Zoologischer Anzeiger 276 (2018) 42–49

Contents lists available at ScienceDirect

Zoologischer Anzeiger

journal homepage: www.elsevier.com/locate/jcz


Research paper

# Fluctuating asymmetry indicates levels of disturbance between agricultural productions: An example in Croatian population of *Pterostichus melas melas* (Coleptera: Carabidae)

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A R T I C L E I N F O

*Article history:*
Received 27 January 2018
Received in revised form
17 June 2018
Accepted 9 July 2018
Available online 27 August 2018

*Keywords:*
Carabid beetles
Beneficial insects
Fluctuating asymmetry
Geometric morphometrics
IPM practices

A B S T R A C T

This study evaluated the use of fluctuating asymmetry (FA) of shape, as a bio-indicator developmental stability (DS) in multiple populations of two different agricultural productions a) perennial (orchard) and b) annual (arable) crops on the carabid beetle *Pterostichus melas melas* (Creutzer, 1799) morphology. Shape variation and FA levels were estimated using geometric morphometrics. The results obtained using geometric morphometric analyses such as regressions (FA scores vs Shape) and partial least squares showed that carabids that inhabited the perennial agro-ecosystem seem to have adapted to the strong anthropogenic influence (i.e. IPM practices) at the phenotypic level, while the carabids inhabiting annual agro-ecosystems experience more unstable environments and their phenotypes seem to have been changed more recently. It was expected that phenotypes of the annual agro-ecosystems would be more variable than the long-established ones. Different IPM practices in agro-ecosystems generate different disturbance degrees in insect communities, and these effects can be successfully quantified by applying geometric morphometric techniques.



## 1. Introduction

Carabid beetles are often used as indicators of habitat change due to their fast response to environmental changes, well-known biology and ecology, as well as requiring easy sampling methods (Thiele 1977; Wallin 1985; Lövei & Sunderland 1996; Burel et al. 1998; Avgin & Luff 2010; Kotze et al. 2011). Agricultural productions in intensive agricultural production often create a modified environment, which generates different degrees of stress in insects (Holland & Luff 2000). The tendency of a development system to produce morphological modifications as a response to random perturbations is usually called developmental instability (DI) or developmental noise (DN) (Polak 2003). One of the most common techniques applied to analyze the impact of DI on a particular morphological feature is fluctuating asymmetry (FA) (Van Dongen 2006; Benítez et al. 2014a; Klingenberg 2015). FA measures the small random deviations occurring between the right and left sides of bilaterally symmetrical traits (Van Valen 1962; Parsons 1992). The FA has been widely applied as a tool to measure DI levels in populations experiencing different environmental stress factors such as temperature, nutrition, radiation, chemical substances, population density, noise, parasitism, light conditions, predation risk and habitat structure (Møller 1997; Alibert et al. 2001; Hoffmann & Woods 2003). The different mechanism that have been advanced to assess environmental stress levels have indicated that higher FA levels are expected in populations under environmental stresses when compared to control populations (Graham et al. 2000). The use of FA as a general and sensitive measure of DI due to environmental stress is supported by decades of studies in different organisms particularly arthrophds (Clarke 1995; Floate & Fox 2000; Hoffmann & Woods 2003; Hendricks et al. 2003; Weller & Ganzhorn 2004; Garnier et al. 2006; Benítez

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https://doi.org/10.1016/j.jcz.2018.07.003
0044-5231/

& Parra 2011; Klingenberg 2015). Therefore, FA would represent a general and straightforward biomonitor that allows the identification of populations under environmental stress, due to its apparent generality with respect to the species, characters, and/or environmental stressor under analysis. Several authors have pointed out that FA can be used as a reliable tool to measure environmental stress, due to its sensitivity and simplicity as compared to other indicators; FA allows an easy quantification since it is straightforward and inexpensive technique (Clarke 1995; Floate & Fox 2000; Hoffmann & Woods 2003; Hendricks et al. 2003; Piscart et al. 2005; Benítez & Parra 2011; Klingenberg 2015; Coda et al. 2016). Previous research has suggested that different degrees of phenotypic disturbance reflect the ability of an individual to overcome the effects of stress. This phenomenon emerges because bilaterally symmetric traits shared the same genetic basis and developmental pathways; hence, they develop under the same or relatively similar environmental conditions (Leamy & Klingenberg 2005; Klingenberg 2015). Furthermore, a recent study has shown that FA can be even used as an index to measure the impact of conventional farming as compared to organic agriculture because FA seems to be able to infer habitat quality (Coda et al. 2016).

Variation in carabid morphology, life history strategies and small-scale abiotic and biotic requirements are extensively documented (e.g. Lindroth 1961–1969; Lindroth 1985–1986). Carabids also respond predictably to not only small-scale but also to landscape and even continent level phenomena being sensitive to disturbances at global scale (e.g. Hengeveld 1987; Kotze & O'Hara 2003; Koivula & Spence 2006).

