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# Phenotypic Variability of *Aphantopus hyperantus* and *Coenonympha arcania* (Lepidoptera: Nymphalidae) in the Vicinity of the Middle Ural Copper Smelter. Part 2. Wing Shape and Eyespot Size

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**Abstract**—We tested the hypotheses that the accumulation of potentially toxic metals (Cu and Zn) in adults of two Nymphalid species (*Aphantopus hyperantus* and *Coenonympha arcania*) correlates with wing shape and eyespot size, as well as increases their fluctuating asymmetry. These traits are less functionally significant compared to wing length, for which no negative impact of pollution was previously found in these species. Therefore, theoretically, their fluctuating asymmetry may better indicate stress. Butterflies were collected at different distances from the Middle Ural Copper Smelter (Revda, Russia). The shape of the forewings and hindwings was analyzed using geometric morphometrics. Eyespot sizes were measured on the ventral side of the forewings and hindwings. Wing shape and its fluctuating asymmetry did not differ between sites in all cases (two species, males and females) but, in one case, correlated with metals (*C. arcania* females). Eyespot size differed between sites in one species (*C. arcania*) and negatively correlated with Cu only in females of this species (only for two out of five analyzed eyespots). The fluctuating asymmetry of eyespot size differed between sites only in one case (*A. hyperantus* males), but it was not highest near the smelter; asymmetry decreased with increasing Zn only in *C. arcania* females. Thus, the tested hypotheses were not unequivocally confirmed: although some pollution effects were found at both the group (differences between sites) and individual (correlation with metals) levels, they were very weak, specific to trait, species, and sex, and therefore, most likely occasional. The results add to doubts about the informativeness of fluctuating asymmetry as an indicator of stress in natural insect populations.

**Keywords:** fluctuating asymmetry, developmental stability, geometric morphometrics, wing pattern, Rhopalocera, diurnal butterflies, air emission, heavy metals, industrial pollution

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## INTRODUCTION

The influence of environmental pollution by emissions from the Middle Ural Copper Smelter on the wing length and its fluctuating asymmetry (FA) in two satyr species, *Aphantopus hyperantus* L. and *Coenonympha arcania* L., was considered in the first part of our work [1]. Within the entire multispecies taxocene of Satyridae butterflies, only these two species persist near the copper smelter. Although we expected to observe an increase in fluctuating asymmetry (FA) along the pollution gradient, no association was found with Cu and Zn concentrations in adult bodies [1]. However, this null result does not preclude the possibility of adverse effects of pollution on the developmental stability of the studied species.

Firstly, the asymmetry values of different traits usually weakly correlate with each other in the same object [2, 3]. Secondly, FA of traits with a high functional load, i.e., closely related to fitness, in particular locomotor organs, are usually lower compared to FA of neutral traits, the development of which is weakly stabilized by natural selection [3, 4]. And thirdly, the assessment of developmental stability based on the FA of several traits may be more reliable compared to the assessment based on a single trait [5, 6].

In the second part of our study, using the same sample, we analyze pollution effects on traits less directly affecting fitness—wing shape and eyespot size in the wing pattern. Developmental stability assessments based on FA of these traits may be more reliable than wing length asymmetry measurements due to their multidimensional nature: we analyze multiple spots simultaneously, while wing shape is characterized by a set of landmarks. Therefore, the FA of wing shape and spot size may theoretically better indicate stress caused by metal accumulation than the FA of wing length.

Wing shape affects speed, maneuverability, and energetic efficiency of insect flight [7, 8]. Within Lepidoptera, wing shape exhibits extreme diversity, yet intraspecific variability is markedly lower than differences observed among genera and families. To assess the discussed indicator, we used geometric morphometrics, an effective tool for biological shape analysis [9–12]. A key advantage of this approach is the independent analysis of shape and size as uncorrelated variables.

The wing pattern in Lepidoptera consists of diverse, usually species-specific elements. In Nymphalidae, these include asymmetric and symmetrical stripes, eyespots, parafocal elements, and a submarginal stripe [13–15]. Eyespots are concentric circles of contrasting colors with a clearly defined central spot (focus). Their number, size, and distribution over the wing cells can vary greatly not only between species, but also between sexes or seasonal generations within a species [16]. Eyespots are well-studied: numerous publications have analyzed variability in their shape and size [14, 17, 18], number [19–21], evolution [15, 22], and development mechanisms [16, 23, 24]. Eyespot variation (number, size, and shape) has also been described for the species under consideration: *A. hyperantus* [25–27] and *C. arcania* [28].

The functions attributed to eyespots depend on their size and location on the wing. Several authors [29–31] suggest that large single spots may deter potential predators. Small spots on the ventral side of wings increase the likelihood of losing only the outer part of the wing during a predator attack, diverting attention from the victim's vital body parts [32]. Besides their protective role, eyespots also serve a communicative function [15, 16, 33].

As in the first part of our study, we analyzed the effects of pollution at two levels: individual (the relationship between the indicator and metal concentrations in adult bodies) and group (comparison of sites with varying pollution levels). The tested hypotheses also partially coincide. As with wing length, in the second part, we tested the hypothesis that metal concentrations in adult bodies positively correlates with FA in wing shape and eyespot size. The hypothesis regarding pollution's effect on wing shape and eyespot size cannot be framed in terms of correlation direction with pollution levels. Thus, for these parameters, it was reformulated simply as the presence of a correlation, regardless of sign: we proposed that wing shape and eyespot size correlate with metal concentrations in adult body. This hypothesis was based on the following rationale: wing shape, size, and eyespot positioning are determined by a gene cascade operating throughout the larval development [16, 34–36]. Thus, we cannot exclude that pollution-induced stress during wing development may cause detectable alterations in adult wing shape and eyespot size.

