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Transgenerational Sub-Lethal Pyrethroid Exposure Affects Shape Variation and Fluctuating Asymmetry in Seed Beetles

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Abstract: One method of crop protection is the application of a widely used group of pesticides—pyrethroids. As xenobiotics, sub-lethal doses of insecticides cause stress in pests, resulting in a change in the shape and size of their organs or bodies. The stress caused by pesticides may lead to acute destabilization of development, but also to transgenerational canalization through the process of genetic assimilation. Fluctuating asymmetry (FA), small random deviations between the right and left sides of bilaterally symmetrical traits, is an outcome of developmental instability and is a measurable indicator of phenotypic response to stress. We exposed four populations of the seed beetle *Acanthoscelides obtectus* to sub-lethal doses of cypermethrin for ten generations in a laboratory evolution experiment. Using geometric morphometrics, we analyzed size and shape changes and the level of fluctuating asymmetry in untreated beetles and in samples from the fifth and tenth generation. Exposure over ten generations led to an increase in the body size of the beetles, shortening of their pronotum and elongation of their thorax and abdomen. After ten generations of exposure to cypermethrin, FA levels decreased, indicating a canalization of development. This study provides new insights into the phenotypic markers of environmental pollution from agricultural activities.

Keywords: *Acanthoscelides obtectus*; geometric morphometrics; experimental evolution; developmental instability; fluctuating asymmetry; insecticide resistance; cypermethrin

1. Introduction

The common method of crop protection is the use of pyrethroids, neurotoxins that bind to voltage-gated sodium channels in the central nervous system of pests and cause their death [1]. It is known that the intensive and inappropriate use of pesticides has led to the development of resistance in target species, which has reached high levels in European countries [2]. In addition, pests can come into contact with sub-lethal doses of pesticides in various ways (e.g., through improper application or through degradation of the insecticide due to abiotic factors such as sunlight, precipitation or temperature), which represent a significant stress factor but do not cause death [3]. In this way, pesticides can affect growth, reproduction or morphological parameters [4–6], which in turn could affect their locomotion [7], mating behavior [8] and life history strategies [9]. Recently, it has been shown that changes in morphological parameters, especially the size and shape of insects, can serve as a useful phenotypic marker of environmental pollution caused by agricultural activities [10,11]. In particular, in flying insects, the wing or body shape and size can even be used to detect differences between non-resistant and resistant variants [12]. For example,

the size of the wings of *Triatoma infestans* increases with increasing exposure to sub-lethal doses of deltamethrin [13], while pesticide treatment decreases the size of the head and thorax in ants [14]. Changes in wing shape due to sub-lethal doses of insecticide have also been detected in *Chironomus columbiensis* and *Triatoma infestans* [13,15], but also in different stored products of Coleopteran such as *Prostephanus truncatus* and *Tenebrio molitor* [16].

Continuous exposure to pesticides over generations can lead to the canalization of development through the process of genetic assimilation [17,18], resulting in stress tolerance and facilitating pest invasion. Thus, stress-induced changes in development can be of adaptive significance, as they can persist in the next generations through transgenerational effects [19]. Studies addressing the effects of pesticides on the development of pests are sometimes based on estimating and quantifying the degree of fluctuating asymmetry (FA) between susceptible and resistant strains [20–24]. Fluctuating asymmetry is considered a measure of developmental instability due to the influence of various environmental stressors and is reflected in morphological changes [25]. It is defined as subtle differences in size and shape between the left and right sides of a bilaterally symmetrical object [26]. As mentioned above, insecticide exposure can disrupt development and thus lead to an increase in fluctuating asymmetry [21,27–29]. For example, increased FA has been observed in resistant strains of the mosquito *Culex quinquefasciatus* [20] and the moth *Heliothis virescens* [30] compared to susceptible strains. However, the relationship between the level of FA and the changes in development as a result of the application of sub-lethal doses of insecticide is controversial. This controversy is based on the fact that while initial insecticide exposure may lead to increased developmental noise and thus asymmetry, subsequent selection has the potential to restore normal developmental processes and lead to a concomitant decrease in asymmetry [21,31]. If this is the case, the development of surviving individuals after generations of exposure to sub-lethal doses of insecticides can be expected to be more canalized. This is confirmed in several studies in which resistant strains have shown lower FA levels compared to susceptible strains when the former were exposed to long-term selection pressure from insecticide use, whereas FA levels increased when this pressure was absent [21,22]. Accordingly, fluctuating asymmetry can be considered as one of the markers of developmental changes due to the effects of sub-lethal doses of insecticides and insecticide resistance, as well [25,32]. Stress tolerance and adaptation to pesticide exposure are among the key factors for pest success. Therefore, understanding the ecological and evolutionary factors that influence the spread of pests is crucial for crop protection. One of the most appropriate methods for observing morphological and developmental changes due to transgenerational exposure to insecticides is experimental evolution, in which phenotypic changes are observed across generations under environmental conditions determined by an experimenter [33,34]. Although the transgenerational effect of insecticides is being studied, for example, in Diptera [15], the full potential of experimental evolution as a contribution to pest control has not yet been realized, especially with regard to crop pests as target species.