The present paper examined the effects of different agro-ecosystems on the carabid species *Pterostichus melas melas* (Creutzer, 1799) population in an integrated plant production of annual crops (arable) and perennial crops (apple orchard). *P. melas melas* have been used as model species in this survey because it is a dominant species in Central and Southern Europe, inhabiting pastures, open forests, forest edges, forests, agricultural land and meadows. *P. melas melas* is a generalist predator, eurytopic and thermophilous species of clay soils that shows a bimodal period of activity (Giglio et al. 2011). This species is very common in Croatian agroecosystems and acts as a polyphagous predator of insect pests, including aphids, lepidopterans, slugs and dipterans (Freude et al. 2006). The aim of the present study is to analyze the present morphological diversity in *P. melas melas*, as well as to measure the levels of FA in shape that might be affected by different production practices of arable crops and among annual and perennial crops. Different agricultural productions should generate different levels of disturbance in the insect and these effects will be quantified by applying geometric morphometric techniques.

## 2. Material and methods

### 2.1. Study sites

Beetles were collected between May and July 2015 two different agro-ecosystems in Croatia where multiple location where selected in every main agro-ecosystem that are away from each other by around 150 km. The first chosen agro-ecosystem was located at the Krapina-Zagorje County in northwest Croatia (Latitude: 46° 07′ 30.00″ N, Longitude: 15° 48′ 25.20″ E), corresponding to an orchard production. The location under study includes Integrated Plant Managed (IPM; all activities undertaken in accordance with EU Directive (Eur-Lex 2009)) apple orchard common for this area. The IPM orchard was regularly treated with a variety of pesticides and no tillage procedures were performed (grass covered). Weeds in the orchard are managed by carrying out mulching treatments between rows several times during the season. When the beetles were collected only one mulching treatment was performed in the orchard. The second area comprised arable agro-ecosystems located at the Virovitica-Podravina County in northern Croatia (Latitude: 45° 52′ 22.80″ N, Longitude: 17° 30′ 18.00″ E). The data collection locations considered four IPM arable fields that were chosen to represent common cultivation practices, as well as the agro-technical measures typically applied in this area. The distance between arable fields were approximately 5 km. By considering both soil type and soil characteristics, the tillage in the arable systems was adapted to the given conditions and performed as follows: ploughing on a depth of 20–25 cm followed by the furrow closure for moisture conservation; chisel ploughing and tillage with the rotary harrow; after harvest disk harrowing and again chisel ploughing.

A population (A0) not affected by pesticides was used as control group since pesticide treatments are yearly applied and depend on the cultivated crops. This population occupied a wheat arable field that was replaced by sugar beet in 2015. The abundant number of beetles found at the pitfall traps provided extra evidence to confirm an absence of pesticides. Further details regarding common production practices in both agro-ecosystems are provided in Table 1. A description of the regional physical and chemical soil properties of the investigated areas are provided in the Supplementary Table 1 (for further details about the different pedological procedures that were carried out also see Kozina et al. 2015).

### 2.2. Climatic factors

The climate data that was used in this study (i.e. mean weekly air temperature, mean weekly soil temperature and the total amount of rainfall per week) were obtained from the Croatian

**Table 1**
General information about agro-ecosystems from where *Pterostichus melas melas* have been collected, O (orchard); A0 (control); A1 (wheat); A2 (corn); A3 (oilseed rape).

| Crop type (label) | Orchard | Arable crops | | | |
|---|---|---|---|---|---|
| | O | A0 | A1 | A2 | A3 |
| Fertilization | 75 kg N<br>75 kg P<br>75 kg K | 85 kg N<br>105 kg P<br>135 kg K | 168 kg N<br>60 kg P<br>90 kg K | 120 kg N<br>52 kg P<br>52 kg K | 74 kg N<br>60 kg P<br>90 kg K |
| Insecticide treatment (active ingredient) | Mineral oil – 12,000 g/ha<br>Thiacloprid – 48 g/ha<br>Deltamethrin 12, 5x2x – 25 g/ha<br>Acetamiprid – 30 g/ha<br>Pirimicarb – 78 g/ha<br>(foliar treatment) | No insecticide pre-crop | Lambda-cyhalothrin – 5 g/ha (foliar treatment) 1st and 2d year | no treatment (1st) and Imidacloprid – 78 g/ha | Thiacloprid – 72 g/ha (foliar treatment) no insecticide treatment second year |
| Pre-crop (2014) | – | Wheat | Sugar beet | Wheat | Wheat |
| Pre-crop (2013) | – | Sunflower | Corn | Sugar beet | Sunflower |
| Pre-crop (2012) | – | Corn | Corn | Fallow | Sugar beet |