## MATERIALS AND METHODS

**The study area.** The study area has been described in detail previously [1]. Briefly, four sites with grassy vegetation were selected: a control site, 75 km southeast of the Middle Ural Copper Smelter (MUCS, Revda), a background site (regional pollution level, 14 km west of the smelter), a buffer site (moderate pollution, 4–5 km), and an impact site (heavy pollution, 1–2 km).

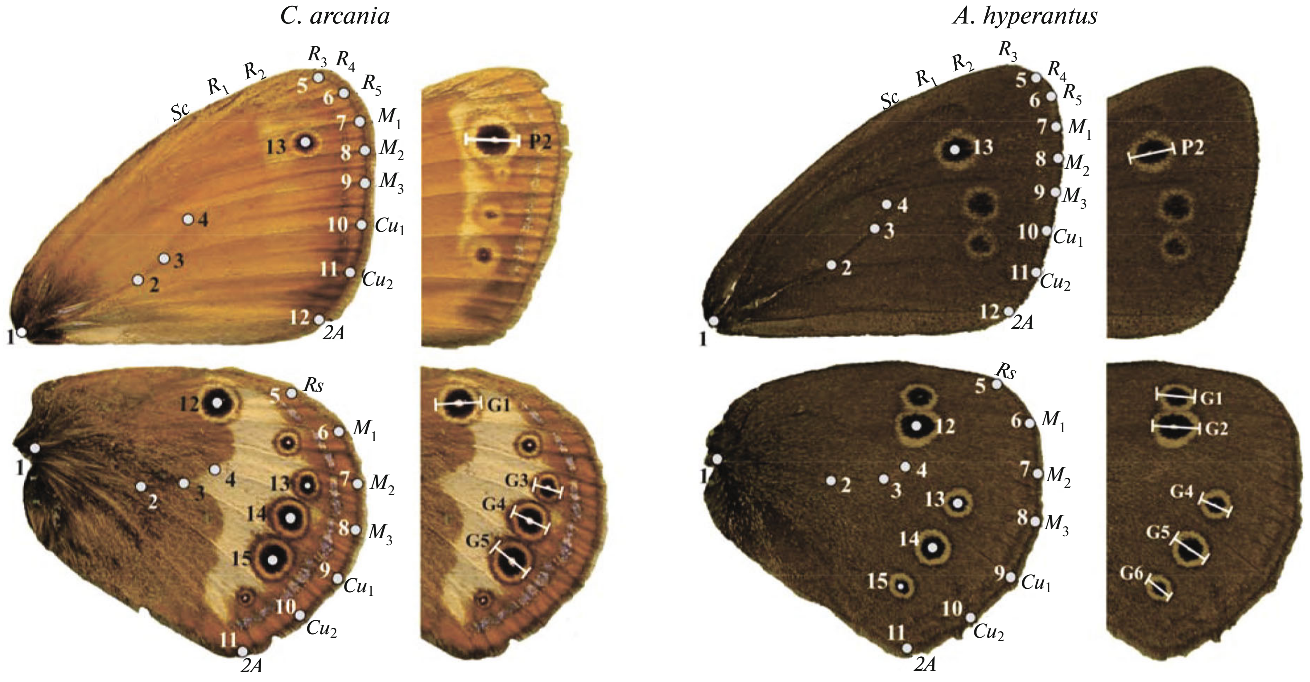
**Sampling methodology.** The collection protocol has been described in detail previously [1]. Briefly, during 29 June–7 July 2003, we captured 225 *C. arcania* and 559 *A. hyperantus* adults across all study sites. For subsequent analysis, we randomly selected 10–20 individuals per sex for each species from every site, yielding a total sample size of 183 specimens.

**Metal concentrations.** Cu and Zn concentrations were measured in the abdomen of each specimen. The rationale for selecting these specific metals and body parts, along with detailed protocols for sample preparation and analytical procedures, have been described previously [1].

**Wing preparation and imaging.** Wings were dissected from the thorax and photographed ventrally using a Canon Eos 600D camera equipped with a Canon Macro Lens EF-S 60 mm f/2.8 USM. Shooting height and angle were fixed using a tripod. We analyzed wing images at 1100 dpi resolution, with landmarks digitized using tpsDig 2.31 [37]. Eyespot diameter was measured from wing images using the ImageJ 1.48v program [38].

The same operator performed all landmark digitization and eyespot measurements twice, with replicate measurements separated by 14 days. To prevent observer bias, the operator was blinded to collection sites. Measurement errors were 0.07 mm for landmark placement and 0.05 mm for eyespot size measurements.

**Wing shape measurements.** A total of 13 landmarks were placed on the forewing and 15 on the hindwing (Fig. 1). These landmarks delineated the wing contour and marked the roots of the veins $M_3$, $Cu_1$, and $Cu_2$ extending from the medial cell, as well as the focus of the eyespots (or their center if absent). Since geometric morphometric methods require identical landmark sets across all specimens, landmarks were only placed on spots present in every individual of the sample. These included forewing spot P2 in both species, hindwing spots G1, G3, G4, and G5 in *C. arcania*, and hindwing spots G2, G4, G5, and G6 in *A. hyperantus* (Fig. 1). Nomenclature of eyespots (P2, G1–G6) follows [39], and that of veins follows [40]. Although hindwing eyespots (landmarks 12–15) differed between species, this did not affect the analysis, as landmark homology was maintained within each species, and interspecific comparisons were beyond the scope of this study.


*C. arcania*

*A. hyperantus*

**Fig. 1.** Landmark configuration for geometric morphometrics of wing shape (left) and linear measurements of eyespots (right) in *C. arcania* and *A. hyperantus*. Nomenclature of eyespots follows [39], and that of veins follows [40].

**Eyespot size measurements.** For *C. arcania*, we measured the diameter of eyespots P2, G1, G3, G4, and G5; for *A. hyperantus*, we measured P2, G1, G2, G4, G5, and G6 (Fig. 1). These spots were present in all captured specimens.