To our knowledge, this study is the first to provide results on whether sub-lethal pyrethroid exposure has transgenerational effects on the morphological traits and development of *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchinae) (Figure S1). Native to Central America, *A. obtectus* has become a cosmopolitan species. This pest, which lives mainly on field bean (*Phaseolus vulgaris* L.) seeds, can cause damage that can account for up to 40% of annual bean yields [35]. *A. obtectus* also develops on other species of the Fabacae family such as chickpeas (*Cicer arietinum* L.), green beans (*Vigna radiata* L.), broad beans (*Vicia faba* L.) or common peas (*Pisum sativum* L.) [36], and significantly increases economic losses. The larva of *A. obtectus* develops in a single dry seed, where it is supplied with water and nutrients. After its hatching from the seed, the adults stop feeding the larva and immediately start mating. Unlike many bruchids, the females of *A. obtectus* disperse their eggs between the seeds instead of attaching them to a surface. The hatching larvae can move between the seeds and eventually burrow into them. The development process of this species, including larval growth and pupation, takes about 30 days and culminates in the emergence of the adults. The rapid transition to reproductive capacity

and the relatively short generation time make *A. obtectus* particularly valuable for long-term laboratory experiments. This efficiency makes it an excellent model organism for the study of evolutionary dynamics under controlled laboratory conditions. The similarity to the conditions in legume storage facilities further emphasizes the importance of laboratory evolution as a tool for studying evolutionary processes under controlled conditions.

Recently, the method of geometric morphometrics has been used to analyze the morphological variability and phenotypic plasticity of different pests [37]. In the context of experimental evolution, geometric morphometrics can be used as a monitoring technique to detect morphological variation in insect populations. Using these methods, we observed changes in shape and size between untreated beetles and the 5th and 10th generations of pyrethroid-exposed beetles. We also tested the hypothesis that the fluctuating asymmetry is lower after ten generations of exposure to the selection pressure of sub-lethal doses of pyrethroid, i.e., that the surviving individuals become more stress-resistant and canalize their development.