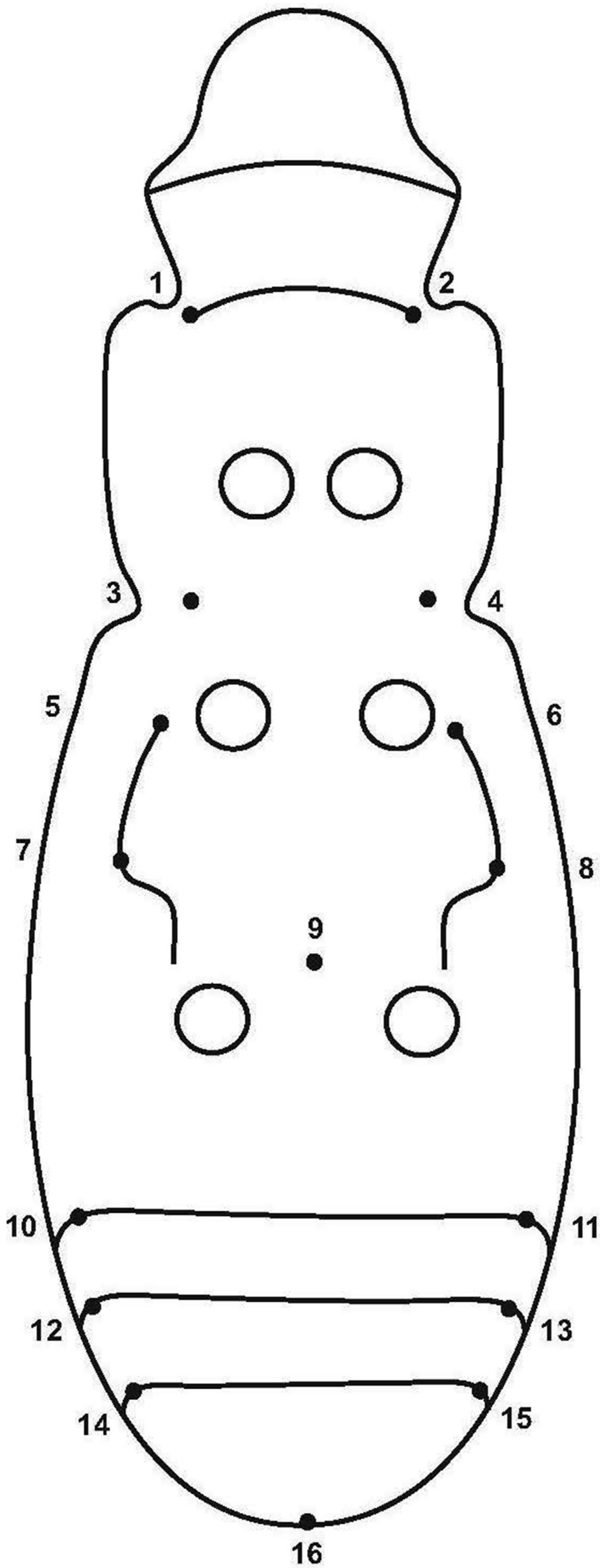


**Fig. 1.** Representation of the 16 morphological landmarks identified on the ventral body shape of *Pterostichus melas melas*, 1: left vertex of pronotal epimere, 2: right vertex of pronotal epimere, 3: left vertex of pronotal carina, 4: right vertex of pronotal carina, 5: upper left point of mesostern, 6: upper right point of mesostern, 7: lower left point of mesostern, 8: lower right point of mesostern, 9: mean point of metastern, 10: left lateral vertex of abdominal segment 3, 11: right lateral vertex of abdominal segment 3, 12: left lateral vertex of abdominal segment 2, 13: right lateral vertex of abdominal segment 2, 14: left lateral vertex of abdominal segment 1, 15: right lateral vertex of abdominal segment 1, 16: pygidium.

Meteorological and Hydrological Service and are available in Supplementary Table 2.

### 2.3. Pitfall trapping

Beetles were sampled using epigeic covered pitfall traps. Polythene pots (Ø = 12 cm, h = 18 cm) were incorporated 18 cm into the soil and covered with PVC lids (Ø = 16 cm) approximately 2–4 cm above ground level. Traps were half filled with saline water (20% solution) for capture conservation; with the addition of few drops of detergent to reduce surface tension (no other chemicals were added). The identification of the collected ground beetles to species level was done based on standard determination keys and morphology (Auber 1965; Bechyne 1974; Harde & Severa 1984; Freude et al. 2006).

### 2.4. Study species

*P. melas melas* was selected to perform further analyses because it represents the dominant species in both agro-ecosystems during the collection period. *P. melas melas* is a nocturnal generalist predator that eats a variety of insects, including agricultural pests. The specimens collected ($n = 250$; $n_{orchard} = 112$, $n_{arable} = 138$) were preserved in 70% ethanol and sex was determined through the examination of bottom side structure on front legs, discrimination by sex was made by the sex combs which are present only at first legs tarsi on males and serve to attach to females during copulation.