A typical eyespot consists of three elements: a white central focus, surrounded by a black ring and an outer yellow ring. If all elements were present, we measured the yellow ring diameter along the wing cell midline (Fig. 1). If the yellow ring was reduced, we measured the black ring diameter. For spots consisting of only a few scales differing in color from the wing background, we measured the remaining element.

Since eyespots were often elliptical or had irregular contours, the chosen measurement did not always correspond to the maximum spot dimension. However, using a standardized direction (along the wing cell midline) reduced measurement subjectivity.

**Wing shape analysis.** Original *XY* coordinates of landmarks contain information about position, orientation, size, and shape of an object. For shape analysis, however, some of this information becomes redundant and must be removed. This is achieved through configuration superimposition using Generalized Procrustes Analysis (GPA) [9–12]. Resulting Procrustes coordinates can be used directly as variables in multivariate analysis and for visualizing shape variation. Among several alternative superimposition methods, GPA emerged as the standard approach and the method of choice for FA shape analysis [41].

We performed superimposition procedure separately for males and females of each species. For each specimen, we obtained Procrustes coordinates of right and left wing landmarks in two replicates. We then averaged conspecific landmark coordinates from both sides to obtain generalized (symmetrized) wing shape per specimen [42]. This parameter was used when analyzing shape dependence on metal concentrations in imago bodies. If at least one of the two wings had damage preventing proper landmark placement, then both wings were excluded from analysis. Thus, from 183 specimens we analyzed only 172 forewing pairs and 168 hindwing pairs (Table 1).

For two-dimensional objects, Procrustes coordinates produce $2k - 4$ shape variables, where $k$ equals number of landmarks. Due to the small sample size, especially for female *C. arcania*, statistical power would be insufficient for reliable results for such a number of variables. We therefore reduced data dimensionality using principal component analysis, and in subsequent calculations used only the first few principal components that captured the majority of shape variation. Using broken stick criterion [43], we determined number of principal components separately for each sample. As a result, wing shape variation was analyzed using the first 4–7 principal components, which collectively accounted for 73–83% of the total variance.

**Eyespot size analysis.** To assess the relationship between the size of each individual eyespot and metal concentrations, we used the mean values of measure-

**Table 1.** Sample size and structure

| Site | *C. arcania* | | | | *A. hyperantus* | | | |
|---|---|---|---|---|---|---|---|---|
| | males | | females | | males | | females | |
| | forewing | hindwing | forewing | hindwing | forewing | hindwing | forewing | hindwing |
| Control | 10/9 | 10/9 | 10/10 | 8/10 | 8/10 | 9/10 | 9/10 | 9/10 |
| Background | 10/10 | 10/10 | – | – | 10/10 | 9/10 | 9/10 | 10/10 |
| Buffer | 9/10 | 9/10 | 9/9 | 8/9 | 10/9 | 9/9 | 9/10 | 10/10 |
| Impact | 20/20 | 19/20 | 14/14 | 14/14 | 20/20 | 19/20 | 15/15 | 15/15 |

Numerator indicates wing pairs used for shape analysis; denominator for eyespot analysis.

ments from the right and left wings for the same spot. We excluded specimens with wing damage that prevented a complete set of eyespot measurements, as well as those where the inter-wing difference in size for any given eyespot statistically significantly exceeded the sample mean. In accordance with the recommendation [6], statistical outliers were identified and removed using Grubbs' $T_G$ test. As a result, eyespot size was analyzed for 176 specimens (Table 1).

**Analysis of trait asymmetry.** Eyespot size asymmetry was analyzed following the standard protocol [6] previously applied to wing length measurements [1]. We implemented a mixed model two-way ANOVA, with body side (right or left wing) as a fixed effect and individuals as a random effect. Directional asymmetry (DA) was inferred from the statistical significance of the "side" effect, and fluctuating asymmetry (FA) from the "individual × side" interaction. For wing shape asymmetry analysis, we used a similar approach adapted for Procrustes coordinates [41]. The statistical significance of both DA and FA was assessed separately for each spot, forewing, and hindwing shape. If statistically significant DA was detected, its magnitude was compared with the FA4a index [6]. DA is considered negligible if its value does not exceed FA4a. In cases where statistically significant shape DA was identified, we subtracted the sample-averaged asymmetry from the Procrustes coordinates of landmarks on one side, thereby removing DA from the dataset [44]. Repeatability of FA estimates was assessed using the ME5 index [6].

When the FA value was statistically significant (i.e., significantly exceeded the measurement error), a measure of individual asymmetry was calculated for each specimen. For eyespots, we used the FA17 index = $\Sigma|\ln(R_j/L_j)|/T$, where $R_j$ and $L_j$, are the diameters of the $j$-th spot on the right and left sides, $T$ is the total number of spots [6]. This metric requires that asymmetry manifestations across traits remain uncorrelated [6, 45]. Preliminary testing showed that the differences $(R_j - L_j)$ of the analyzed eyespots did not correlate with each other (Pearson's coefficients ranged from –0.33 to 0.33, all $p > 0.05$). Although some information is lost when using FA17 (i.e., when reducing multivariate to univariate analysis), this is compensated for by greater statistical power and enhanced interpretability.

Individual shape asymmetry was assessed as the Procrustes distance between right and left wing landmark configurations [41, 42], computed as the square root of the sum of squared differences between Procrustes coordinates of homologous landmark pairs. In [6], the Procrustes distance is given as the FA18 index. It characterizes the absolute difference in shape between the right and left wings [41, 44]. As our study analyzed both forewings and hindwings, two FA18 values were obtained for each specimen. We obtained total individual shape asymmetry measure ($FA18_{tot}$) by summing FA18 values across forewings and hindwings for each specimen. To our knowledge, our proposed measure of individual asymmetry does not have a commonly used name. We use it by analogy with the FA11 index for traditional measurements, which is calculated by the formula FA11 = $\Sigma|R_j - L_j|$ [6].

