mm, left antenna length of 1.135 ± 0.125 mm, right tibia length of 0.957 ± 0.102 mm and left tibia length of 0.963 ± 0.103 mm. In the presence of the contaminant, the mean total body length was 1.913 ± 0.171 mm, the right antenna length was 1.130 ± 0.115 mm, the left antenna length was 1.125 ± 0.121 mm, the right tibia length was 0.987 ± 0.084 mm and the left tibia length was 0.977 ± 0.088 mm. The lengths of the left and right sides of both appendages were highly correlated (0.95 with $p < 0.001$ for the antenna; 0.97 with $p < 0.001$ for the tibia). Therefore, we used the measures of the right antenna and tibia for the morphometry and allometry analyses. The morphometric analysis comparing the size of the appendages and total body length between individuals (Fig. 2) did not show a significant difference between the treatments ($p > 0.05$ for all models).

For the allometry analysis, we verified in both treatments the occurrence of hypoallometry, i.e., an allometric coefficient lower than 1. The antenna presented an allometric coefficient of 0.62 in the absence of Pb and 0.78 in the presence of Pb (Fig. 3), with an expressive increase in the confidence interval in the presence of the contaminant. The antenna had a confidence interval of 0.236 ± 1.215 mm in the absence of Pb, while in the presence of the contaminant the confidence interval was 0.194 ± 2.140 mm. The tibia had an allometric coefficient of 0.26 in the absence of Pb and 0.49 in the presence of Pb (Fig. 3). The confidence interval increasing slightly in the presence of Pb; in the absence of the contaminant, the confidence interval was 0.177 ± 0.915 mm, while in the presence of the contaminant, the confidence interval was -0.067 ± 0.649 mm.

Regarding FA, we did not observe the tendency toward larger antenna and tibia and an increase in FA in these organs, and therefore, we did

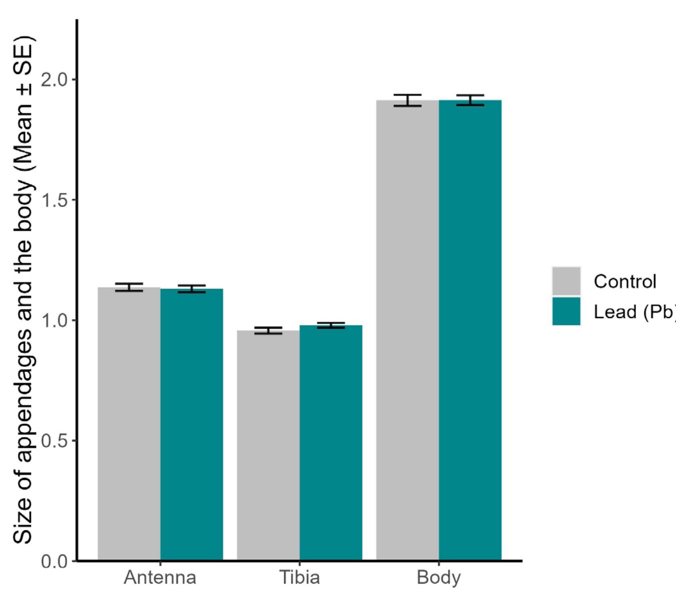

Figure 2 Mean length and standard error of the antenna, tibia and body length of *Brevicoryne brassicae* in the presence (Lead (Pb)) and absence (Control) of lead.

not identify bias. FA was not detected in either the individuals exposed to Pb or the control individuals, indicating no significant differences between the treatments for either of the measured organs. ($p > 0.05$ for both models) (Fig. 4).

Discussion

While measuring the body size and structures (such as tibia and antenna) of *B. brassicae* individuals that developed on kale plants cultivated in Pb-contaminated soil and control plants, we observed that the measured organs showed no significant differences in size between the treatments or notable variations in bilateral symmetry. Our findings have demonstrated that allometry, morphometry, and FA are not effective for detecting environmental stress caused by field exposure of aphids to Pb in the soil.