### 2.5. Shape analysis

Geometric morphometric analyses were performed using an image of the ventral body side of individuals taken by Leica DFC295 digital camera on a trinocular mount of a Leica MZ16a stereomicroscope and saved as .JPEG files using Leica Application Suite v3.8.0 (Leica Microsystems Limited, Switzerland), all the specimens were photographed in the same position (on microscope slide using clay mold). Sixteen landmarks were digitized (LMs: anatomical homologous coordinates) using the tpsDIG v2.17 software (Rohlf 2013) (Fig. 1). X-Y coordinates were obtained and the shape information was extracted using a full Procrustes fit (Rohlf & Slice 1990; Dryden & Mardia 1998). In order to analyze the symmetry of the structure, reflection was removed by including the original and mirror image of all configurations under analysis and simultaneously superimposing all of them (Klingenberg et al. 2002).

Measurement error (ME) is of crucial importance when analyzing FA, due to possible errors in the landmark acquisition procedure (e.g. Palmer and Strobeck 1986). In order to evaluate the significance of FA, all individuals were digitized twice. An analysis of variance (ANOVA) was applied on centroid size to asses possible size differences, while a Procrustes ANOVA was performed to assess shape differences, taking into account the comparison of the values of $MS_{ind}$ (MS of individual variation of the ANOVA) with Error 1. These analyses were performed between sexes separately to analyses the influence of sex to overcome the shape asymmetry.

The main patterns of shape variation were visualized by carrying out a principal component analysis (PCA), computed from the covariance matrix of the averaged population symmetric component of shape (Klingenberg et al. 2002). In order to statistically

**Table 2**
Procrustes ANOVA performed to assess fluctuating and directional asymmetry for both centroid size and shape of the *Pterostichus melas melas* individuals that inhabit the arable crops and orchard. Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances (dimensionless).

| Effect | SS | MS | df | F | P |
|---|---|---|---|---|---|
| **Centroid size** | | | | | |
| Individual | 19818180 | 79272.7195 | 250 | 143.21 | <0.0001 |
| Error 1 | 133958.34 | 553.546873 | 242 | | |
| Effect | SS | MS | df | F | P |
| **Shape** | | | | | |
| Individual | 0.7314601 | 0.00024382 | 3000 | 2.74 | <0.0001 |
| Side | 0.0137833 | 0.0011486094 | 12 | 12.89 | <0.0001 |
| Ind[a] side | 0.2673201 | 0.0000891067 | 3000 | 3.21 | <0.0001 |
| Error 1 | 0.0613491 | 0.0000277805 | 5808 | | |

[a] This analysis takes into account the object symmetry in the data.

assess whether there were differences between the different locations, a canonical variate analysis (CVA) of the symmetric component of shape variation was also performed and Mahalanobis distances were computed (Campbell & Atchley 1981). CVA maximizes the differences between groups relative to the variation within groups and it is consequently one of the most applied tools to discriminate among groups (Campbell & Atchley 1981). In order to analyze the relationship between the shape and the levels of FA a multivariate regression including its respective confidence ellipses (90%) per group were performed using the shape and the FA scores and statistically tested with 10,000 iterations permutation analysis. To analyze the correlation between the climate data and shape, a Partial Least Square analysis was performed using temperature and rainfall as covariates (Benítez et al. 2014b).

All statistical and morphometrics analyses were performed using the software MorphoJ 1.06e (Klingenberg 2011).

A morphological affinity dendrogram was generated by applying Ward's method for agglomerative-hierarchical cluster analysis using the PCs from a PCA of the average shapes obtained from of each arable crop and orchard category (Euclidean distances were used as similarity index) (Hammer & Harper 2008). This analysis was performed in R v.3.3.2 (https://www.r-project.org/).

## 3. Results

The Procrustes ANOVA applied to assess ME and FA levels showed that ME was negligible ($MS_{invid} > ME$) (Table 2).

The PCA of the ventral body shape showed that the first three PCs accounted for 64.1% (PC1 = 25.6%; PC2 = 22.1%; PC3 = 16.3%) of total shape variation, thus providing a reasonable approximation of