**Analysis of Trait-Metal Concentration Relationships.** To examine relationship between univariate traits (FA17 and $FA18_{tot}$) and individual toxic load, univariate analysis of covariance (ANCOVA) was used, and multivariate analysis of covariance (MANCOVA) was used for multivariate traits (set of spots, first principal components of shape). Both models shared identical design: two covariates—($\log_{10}$-transformed copper ($\log_{10}$Cu) and zinc ($\log_{10}$Zn) concentrations), and one categorical factor (site). The covariates did not correlate with each other [1]. We quantified effect sizes using partial correlation ratios $\eta^2 = SS_{effect}/(SS_{effect} + SS_{error})$, where $SS_{effect}$ is the sum of squares explained by the factor and $SS_{error}$ denotes residual variance.

Since in our case MANCOVA operates on only the first few shape principal components, this precludes direct identification of landmark-specific contributions to wing shape changes associated with individual toxic load. Therefore, to visualize the shape changes with increasing metal concentration, a multivariate regression analysis of Procrustes coordinates was additionally performed on the full set of landmarks. To account for potential site-specific effects on wing morphology, we maintained the ANCOVA framework

**Table 2.** MANCOVA results for wing shape

| Wing | Predictor | Partial $\eta^2$ | Wilks' $\Lambda$ | $F$ | $df$1 | $df$2 | $p$ |
|---|---|---|---|---|---|---|---|
| | | | Males *C. arcania* | | | | |
| Forewing | Site | 0.17 | 0.60 | 1.45 | 15 | 108.1 | 0.14 |
| | $\text{Log}_{10}\text{Cu}$ | 0.08 | 0.92 | 0.66 | 5 | 39 | 0.66 |
| | $\text{Log}_{10}\text{Zn}$ | 0.08 | 0.92 | 0.66 | 5 | 39 | 0.66 |
| Hindwing | Site | 0.14 | 0.66 | 1.13 | 15 | 105.3 | 0.34 |
| | $\text{Log}_{10}\text{Cu}$ | 0.09 | 0.91 | 0.72 | 5 | 38.0 | 0.61 |
| | $\text{Log}_{10}\text{Zn}$ | 0.10 | 0.90 | 0.87 | 5 | 38.0 | 0.51 |
| | | | Females *C. arcania* | | | | |
| Forewing | Site | 0.18 | 0.68 | 1.33 | 8 | 50.0 | 0.25 |
| | $\text{Log}_{10}\text{Cu}$ | 0.34 | 0.66 | 3.27 | 4 | 25.0 | 0.03 |
| | $\text{Log}_{10}\text{Zn}$ | 0.14 | 0.86 | 0.99 | 4 | 25.0 | 0.43 |
| Hindwing | Site | 0.10 | 0.81 | 0.47 | 10 | 42.0 | 0.90 |
| | $\text{Log}_{10}\text{Cu}$ | 0.25 | 0.75 | 1.42 | 5 | 21.0 | 0.26 |
| | $\text{Log}_{10}\text{Zn}$ | 0.19 | 0.80 | 1.03 | 5 | 21.0 | 0.43 |
| | | | Males *A. hyperantus* | | | | |
| Forewing | Site | 0.15 | 0.63 | 1.17 | 15 | 97.0 | 0.31 |
| | $\text{Log}_{10}\text{Cu}$ | 0.10 | 0.90 | 0.78 | 5 | 35.0 | 0.57 |
| | $\text{Log}_{10}\text{Zn}$ | 0.11 | 0.89 | 0.86 | 5 | 35.0 | 0.52 |
| Hindwing | Site | 0.22 | 0.50 | 1.33 | 21 | 101.1 | 0.17 |
| | $\text{Log}_{10}\text{Cu}$ | 0.10 | 0.90 | 0.58 | 7 | 35.0 | 0.77 |
| | $\text{Log}_{10}\text{Zn}$ | 0.19 | 0.81 | 1.16 | 7 | 35.0 | 0.35 |
| | | | Females *A. hyperantus* | | | | |
| Forewing | Site | 0.19 | 0.71 | 0.77 | 15 | 88.7 | 0.71 |
| | $\text{Log}_{10}\text{Cu}$ | 0.11 | 0.89 | 0.81 | 5 | 32.0 | 0.55 |
| | $\text{Log}_{10}\text{Zn}$ | 0.18 | 0.82 | 1.41 | 5 | 32.0 | 0.25 |
| Hindwing | Site | 0.15 | 0.65 | 1.08 | 15 | 94.3 | 0.39 |
| | $\text{Log}_{10}\text{Cu}$ | 0.05 | 0.95 | 0.39 | 5 | 34.0 | 0.85 |
| | $\text{Log}_{10}\text{Zn}$ | 0.15 | 0.85 | 1.22 | 5 | 34.0 | 0.32 |

(metal concentration as covariate, site as categorical factor). A detailed description of the method is given in [46, 47].

Ordination of samples by spot size was performed using canonical variates analysis (CVA).

Calculations were performed in Statistica 10.0 (Statsoft, Inc.), Past [48], and MorphoJ [49]. Original wing images are archived in Figshare (https://doi.org/10.6084/m9.figshare.26531386.v1).

## RESULTS

**Wing shape.** No statistically significant differences in wing shape were detected among study sites (Table 2). Also, wing shape did not depend on Zn concentration. A statistically significant relationship between shape and Cu concentration was found only in one case: the forewings of female *C. arcania*. For this sample, the first four principal components were used, which together explained 80% of the shape variance. Multivariate regression of Procrustes coordinates explained only 5.1% of the variance and was statistically insignificant ($p = 0.119$). The detected shape changes were exceptionally subtle—visible only when magnified through extrapolation to extreme (non-observed) Cu concentrations (Fig. 2). At these exaggerated scales, female *C. arcania* wings become slightly narrower, and their apex becomes slightly rounded.