The absence of an effect from Pb on the morphometric trait size variation in *B. brassicae* may be related to the bioaccumulation and detoxification processes of the individuals. Merrington et al. (2001) demonstrated that bioaccumulation in aphids was lower than that in host plants in soil contaminated with Cd and Zn, evidencing a smaller catchment of the metal and possible creation of a barrier for its movement through the food chain. The removal of Pb through honeydew by aphids is a possible detoxification mechanism, controlling its transfer to other trophic levels (Naikoo et al., 2019). Increased Pb elimination via aphid excretion was reported in a dose-dependent manner in *Aphis fabae* Scop. and *Lipaphis erysimi* (Kaltenbach) species in *Vicia faba* L. and *Brassica juncea* (L.), respectively, indicating a possible detoxification mechanism responsible for the reduction of this element in the body of these insects (Dar et al., 2015; Naikoo et al., 2019). However, it is argued that this mechanism may not apply to all aphid species (Dar et al., 2015). For example, Pb was biomagnified in the aphid *Acyrthosiphon pisum* (Harris) on pea (Woźniak et al., 2017). All these works, as well as others in the area (e.g. Dar et al., 2017; Shi et al., 2020), addressed the availability of heavy metals to aphids through leaf chemical analyses. We need specific works reporting the levels of heavy metals in the phloem of plants aphids' food sources to understand the process of accumulation and detoxification of metals. Other aphid detoxification mechanisms may also be involved in this response; however, studies that are more detailed are needed to observe the physiological processes of individuals exposed to heavy metals.

Pb treatment did not affect the levels of FA, and it was not possible to verify instabilities in the development of aphid individuals in this context. Görür (2006) conducted an experiment under controlled laboratory conditions and verified that copper (Cu; as $CuS0_4 + 5H_2O$) and Pb (Pb; as $Pb(NO_3)_2$) applied via plant irrigation at concentrations of 3.14 mg.L^{-1} and 1.28 mg.L^{-1}, respectively, increased the FA of *B. brassicae* associated with cabbage and radish. However, the indices created by the author to relate the instability by FA to a reduction in fitness did not present significant values. Frequently, the relations between stress, FA and fitness demonstrated to be weak and heterogeneous because they present a large variation between groups and measured organs (Leung and Forbes, 1996). The difference in plant species variety may have contributed to the difference in the findings of our work with those of Görür (2006). Furthermore, the methods used by Görür, such as the daily irrigation of seedlings with contaminant solution, could explain the differences from our results, since recurrent contamination could affect aphid detoxification mechanisms. Unlike the work cited, we conducted direct soil contamination only once to simulate a local contamination scenario and to assess the effect of the maximum allowable Pb concentration for soils in Brazil, and there was natural infestation by insects in an open greenhouse, increasing the aphid population variability and simulating natural conditions. In addition, studies have not used a range of heavy metals concentrations on insects. We suggest that future studies consider other concentrations to evaluate the effects on fauna and also compare laboratory and field conditions.

Bjorksten and Pomiankowski (2000) discovered that flies of the species *Teleopsis dalmanni* (Wiedemann) did not present differences in FA when the larval stadia were exposed to food stress in the form of nutritionally poor resources, despite the trait size reduction in individuals in this treatment. Relating to the heavy metals, the size and symmetry evaluation of ants *L. flavus* eyes along a gradient of post-mining pollution also demonstrated the reduction of size and did not indicate AF (Grześ et al., 2015). These studies demonstrate that FA may not be a good indicator of stress in the development of individuals. Wadhwa et al. (2017) verified that although there is a reduced body size of isopods found in soils containing high levels of heavy metals, the FA of these populations is lower than that of populations found