2. Materials and Methods

2.1. Rearing Conditions of A. obtectus and Experimental Set-Up

This research was conducted using a laboratory population of *A. obtectus,* referred to as the base population, that had been maintained for over 35 years (301 generations). This population was initially established from beetles that hatched from infected bean seeds obtained from three legume storages from Belgrade [38]. For this experiment, approximately 300 newly hatched adults were randomly selected from the base population to create four replicas, each placed in four transparent cylindrical glass jars (volume: 720 mL; approximately 700 seeds per jar). In order to compare transgenerational sub-lethal effects of the insecticide, beetles from the untreated generation in each replica were sampled for morphometric analyses (referred to as the "untreated zero generation"—G0). The adults of the first generation were exposed to a sub-lethal concentration of the synthetic pyrethroid cypermethrin. In this experiment, we used the concentration that would kill 20% of individuals, i.e., the concentration that had a survival rate of about 80% in each generation ($LD_{20} = 0.1\ \mu g/cm^2$). Experimental beetles were exposed to cypermethrin in 9 cm diameter Petri dishes for 24 h. The surviving individuals were collected and grown on fresh bean seeds. In order to analyze the transgenerational effects of cypermethrin exposure among the beetle populations, this experimental procedure was repeated approximately every 30 days with newly hatched adults for the ten generations, consecutively (Figure 1). To prevent contamination, fresh seeds were frozen before they were used in the experimental procedure. Since *A. obtectus* is facultatively aphagous, no food or water was provided to the adult beetles. All experimental beetles were kept in a dark incubator at a constant temperature ($30 \pm 1\ °C$) under environmental humidity.
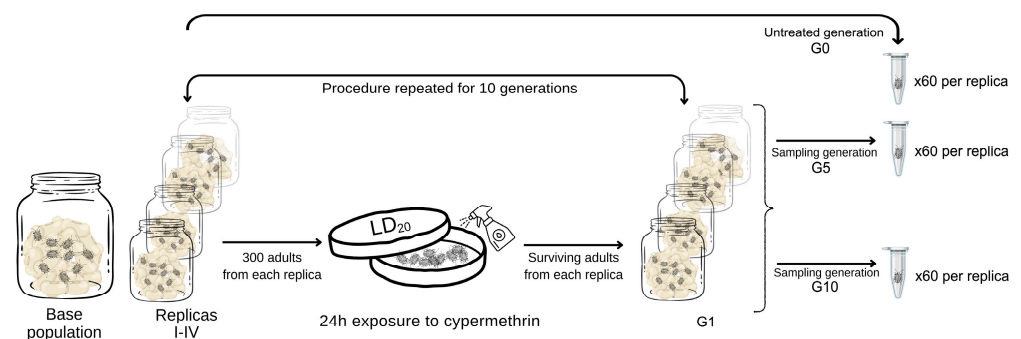

Figure 1. Experimental design using an experimental evolution approach in which beetles were exposed to a sub-lethal dose of cypermethrin (LD_{20}) for ten generations. Beetles were sampled from the base population and after five and ten generations of cypermethrin exposure.

Within 24 h of their adult life, we collected 30 females and males per replica from G0 and from the 5th (G5) and 10th (G10) cypermethrin-exposed generations (Figure 1). The beetles were collected in 1.5 mL Eppendorf tubes and stored at −20 °C. Adults were placed individually on a plasticine mold glued to a microscope plate and photographed against a 10 mm scale on the ventral side using a Nikon Digital Sight Fi2 camera (Nikon, Tokyo) attached to the Nikon SMZ800 (Nikon, Tokyo). The distance and magnification were kept constant during photography.

2.2. Digitizing Landmarks and Geometric Morphometrics

Digital photographs of the beetles were used to digitize landmarks in order to characterize the shape of the beetle body [39]. We selected configurations of 22 landmarks of objects (12 landmarks for the thorax and 10 landmarks for the abdomen, as shown in Figure S2; for details on the positions and descriptions of landmarks, see [40]). In order to estimate measurement error, the landmarks were digitized twice and by one person using the software TpsDig2 [41].

To obtain information on the body shape of the beetles from the landmark configurations, the method of geometric morphometrics was used. The effects of position, rotation and orientation were eliminated using a Generalized Procrustes Analysis (GPA) [42,43]. The centroid size, i.e., the square root of the sum of the squared distances of all landmarks from their centroid, was used as a measure of size. The Procrustes distance, i.e., the square root of the sum of squared differences between the positions of landmarks in two optimally (according to least squares) superimposed configurations at centroid size, contained the information about shape variation, and these values were used as input data for all further statistical analyses [44].

2.3. Statistical Analyses

To test for differences in size between G0 and the cypermethrin-exposed populations for females and males separately, a one-way ANOVA was used. Means in size between the experimental groups were separated using Tukey's (HSD) test [45].

To test whether variation in the shape of the beetles represents a consequence of the allometric growth, a multivariate regression analysis of the Procrustes distances on the log centroid size was applied [46]. A statistically significant regression would indicate that the variation in shape is influenced by the variation in size. If this was the case, morphometric analyses of the shape variables corrected for the size effect were performed to eliminate the influence of allometry on the shape variation.

A principal component analysis (PCA) was performed to examine the overall pattern of shape variation, while differences in mean shapes between the untreated and cypermethrin-exposed beetles were analyzed using canonical variate analyses (CVAs). The statistical significance of pairwise differences in mean shapes was assessed with a permutation test using the Procrustes distances (10,000 permutations per test). Discriminant function analyses (DFAs) were performed to visualize shape changes between untreated and cypermethrin-exposed beetles.