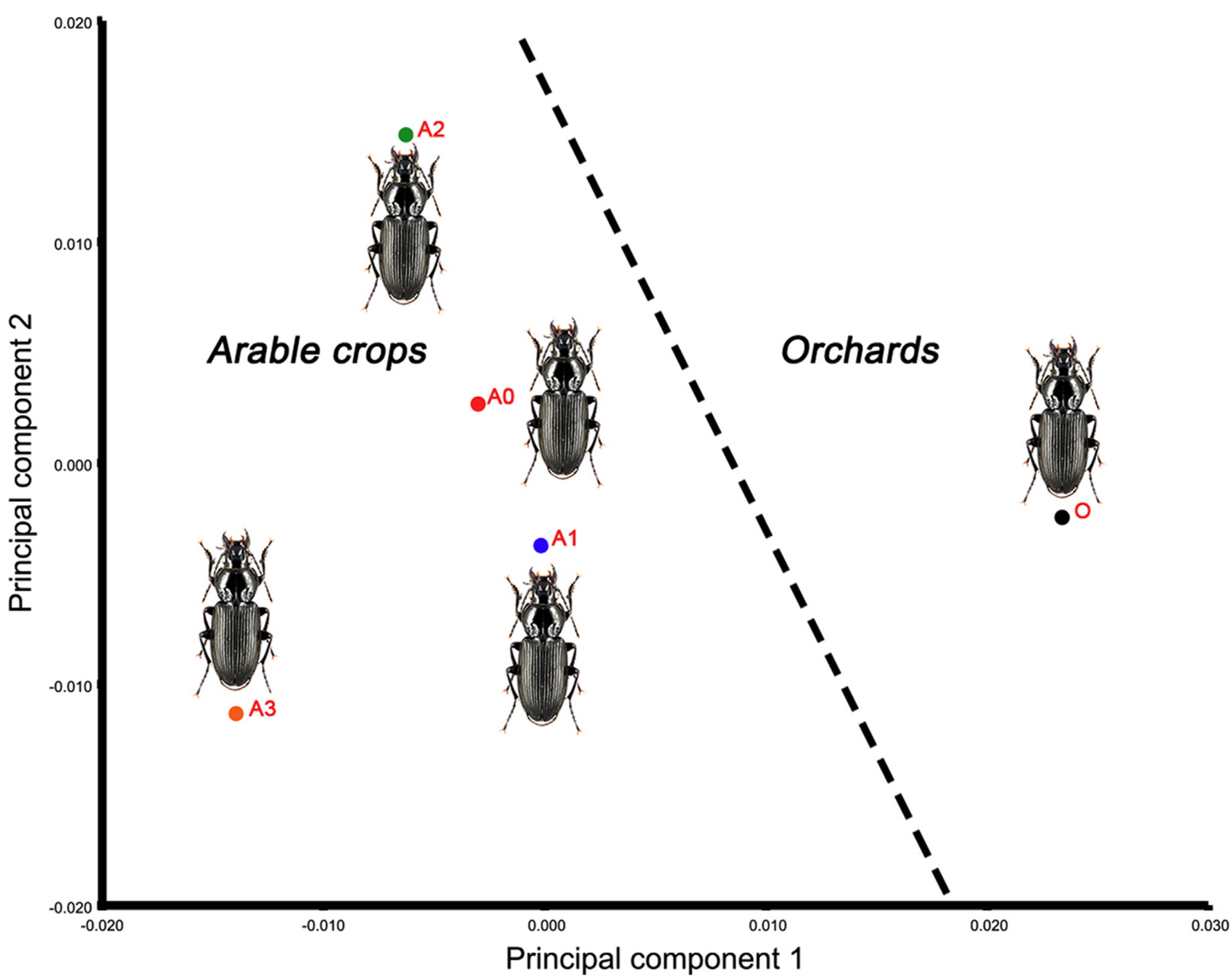


**Fig. 2.** Principal component analysis for the average body shape in *Pterostichus melas melas*. The first two principal components (PCs) are used to display most of the variation of shape.

the total amount of variation observed in the sample. The PCA plot confirmed the presence of a particularly dissimilar shape for the orchard beetles (Fig. 2). The morphological affinity dendrogram showed clear differences between the beetles inhabiting arable A0 (control): red; A1 (wheat): green; A2 (corn): green; A3 (oilseed rape) and those occupying the apple orchard (O) (Fig. 3). Noticeable FA levels were found in all the specimens inhabiting the arable crops, higher levels of FA were found in females than males (A1, A2, A3) as compared with the control, nevertheless, in the orchard males have higher FA than females (Fig. 4). The multivariate regression of the symmetric shape component on the FA scores, it was found a significant ($P = 0.004$) but not particularly strong relationship ($r^2$: 0.386) between the beetles raise at different agro-ecosystems. The individual inhabiting the orchard exhibited a particularly dissimilar shape in comparison with the three other groups raised in arable crops that shows a more variable shape (Fig. 5). Similar results were found when applying the CVA. This analysis showed a significant differentiation among arable crops and orchard populations based on Mahalanobis distances (Table 3). Finally, the two-block partial least-squares analysis indicated a strong association between shape variables and the combined environmental conditions (temperature and rainfall) ($r^2 = 0.778$, p-value <0.001).