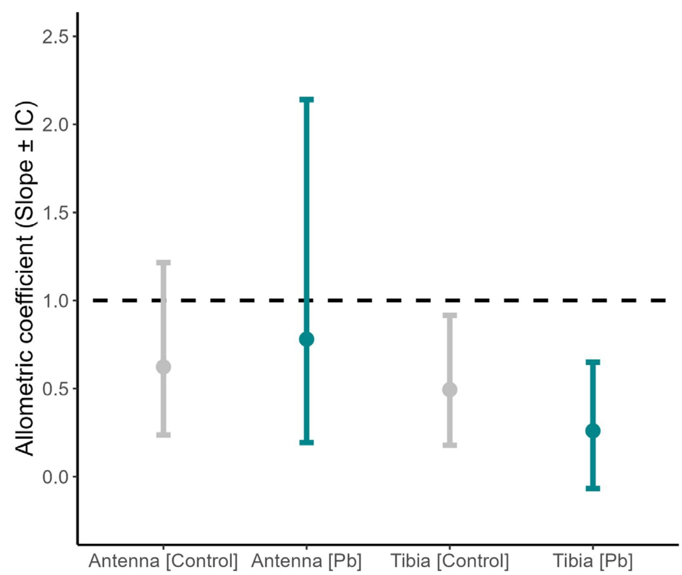

Figure 3 Negative allometry represented by the allometric coefficients of both the antenna and tibia and their confidence intervals; the values are related to the body length of *Brevicoryne brassicae* in the presence (Lead (Pb)) and absence (Control) of lead.

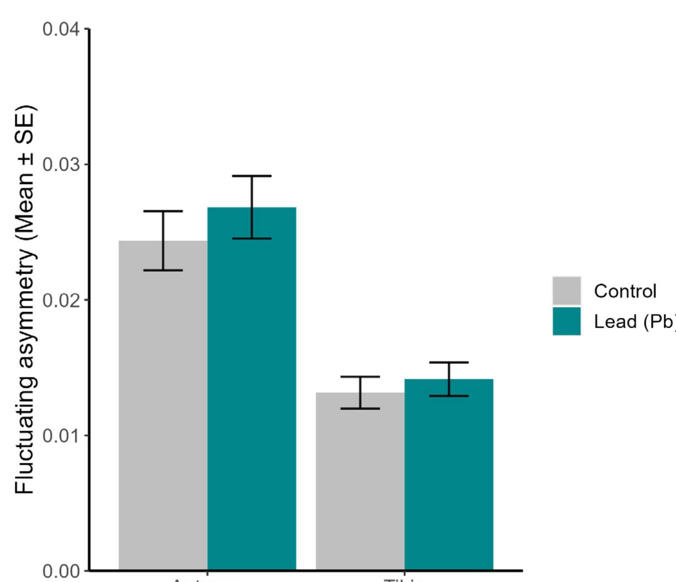

Figure 4 Fluctuating asymmetry (mean and error deviation) observed in the antenna and tibia of *Brevicoryne brassicae* in the presence (Lead (Pb)) and absence (Control) of lead.

in places with lower rates of heavy metals. Indeed, Floate and Fox (2000) raised the hypothesis of differential mortality to explain why exposure to the pesticide ivermectin during the development of *Musca domestica* L. does not affect the levels of FA in the wings of this species. The flies that survived the pesticide represent a more tolerant subset of the original population; in this subset, symmetry was not affected by the stressful conditions. Levels of FA can be biased in stressful conditions by mortality of individuals with unstable development. Therefore, the interaction between the FA of individuals and the adequacy of the stressor may confound the effect of heavy metals on symmetry (Hendrickx et al., 2003). Not many studies have evaluated fluctuating asymmetry in arthropods. Some of them currently use wing measurements to detect FA, but for this, they employ geometric morphometrics and area measurements (Benítez, 2013; Benítez et al., 2020; Vilaseca et al., 2022). In our study, since we measured apterous individuals, we obtained linear measurements of the traits. Therefore, we assessed the fluctuating asymmetry using the method described by Palmer and Strobeck (1986). When considering developmental stresses associated with heavy metal pollution, fluctuating asymmetry has not proven to be a reliable indicator, even in the case of plants (Zverev et al., 2018; Sandner et al., 2019). In this study, it was not possible to detect this mechanism in *B. brassicae*, since the measurement of individuals did not extend over generations and we did not record mortality rates with measures of single-generation individuals. However, we observed a drastic reduction in the population density of this species in the presence of Pb (Morales-Silva et al., 2022), indicating that this population experienced stress and that differential mortality could have occurred. We suggest that future work seeks to identify this effect.