To test the statistical significance of the different types of asymmetries, a Procrustes ANOVA was performed with the main effects being the individual, the side and their interaction [47]. In addition, we estimated variation components for each factor [48,49]. The FA_{10a} index was used to quantify the fluctuating asymmetry for the untreated and cypermethrin-exposed beetles [50]. This index describes the magnitude of fluctuating asymmetry of shape after measurement error has been partitioned out and is calculated as follows: $FA10a = 0.798 \sqrt{2(MSsj − MSm)/M}$, where MSsj is the mean square of the interaction side's x individuals from the Procrustes ANOVA, MSm is the mean square of error from the Procrustes ANOVA and M is the number of replicate measurements. Comparisons of the FA values between the defined experimental groups were carried out using the F-test. The effects of the experimental groups were consistent between replicate lines, making it unlikely that they were caused by random genetic drift. Furthermore, the

results presented in this work refer to the total sample, as they showed no sex-specific effects, that is, the same trends were recorded for both females and males. Therefore, all analyses were performed with pooled data.

All morphometric and statistical analyses were performed using R v.4.3.1 [51] and MorphoJ software v.1.06d [52].

3. Results

The mean value, standard deviation and variance of the centroid sizes of the untreated and cypermethrin-exposed beetles are shown in Table 1. The one-way ANOVA revealed significant differences in their sizes, while Tukey's test confirmed that all comparisons between G0 and the cypermethrin-exposed populations were highly significant ($p < 0.001$). The beetles from G0 had the smallest centroid size, while the G10 beetles, that is, beetles sampled after ten generations of continuous cypermethrin exposure, were the biggest (Mean$_{G0}$ = 7.68, Mean$_{G5}$ = 7.93, Mean$_{G10}$ = 7.97). The multivariate regression of beetle shape variation on log centroid size was found to be highly statistically significant ($p < 0.0001$), so all further analyses were performed on two types of data—those that included allometry and those that excluded allometry.

Table 1. Centroid size mean, standard deviation (Std Dev) and variance for sampling populations of *A. obtectus* over the course of ten generations of cypermethrin treatment.

Sampling Generation	N	Mean	Std Dev	Variance
G0	240	7.6765	0.0669	0.0045
G5	239	7.9272	0.0622	0.0039
G10	240	7.9670	0.0590	0.0035

The overall morphological variation in the beetles is shown in Figure 2, with the first two PC axes accounting for 71% of the total shape variation. The main shape gradient along the first PC before size correction (Figure 2a) ranges from G10 beetles with an elongated pronotum and a shorter thorax to G0 beetles with a short pronotum and a wide and elongated thorax. Along the same PC after correction for size, the main shape gradient spans from G5 beetles with a short pronotum and a wide and elongated thorax to G0 beetles with an elongated pronotum and a narrow thorax (Figure 2b). Along PC2, however, the morphology is similar between the data with and without allometry. In both cases, there is a shift from G0 beetles with shorter thoraxes and elongated abdomens to G5 beetles with elongated thoraxes and short abdomens (Figure 2a,b).
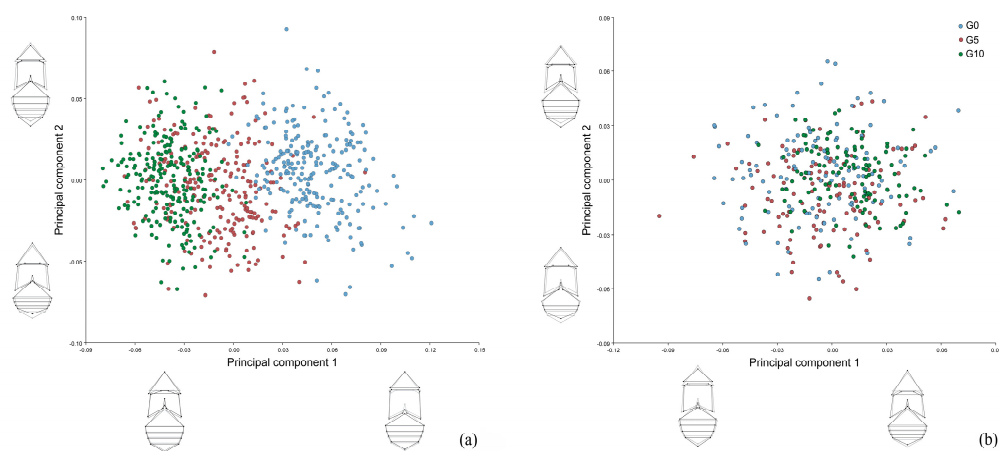