**Wing shape asymmetry.** In all samples, statistically significant FA in wing shape was detected (Appendix, Tables S1, S2), confirming sufficient landmark placement accuracy for FA analysis. The mean landmark placement repeatability (i.e. *ME5*) was 0.77 (from 0.68

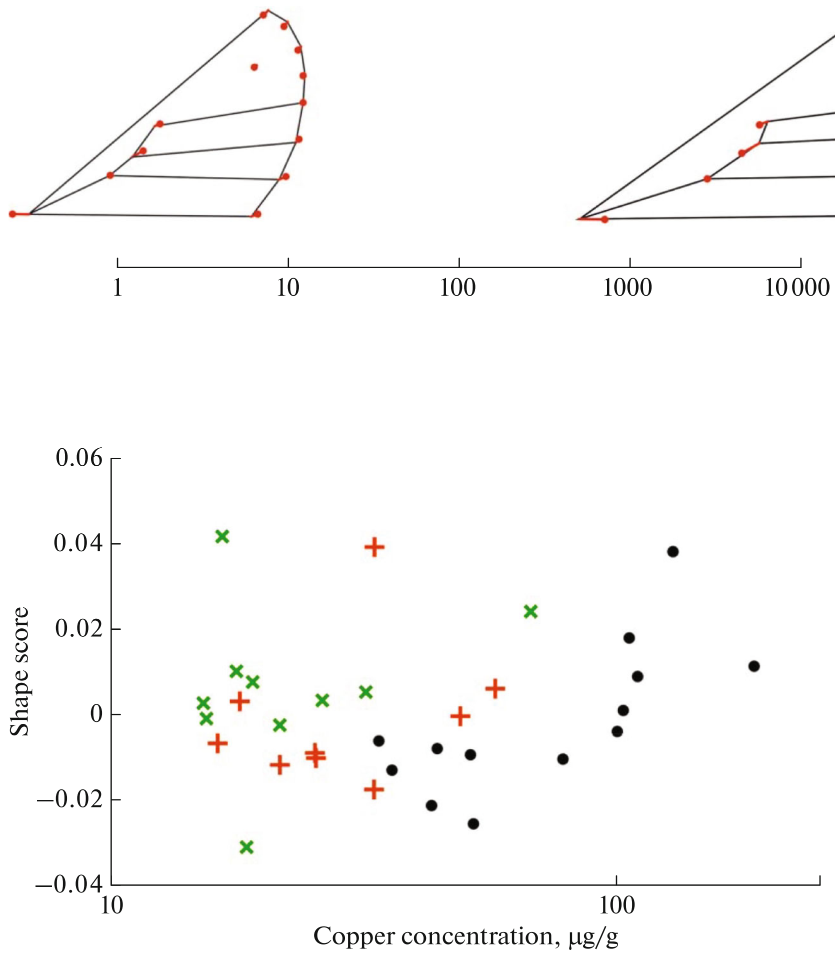


**Fig. 2.** Wing shape variation in female *C. arcania* in relation to copper concentration: the control site is shown in green, the buffer site in red, and the impact site in black. Shape scores represent individual specimen projections along the multivariate regression axis—the direction in shape space that maximizes covariation with copper concentration [43, 44]. The differences in wing configuration corresponding to a 10 000-fold difference in copper concentration are shown at the top.

to 0.83), indicating acceptable reliability of the results (Appendix, Tables S1, S2).

For most samples (forewings and hindwings of males and females of *A. hyperantus*, forewings of females of *C. arcania*), statistically significant shape DA was detected. In half of the cases, the DA value exceeded FA4a. Since the presence of a weak (albeit statistically significant) shape DA in animals is considered to be the norm rather than an anomaly [44], traits with DA were not excluded from the analysis. In accordance with the recommendations [41], we removed DA from the data before analyzing the influence of factors on the wing shape FA.

Wing shape FA in *A. hyperantus* (both sexes) and male *C. arcania* showed no dependence on either site or metal concentrations. Female *C. arcania* demonstrated significant positive correlations between wing shape FA and zinc (but not copper) concentrations across all sites (Table 3, Fig. 3). A statistically significant effect of the “site” factor was also found, but the “site × $\log_{10}$Zn” interaction was not significant ($F$ (2, 24) = 0.41, $p = 0.67$), i.e., the regression slope coefficients did not differ between sites. The contribution of the “site” factor to the wing shape FA ($\eta^2 = 0.26$) was comparable to the contribution of $\log_{10}$Cu ($\eta^2 = 0.28$).

**Eyespot size.** Statistically significant differences between the sites in terms of eyespot size were found only in *C. arcania* (Table 4). Canonical analysis was performed separately for males and females of this species (Fig. 4). In males, individuals from the control site are most strongly distanced from other samples: their P2 eyespot is relatively smaller, while G4 is larger. In females, individuals from the buffer site are somewhat distanced from other samples: they have a larger P2 eyespot, while in the control site all eyespots are smaller.

Zn concentrations did not correlate with eyespot size, whereas for Cu a correlation was found in one case – in females of *C. arcania* (Table 4). The effect size for site ($\eta^2 = 0.36$) was comparable to that of Cu