The negative allometry found in both treatments suggests that the body length increase does not match a proportional increase in the appendices of *B. brassicae*. Negative allometry is commonly found in insects. Studies that evaluated the effect of different types of stress on insects, such as insecticide-induced stress in parasitoids (*Trichogramma pretiosum* Riley) (Souza et al., 2018) or resource competition stress in seed-feeding insects (Bruchinae beetles and Braconidae wasps) (Silva et al., 2017; Oliveira et al., 2020), have found negative allometry for both insects that developed under stress and insects in control groups. Furthermore, similar to our study, these studies found an increase in the variation of the allometric coefficient and confidence intervals when insects were exposed to these types of stresses, suggesting that allometry could be a more sensitive tool for detecting body variations caused by different types of stress. However, studies considering the effects of heavy metals on the allometry of invertebrates in general are rare, and this aspect requires further exploration. Regarding the measured organs, the antenna of *B. brassicae* presented a greater variation than the tibia in the allometric coefficient. As demonstrated by Mirth et al. (2016), the parameters of the morphological size relations, such as the allometric coefficient and intercept, may vary for different organs in the same species. In addition, the allometric relations may be dependent on the type of heavy metal used and the parts measured, as different organs can respond in different ways (Hédouin et al., 2006).

Suhendrayatna et al. (2019) demonstrated that Pb and Zn in sediments from mangroves at concentrations of 8.246 - 12.995 Pb mg.kg^{-1} and 32.371- 45.045 Zn mg.kg^{-1} created a negative allometric pattern in the shells of individuals of the mollusk species *Geloina erosa* (Solander) that lived and grew in this habitat. They found a hyperaccumulation of Zn in the mollusks, but these individuals did not incorporate Pb. These findings demonstrate that there may be consequences on the development of organisms due to long-term heavy metal incorporation. However, the relationship between changes in the development of individuals and the incorporation of metals, in general, remains unclear. As noted, different metals can have different mobility, depending mainly on the organism and the environment, as discussed previously. For example, the beetle *Pterostichus cupreus* L., when reared on copper-contaminated soil and food (0.5 mg Cu^{2+}/g^{-1} dry weight of soil and 0.5 mg Cu^{2+}/g $^{-1}$ fresh weight of food), exhibited significantly reduced locomotor behavior, despite having copper levels in their bodies that were either equal to or slightly higher than those in the beetles in the control group (Bayley et al., 1995). Therefore, the effect of metals on the development of individuals can be observed regardless of the incorporation of the element.

Conclusions

In this study, we demonstrated that allometry, morphometry, and FA were not efficient tools for detecting heavy metal stress in aphids exposed to Pb through soil under field conditions. The drastic reduction in population density of these insects due to exposure to Pb concentrations allowed by soil legislation did not reflect changes in morphometric traits of these individuals. Thus, we have demonstrated that studies investigating stress from environmental contamination under field conditions may yield different responses compared to experiments conducted under more controlled conditions. Therefore, considering the variation in findings among studies using morphometric traits, we discourage the use of morphometric analyses in investigating environmental stress in insects caused by field exposure to heavy metals. Instead, we suggest employing population (e.g., population density, fecundity) and community (e.g., species diversity and composition, food web metrics) ecological metrics, as they have proven to be more effective in our research (Morales-Silva et al., 2022, 2023) and are more extensive and relevant from an ecosystem perspective. We also emphasize the need to investigate certain aspects related to this process, such as the relationship between metal bioaccumulation and bodily variations, and the specific mechanisms of detoxification employed by individuals.

Acknowledgments

We thank the Department of Agriculture at the Federal University of Lavras for the space granted in the experimental farm, as well as the "Centro de Estudos em Biologia Subterrânea" (CEBS) for providing access to a stereomicroscope. We thank Letícia A. de Oliveira for the support on figure construction. We are also grateful to Tamires C. T. Oliveira and Diego de Souza, for the suggestions on an earlier version of this paper.

Funding

This work was supported by the "Fundação de Amparo à Pesquisa do Estado de Minas Gerais", (FAPEMIG - APQ-02700-17), the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) (140627/2017-0, 130494/2020-8, and 307889/2021-1).

Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution statement

BCS conceptualization, material preparation, data collection, analysis, investigation, writing. TMS conceptualization, material preparation, investigation, writing. LDBF conceptualization, analysis, investigation, writing, funding acquisition.

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Supplementary material

The following online material is available for this article:

Table S1 -Heating program and operating parameters used for the total digestion of the samples assisted by a digester block.

Table S2 - Instrumental parameters used for Pb determination by ICP OES.

Table S3 - Results obtained in the determination of Pb in leaf samples by ICP OES ($mg.kg^{-1}$)