Figure 2. Morphospaces of seed beetles before (**a**) and after (**b**) size correction along the first two PC axes. The presented shape changes correspond to the minimum and maximum PC scores in the scatterplots and are shown as wireframe diagrams.

Canonical variate analyses revealed a clear separation along CV1 (which accounts for 94.5% of the total shape variation) of G0 beetles from G5 and G10 beetles before correction for size (Figure 3a). However, after correction for size, this separation is no longer remarkable (Figure 3b), but the *p*-values from the permutation test of the Procrustes distances between all groups are highly statistically significant (*p* < 0.001). The G0 beetles had a longer pronotum and a shortened and wider thorax in comparison to G5 and G10 (Figure 4a,b). nly in a G0 and G5 comparison without allometry effect, thorax of G0 beetles is more elongated. Abdomen in G0 vs G5 or G10 comparison is shortened after correction for size.
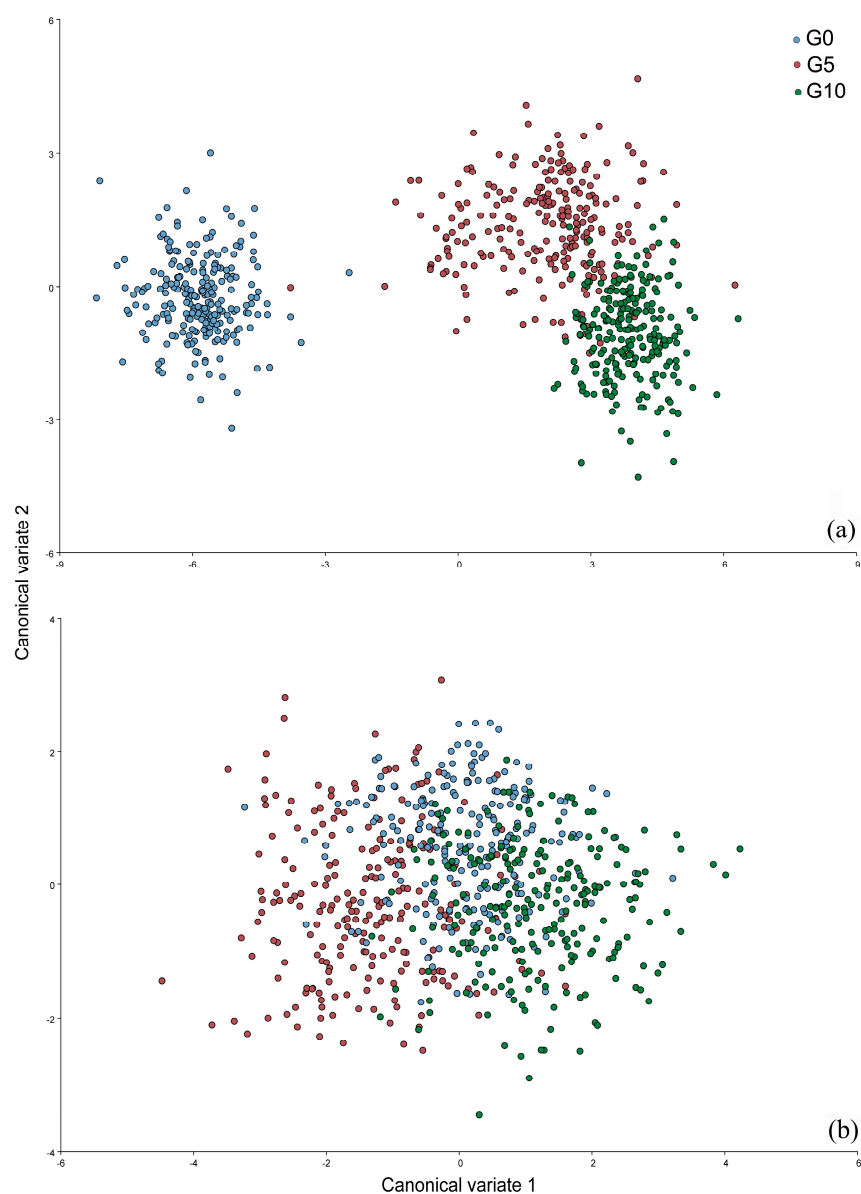