**Table 3.** ANCOVA results for fluctuating asymmetry in wing shape ($FA18_{tot}$)

| Predictor | Partial $\eta^2$ | *SS* | *df* | *F* | *p* | Regression slope (±error) |
|---|---|---|---|---|---|---|
| Males *C. arcania*: $R^2 = 0.13$, $F(5, 42) = 1.29$, $p = 0.29$ | | | | | | |
| Site | 0.13 | 0.00106 | 3 | 2.07 | 0.12 | – |
| $Log_{10}Cu$ | 0.02 | 0.00001 | 1 | 0.07 | 0.80 | 0.002 ± 0.007 |
| $Log_{10}Zn$ | <0.01 | <0.00001 | 1 | 0.01 | 0.94 | 0.001 ± 0.008 |
| Females *C. arcania*: $R^2 = 0.36$, $F(4, 25) = 3.52$, $p = 0.02$ | | | | | | |
| Site | 0.26 | 0.00071 | 2 | 4.30 | 0.02 | – |
| $Log_{10}Cu$ | <0.01 | <0.00001 | 1 | 0.02 | 0.88 | 0.002 ± 0.010 |
| $Log_{10}Zn$ | 0.28 | 0.00080 | 1 | 9.68 | <0.01 | 0.029 ± 0.009 |
| Males *A. hyperantus*: $R^2 = 0.22$, $F(5, 39) = 2.21$, $p = 0.07$ | | | | | | |
| Site | 0.19 | 0.00061 | 3 | 2.97 | 0.04 | – |
| $Log_{10}Cu$ | 0.06 | 0.00017 | 1 | 2.50 | 0.12 | –0.011 ± 0.007 |
| $Log_{10}Zn$ | 0.01 | 0.00002 | 1 | 0.22 | 0.64 | 0.003 ± 0.005 |
| Females *A. hyperantus*: $R^2 = 0.03$, $F(5, 36) = 0.23$, $p = 0.95$ | | | | | | |
| Site | 0.03 | 0.00031 | 3 | 0.33 | 0.80 | – |
| $Log_{10}Cu$ | <0.01 | 0.00002 | 1 | 0.05 | 0.82 | 0.003 ± 0.013 |
| $Log_{10}Zn$ | <0.01 | <0.00001 | 1 | 0.01 | 0.91 | 0.002 ± 0.016 |

**Table 4.** MANCOVA results for eyespot size

| Predictor | Partial $\eta^2$ | Wilks' Λ | *F* | *df* 1 | *df* 2 | *p* |
|---|---|---|---|---|---|---|
| Males *C. arcania* | | | | | | |
| Site | 0.25 | 0.45 | 2.42 | 15 | 108.1 | 0.005 |
| $Log_{10}Cu$ | 0.08 | 0.92 | 0.71 | 5 | 39.0 | 0.620 |
| $Log_{10}Zn$ | 0.11 | 0.88 | 0.95 | 5 | 39.0 | 0.461 |
| Females *C. arcania* | | | | | | |
| Site | 0.36 | 0.41 | 2.73 | 10 | 48.0 | 0.010 |
| $Log_{10}Cu$ | 0.39 | 0.61 | 3.03 | 5 | 24.0 | 0.030 |
| $Log_{10}Zn$ | 0.25 | 0.75 | 1.60 | 5 | 24.0 | 0.199 |
| Males *A. hyperantus* | | | | | | |
| Site | 0.21 | 0.52 | 1.57 | 18 | 108.0 | 0.081 |
| $Log_{10}Cu$ | 0.16 | 0.84 | 1.17 | 6 | 38.0 | 0.344 |
| $Log_{10}Zn$ | 0.19 | 0.81 | 1.47 | 6 | 38.0 | 0.216 |
| Females *A. hyperantus* | | | | | | |
| Site | 0.15 | 0.64 | 0.92 | 18 | 96.7 | 0.552 |
| $Log_{10}Cu$ | 0.17 | 0.83 | 1.20 | 6 | 34.0 | 0.330 |
| $Log_{10}Zn$ | 0.15 | 0.85 | 1.03 | 6 | 34.0 | 0.426 |

($\eta^2 = 0.39$). Only two spots (P2 and G1) showed statistically significant size reduction with increasing Cu concentrations (Fig. 5, Appendix, Table S3).

**Eyespot size asymmetry.** All samples exhibited statistically significant FA in eyespot size (Appendix, Tables S4–S7). Measurement repeatability (*ME5*) averaged 0.69 (from 0.57 to 0.88), which, as in the case of the shape FA, indicates the reliability of the results. In two cases, statistically significant DA was revealed in *A. hyperantus*: in males—G2 spots, in females—P2 (Tables S6, S7). However, a comparison of the DA value with the FA4a index showed that in both cases directional asymmetry can be neglected (for males

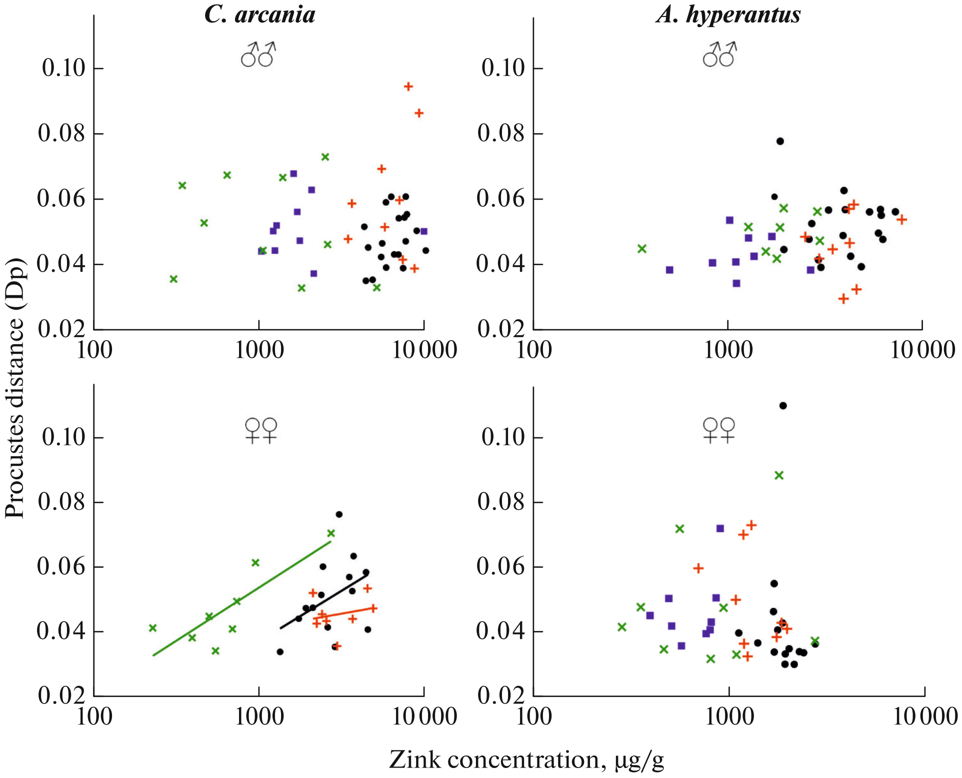


**Fig. 3.** Relationship between fluctuating asymmetry in wing shape ($FA18_{tot}$) and zinc concentration: green indicates control site, blue indicates background site, red indicates buffer site, and black indicates impact site.