## 4. Discussion

This study investigated how different agricultural production practices influence FA levels in *P. melas melas*. The main findings revealed a clear presence of shape FA associated with both agro-ecosystems under study. Nevertheless, there were no significant differences between FA levels from different agro-ecosystems to the control, thus suggesting that *P. melas melas* might have modified their phenotype to each particular agro-ecosystem (apple orchard) (Fig. 5). The results of the multivariate regression of shape on FA confirmed what was outlined above, showing different body shapes depending on the inhabited agro-ecosystem, particularly for the individuals occupying the apple orchard that exhibited a noticeably different body shape (Fig. 4). The average shape was similar for the four arable crops evaluated including the control (A0-A3), while it was particularly dissimilar for the apple orchard (Fig. 3 and Table 3).

It is well-known that FA is a proxy for DS (Clarke 1995; Hoffmann & Woods 2003; Hendricks et al. 2003; Leamy & Klingenberg 2005; Van Dongen 2006; Garnier et al. 2006; Klingenberg 2015; Coda et al. 2016) hence in broader terms, more symmetrical individuals would have better survival chances than those with lower symmetry levels (Møller & Swaddle 1997; Møller 1997; Lens et al. 2002; Benítez et al. 2008). Since FA can directly affect an individual's fitness, our results of FA levels associated with sexual dimorphism has shown that in populations exhibiting high FA levels, females tend to show slightly higher asymmetry levels than males, while in populations exhibiting lower FA levels the opposite effect was found (Fig. 4). Benítez et al. (2008) has showed that beetle populations inhabiting pine plantation forests under environmental stress due to resine accumulation, showed high levels of asymmetry in their antennae, where was higher in females affecting the oviposition behavior. Consequently, the higher FA levels observed in the beetles should suggest a lower quality of the environment under study. However, the similar FA levels suggest a relatively faster adaptive process of the DS of these organisms. Previous research on this topic have shown some relationship between FA levels and mortality levels associated with insecticides (Chang et al. 2007a,b; Ribeiro et al. 2007; Allenbach 2011). Polak (2003) describes numerous examples that relate fitness to instability (i.e. the outcome of many subtle stochastic events that tend to modify the accuracy of development in a certain environment) during larval development. For example, changes in levels of bilateral asymmetry could negatively disturb the search for an adequate oviposition place, mate choice, among others. The morphological affinity dendrogram and the CVA are concordant showing that differences in the intraspecific shape variation for the individuals from the different arable crops and the apple orchard, where the adaptation to the anthropogenic influences could be the consequence. The environmental quality was also reflected in the population density observed at the pitfall traps (control > orchard > arable crops). Ribeiro et al. (2007) suggest that an extended period of insecticide selection could led to the evolution of fitness modifier genes that might improve the performance of the resistant genotypes, thus reducing their FA levels, and leading to their eventual fixation in the population. The PLS results showed that the body shape variation in *P. melas melas* covaries with the environmental factors because of plasticity, nevertheless a deeper

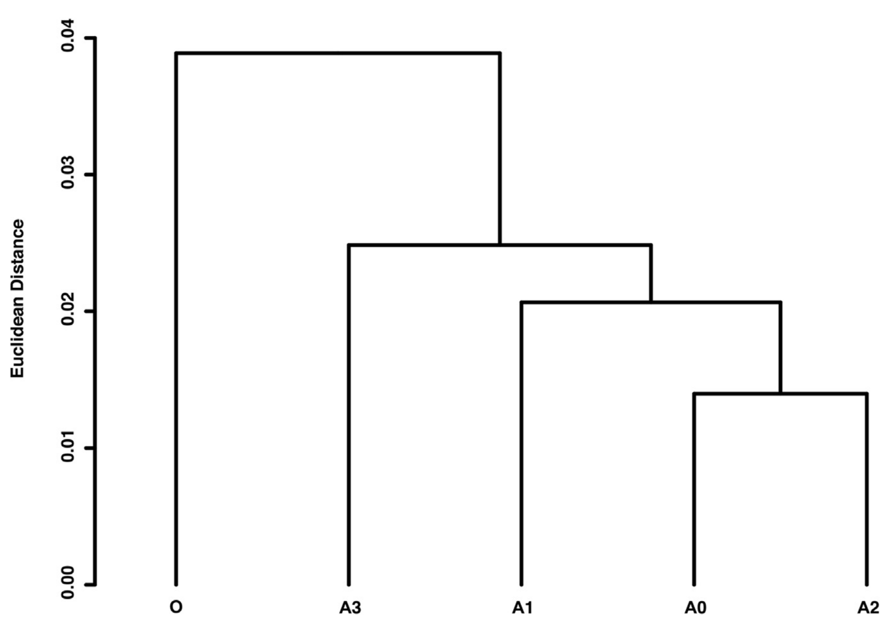


**Fig. 3.** Morphological affinity dendogram generated using Ward's method of the average shapes computed for each location under study.