Figure 3. Effects of sub-lethal doses of cypermethrin after five and ten generations of exposure on the shape of beetles before (**a**) and after (**b**) correction for size, shown in a scatterplot with canonical variables.

The Procrustes ANOVA of shape variation showed that all causal effects were highly statistically significant for all three experimental groups (all *p* < 0.0001) (Table 2). The values of the FA10a indexes demonstrate significant decrease from the G0 beetles to the beetles of G10, as shown in Figure 5. The F-test showed that the FA values ($FA_{10aG0} = 0.00523$; $FA_{10aG5} = 0.00470$; $FA_{10aG10} = 0.00436$) induced by cypermethrin exposure differed signif-

icantly between all three experimental groups ($P_{G0 \text{ vs. } G5}$ = 0.00064; $P_{G0 \text{ vs. } G10}$ < 0.0001; $P_{G5 \text{ vs. } G10}$ < 0.01, at α = 0.05) (Table 3, Figure 5).
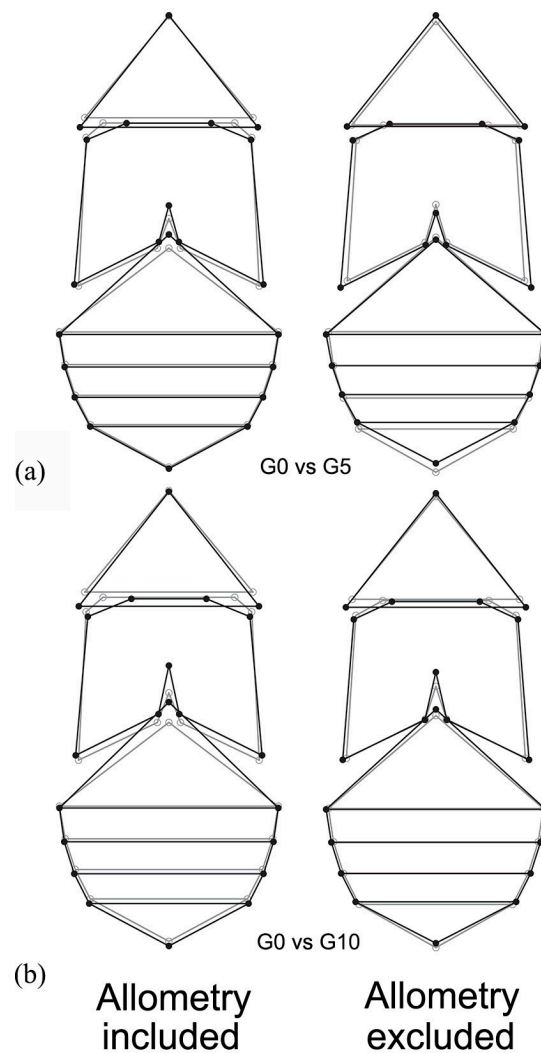

Figure 4. Shape changes (**a**) between G0 and G5 experimental groups and (**b**) between G0 and G10 experimental groups, shown as wireframe diagrams before and after size correction. G0 is presented as a black diagram, while G5 and G10 are presented as a gray diagram.

Table 2. Procrustes ANOVA of shape in three sampling populations of seed beetles with the individual effect, the side effect, the effect of individual $\times$ side interaction and measurement error.