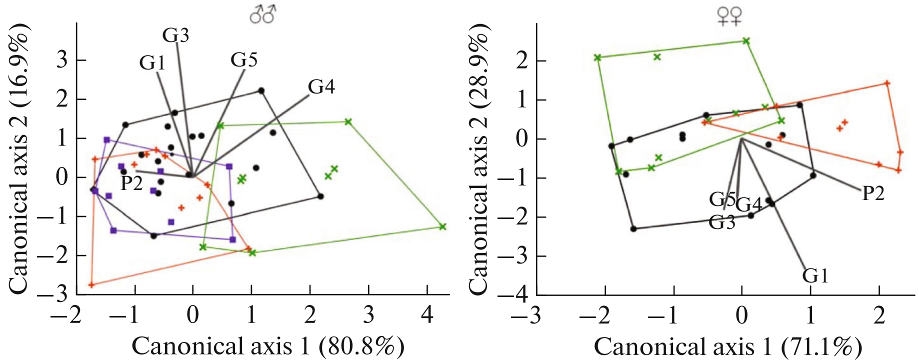


**Fig. 4.** Canonical variate analysis of eyespot size variation in male and female *C. arcania*: green indicates the control site, blue indicates the background site, red indicates the buffer site, and black indicates the impact site. Vectors indicate contributions of original traits to canonical axes. Explained variance proportions is in brackets.

DA = 0.05 mm, FA4a = 0.11; for females DA = 0.05 mm, FA4a = 0.13).

Statistically significant differences between sites were found for FA17 in *A. hyperantus* males (Table 5). The index is 0.070 ± 0.009 in the control site, 0.069 ± 0.006 in the background site, 0.105 ± 0.013 in the buffer site, and 0.072 ± 0.005 in the impact site. In other words, the males from the buffer site are the most asymmetrical in terms of eyespot size: only this group is statistically significantly different from the others (Tukey's criterion, $p < 0.05$).

Cu concentrations did not correlate with FA17. Only in one case, the female *C. arcania*, a negative correlation between FA17 and Zn was found, but the overall pattern was statistically insignificant (Table 5 and Fig. 6), which is most likely due to insufficient sample size.

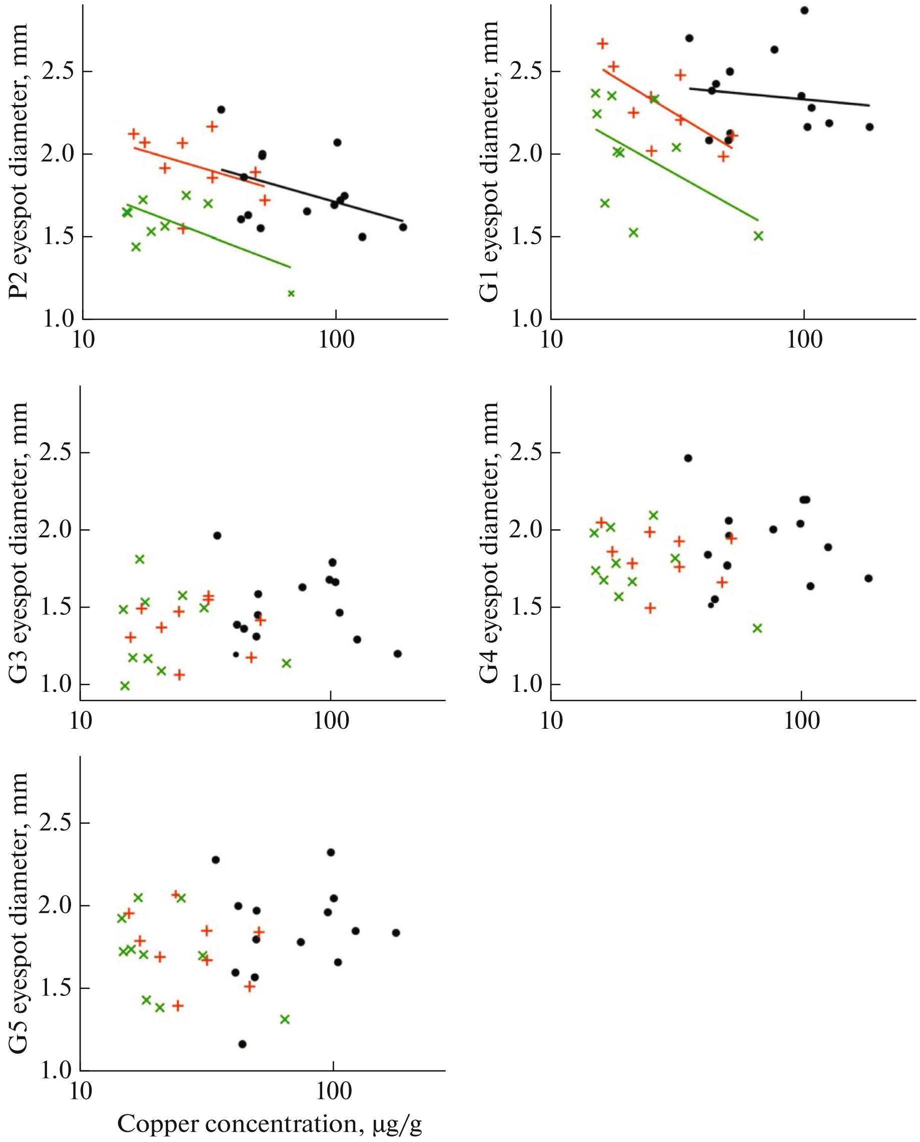


**Fig. 5.** Relationship between eyespot size in female *C. arcania* and copper concentration: the control site is shown in green, the buffer site in red, and the impact site in black.