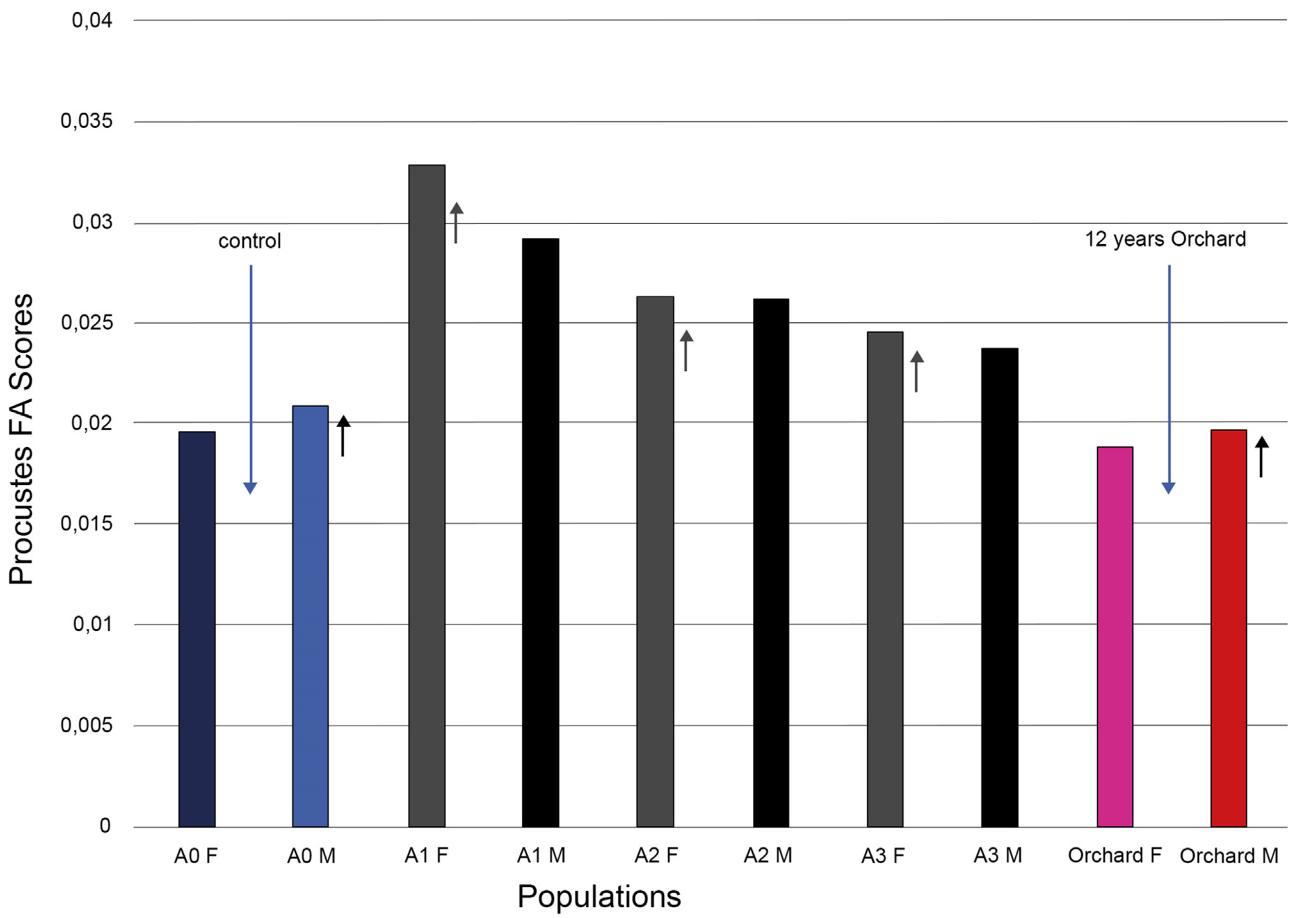


**Fig. 4.** Histogram of the intensity of FA at multiple population and sex the values are calculated using the Procrustes FA Scores blue bars represent the A0 control population without pesticide, A1 (wheat): black/grey; A2 (corn): black/grey; A3 (oilseed rape): black/grey and red one represents the 12 years apple orchard. F = female (blue, grey and pink) and M = male (light blue, black and red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