	SS	MS	df	F	Variation Components	*p*
G0						
Individual (I)	0.98835032	0.0002067678	4780	4.28	77.15%	<0.0001
Side (S)	0.01137571	0.0005687854	20	11.78	0.89%	<0.0001
I $\times$ S	0.23086055	0.0000482972	4780	9.19	18.02%	<0.0001
Error	0.05044233	0.0000052544	9600		3.93%	
G5						
Individual (I)	1.04502723	0.0002195435	4760	5.70	82.26%	<0.0001
Side (S)	0.00503024	0.0002515118	20	6.53	0.39%	<0.0001
I $\times$ S	0.18346033	0.0000385421	4760	10.01	14.44%	<0.0001
Error	0.03679463	0.0000038488	9560		3.89%	
G10						

Table 2. *Cont.*

	SS	**MS**	**df**	**F**	**Variation Components**	*p*
Individual (I)	0.69399213	0.0001451866	4780	4.18	71.72%	<0.0001
Side (S)	0.06079900	0.0030399500	20	87.47	6.28%	<0.0001
I × S	0.16611616	0.0000347523	4780	7.15	17.17%	<0.0001
Error	0.04666647	0.0000048611	9600		4.82%	
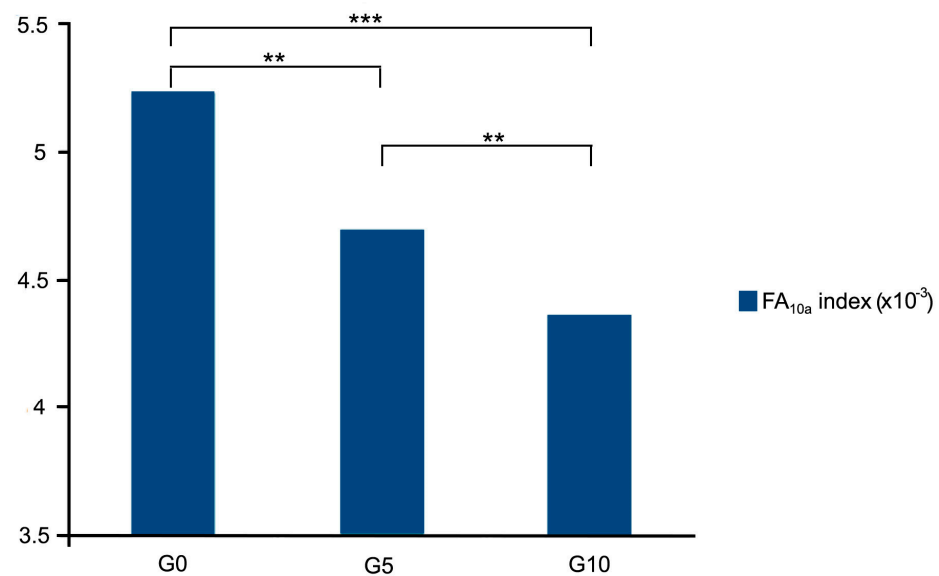
Figure 5. Fluctuating asymmetrical variations in shape for the G0, G5 and G10 experimental groups of *A. obtectus*. Statistically significant differences between the FA10a indexes are marked as *** for $p < 0.0001$ and ** for $p \leq 0.01$.

Table 3. F-test on FA10 indexes for differences in FA between sampling populations of *A. obtectus*.

FA_{10a} **Index**	df_1, df_2	**F**	$p_{(\alpha = 0.05)}$
G0 vs. G5	3774, 3837	1.11	0.00064
G0 vs. G10	3774, 3502	1.20	<0.0001
G5 vs. G10	3837, 3502	1.08	0.01

4. Discussion

A common method of pest control involves the exposure of pests to high doses of pesticides—that is, doses with lethal effects. However, their exposure to sub-lethal doses can lead to morphological variations and changes in developmental patterns as a result of stress and the evolutionary process of resistance. The results of this work allow us to evaluate the morphological responses of surviving beetles to sub-lethal doses of cypermethrin and show that this treatment had significant effects on the size and shape of the beetles as well as on their developmental pathways. The main observed trend during the transgenerational exposure of the beetles to cypermethrin was a significant increase in size—from the smallest G0 beetles to the largest ones after ten generations of continuous pesticide exposure (G10). The size of the beetles had a considerable influence on the variation in their shape, although the shape differences between the experimental groups were present regardless of allometry. Exposed individuals had a more elongated thorax and a more subtly elongated abdomen than the untreated beetles.