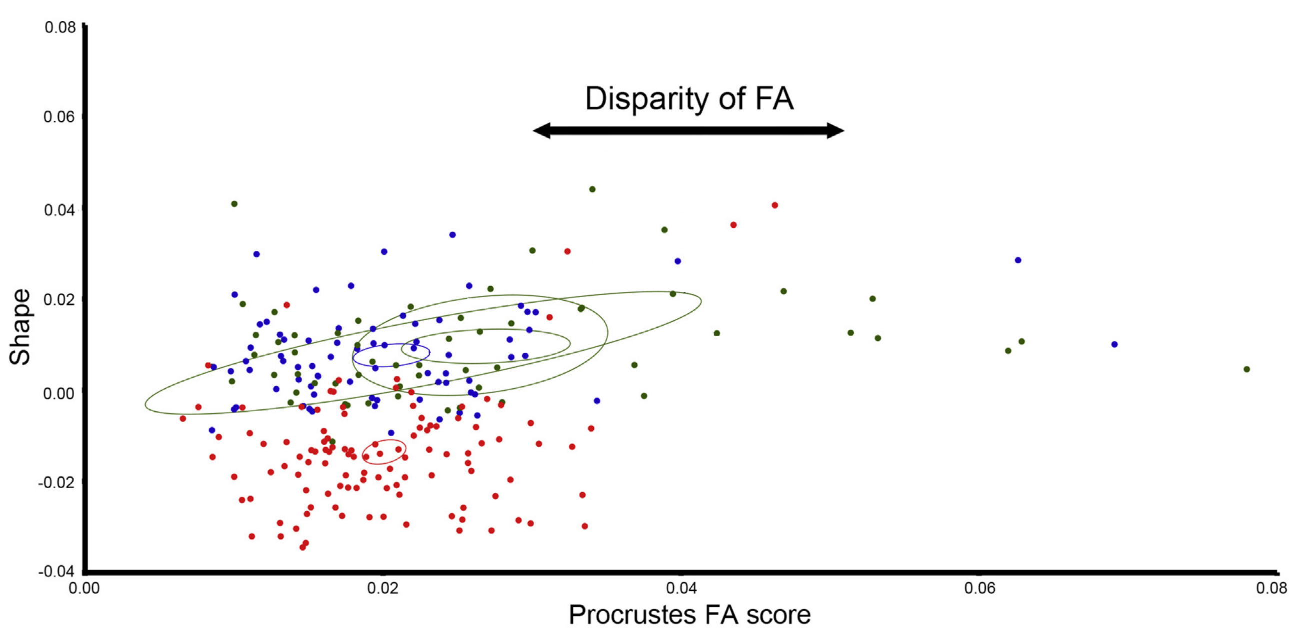


**Fig. 5.** Multivariate regression analysis of shape on Procrustes FA. The different colors represent the different populations under study O (orchard): red; A0 (control): blue; A1 (wheat): green; A2 (corn): green; A3 (oilseed rape): green, M (museum control) black. The 90% confidence ellipses were calculated for the mean of every populations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 3**
Results of the CVA analysis showing the computed Mahalanobis distances and the respective p-values (10,000 iterations).

| Populations Distance P value | A0 sugar beet Mahalanobis P value | A1 wheat Mahalanobis P value | A2 corn Mahalanobis P value | A3 oilseed rape Mahalanobis P value |
|---|---|---|---|---|
| A1 | 1.63<br>0.623 | | | |
| A2 | 1.06<br>0.0027 | 1.88<br>0.397 | | |
| A3 | 1.77<br>0.016 | 1.921<br>0.833 | 2.155<br>0.0021 | |
| O/Orchard | 2.74<br><0.0001 | 3.317<br>0.0007 | 3.34<br><0.0001 | 3.96<br><0.0001 |

comparison is needed to elaborate on the relationship between climatic conditions and exposure to agrochemicals. The apple orchard population has experienced 12 continuous years of foliar pesticide exposure, which would correspond to ca. 12 beetle generations (*P. melas melas* is univoltine species, with one generation per year, its oviposition is in autumn, winter larvae and a new generation appears in the forthcoming spring). Hence it is possible to conclude that this carabid population has inhabited the same area for enough time to particularly adapt to this strong agrochemical exposure both at the genotypic and phenotypic level (e.g. their body shape is particularly dissimilar as compared to the other groups under study). On the other hand, the populations occupying the arable crops experience more unstable environments, because the arable agro-ecosystems are modified each year. This suggests that the phenotypes observed in the arable crops have evolved more recently when compared to the ones observed in the more stable (although with a high agrochemical exposure) environment of the apple orchard. Therefore, it was expected that phenotypes of the carabids from arable agro-ecosystem would be more variable than the carabids from the long-established ones when developmental constraints have evolved through a canalizing selection process, in which similar phenotypic expression is stabilized across a range of genotypes.

## Acknowledgments

The authors are very grateful to the comments of several experts on the topic and principally to the reviewers to provide valuable comments to the final version of this manuscript. We are grateful to the family farm Katančić and family farm Fruk for allowing the data collection. We also thank the taxonomy expert Teun van Gijzen for his help during the identification of the ground beetle species. The Dr. Benitez was financed in part, by a Small Grant from Universidad de Tarapacá, Chile 2017 9719-17. Funding: This study was supported by the European Union from the European Social Fund [grant number: HR.3.2.01-0071] and within the EU projects LIFE+SU.SA.FRUIT [grant number: LIFE13 ENV/HR/000580] and LIFE+INSECTLIFE [grant number: LIFE13 ENV/HU/001092].

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcz.2018.07.003.

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