Similar effects of sub-lethal doses of pesticides on insect size have been found in a number of species. For example, in *Triatoma infestans*, individuals developed in environments with sub-lethal pesticide doses had larger wings, leading to conclusions that the

wing enlargement was the outcome of selection favoring such phenotypes under insecticide exposure [11,53]. In a few other experiments with sub-lethal doses of insecticides, significant changes in body size have been observed in several Coleoptera species [16,54]. Considering the resemblance between different insect species in response to pesticides, it could be hypothesized that the first target of selection is the body size, as it is to be expected that the smaller beetles have a lower probability of surviving when exposed to harmful chemicals. A number of studies have also investigated the acute or transgenerational effects of pyrethroids on the morphological shape of different insect species [11,13,15,16]. The most pronounced results suggested that sub-lethal doses of these insecticides affected the shape of wings or body segments associated with locomotion [13,15] and consequently influenced insect behavior.

In order to survive, insect populations, which are continuously exposed to insecticides, evolve different adaptive mechanisms of resistance that have high energy requirements [55]. This changes the metabolism in different cells, the developmental pathways, the patterns of growth and the morphology, as well as the behavior, of insects. For example, trophocytes—body fat cells with lipid droplets, glycogen and protein components—in the abdomen and thorax increase in size under the influence of insecticides [56]. In particular, it has been shown that resistant strains of *A. obtectus* (but also other insect species, e.g., *Sitophilus zeamais*) contain trophocytes of a greater volume [57,58]. The increase in size and change in thorax and abdomen shapes observed in *A. obtectus* after ten generations of continuous selection pressure could reflect the higher energy demands, as more energy is needed for insecticide resistance, influencing the patterns of development and reproduction.

In general, it has been shown that changes in morphology represent insects' typical strategy for coping with a stressful environment [59,60]. Along with morphology, seed beetles demonstrate modifications in a whole package of different interconnected traits when exposed to environmental stress factors. For example, after undergoing a shift to a novel host plant species, which can have different and often harmful compounds compared to those of the original host on which the population has been adapted, these beetles prolong their development, have a higher body mass and exhibit higher fecundity [61]. Transgenerational cypermethrin exposure, as a continuous stress, leads to evolutionary modifications similar to those associated with achieving the fitness homeostasis. In other words, the relationships between various traits change along with their genetic, metabolic and biochemical backgrounds, enabling beetles to adapt to new environmental factors [18].

One of the measures that suggests the level of developmental stability is the fluctuating asymmetry [26]. FA is an important tool for investigating pest control and has been used intensively in recent years [37]. Theoretically, it is assumed that FA levels increase under stress conditions and that the application of sub-lethal doses of insecticides destabilizes pest development. Indeed, in some cases, fluctuating asymmetry increases proportionally with the level of application rates [21,22,25,27,28,62,63]. Although increased FA is an expected acute response to stress, the adaptation to continuous stress, such as in the transgenerational exposure of beetles to insecticides, could change this relationship through the stabilization of development in order to reach the adaptive maximum in a certain environment. The consequent coordinated changes in various phenotypic traits that enable maximal survival and reproduction are selectively favored and could lead to the canalization of a specific adaptive developmental pathway—this process is named genetic assimilation [18]. This assumption has been confirmed by some studies showing that insecticide-resistant strains show more stable development compared to susceptible strains. For example, insecticide-resistant strains of maize weevil exhibit lower FA than susceptible strains [64], while *T. infestans* develop more symmetrical wings after being sprayed with insecticides [11,32].

In this study, the level of fluctuating asymmetry estimated in the seed beetles decreased significantly through generations of continuous cypermethrin treatment, leading us to conclude that the beetles stabilized their development. The most significant difference in FA levels was observed between the untreated beetles and the beetles evolved for ten generations under the cypermethrin exposure. All morphological changes due to the

intense selection pressure from the insecticide suggest that stabilized development—that is, canalized development—towards a larger body size and an elongated thorax and abdomen shows significant selective advantages.

Overall, this work has shown that the assessment of different morphological aspects (shape, size and symmetry of pests) can serve as a useful phenotypic marker of environmental pollution caused by agricultural activities. In particular, it provides important information on the morphology and development of *A. obtectus* and demonstrates that the assessment of morphological parameters can contribute to successful pest management control.

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