## DISCUSSION

### *Wing Shape*

Two phases, gliding and flapping, alternate in the flight of Lepidoptera. Wing shape affects the efficiency of both phases. Gliding flight is relatively energy-efficient, with its efficiency increasing proportionally to the wing aspect ratio (the ratio of wing length to wing area) [7, 8]. Flapping flight provides greater maneuverability at higher energy costs, and its efficiency depends on the area distribution along the long axis of the wing—that is the position of its center of gravity [8, 50]: the more distal the wing's center of gravity is, the more lift the wings produce per stroke. Both the aspect ratio and the position of the center of gravity characterize its overall proportions. These parameters primarily influence lepidopteran aerodynamics and flight efficiency.

Our analysis focused on microscale wing shape variation that does not alter these fundamental proportions. The relationship between such fine-scale shape differences and aerodynamics remains poorly understood [8], leaving open the question of how minor shape variations might affect flight efficiency and individual fitness. We hypothesize that their influence is weaker than that of overall wing proportions. Similarly, we would not expect fluctuating asymmetry in wing shape to substantially affect flight performance.

For some insect species, changes in wing shape (but not FA) may serve as stress indicators [34]. However, in the satyrid butterflies we studied, we found no

**Table 5.** ANCOVA results for fluctuating asymmetry (FA17) in eyespot size

| Predictor | Partial $\eta^2$ | *SS* | *df* | *F* | *p* | Regression slope (±error) |
|---|---|---|---|---|---|---|
| Males *C. arcania*: $R^2 = 0.11$, $F(5, 43) = 1.03$, $p = 0.413$ | | | | | | |
| Site | 0.07 | 0.001 | 3 | 1.11 | 0.357 | – |
| $\text{Log}_{10}\text{Cu}$ | <0.01 | <0.001 | 1 | <0.01 | 0.984 | –0.001 ± 0.013 |
| $\text{Log}_{10}\text{Zn}$ | 0.05 | 0.001 | 1 | 2.11 | 0.154 | 0.020 ± 0.014 |
| Females *C. arcania*: $R^2 = 0.26$, $F(4, 28) = 2.41$, $p = 0.073$ | | | | | | |
| Site | 0.03 | $453 \times 10^{-6}$ | 2 | 0.49 | 0.615 | – |
| $\text{Log}_{10}\text{Cu}$ | <0.01 | $5 \times 10^{-6}$ | 1 | 0.01 | 0.917 | –0.002 ± 0.020 |
| $\text{Log}_{10}\text{Zn}$ | 0.15 | $2288 \times 10^{-6}$ | 1 | 4.99 | 0.034 | –0.045 ± 0.020 |
| Males *A. hyperantus*: $R^2 = 0.27$, $F(5, 43) = 3.11$, $p = 0.017$ | | | | | | |
| Site | 0.27 | 0.011 | 3 | 5.17 | 0.004 | – |
| $\text{Log}_{10}\text{Cu}$ | 0.01 | <0.001 | 1 | 0.46 | 0.502 | 0.014 ± 0.021 |
| $\text{Log}_{10}\text{Zn}$ | 0.07 | 0.002 | 1 | 3.28 | 0.077 | –0.030 ± 0.017 |
| Females *A. hyperantus*: $R^2 = 0.11$, $F(5, 39) = 0.96$, $p = 0.454$ | | | | | | |
| Site | 0.07 | $1373 \times 10^{-6}$ | 3 | 1.00 | 0.402 | – |
| $\text{Log}_{10}\text{Cu}$ | 0.01 | $114 \times 10^{-6}$ | 1 | 0.25 | 0.620 | 0.006 ± 0.013 |
| $\text{Log}_{10}\text{Zn}$ | <0.01 | $4 \times 10^{-6}$ | 1 | 0.01 | 0.928 | 0.002 ± 0.019 |

clear relationship between the metal content and wing shape. Of the four possible combinations (two species × two sexes), a dependence was detected in only one case (female *C. arcania*), and for only one of the two analyzed metals (Cu). But even in this case, the shape differences corresponding to the difference between background and impact Cu concentrations are so minimal that significant efforts are required to localize them on the wing (Fig. 2). This suggests that the differences in wing shape between clean and contaminated sites are negligible, and in the identified case, given the small size of this sample, most likely random.

A similar pattern emerged for wing shape FA, with only 1 of 4 cases (again female *C. arcania*) showing increased FA in response to metal concentration—specifically Zn rather than Cu. Let us recall that no connection was found between the wing length FA and metal accumulation in adults [1]. On the one hand, this discrepancy aligns with the idea that wing shape as a multidimensional trait with relatively weak direct fitness effects has a higher sensitivity in assessing the effect of a stressor on developmental stability compared to the wing length. On the other hand, the effect was found only for females of one species, which does not allow us to consider the observed relationship between wing shape FA and individual toxic load as a general pattern. Moreover, given the small sample size, there is no reason to consider this effect reliably established. To our knowledge, the only study examining industrial pollution effects on wing shape FA in wild insect populations (Hymenoptera) also found no relationship between FA and emissions from a zinc smelter [51].

### *Wing Pattern Eyespots*

We analyzed the ventral eyespots on forewings and hindwings, which are clearly visible in satyrid butterflies at rest. The functional significance of these spots remains controversial. Several studies have supported the deflection hypothesis (redirecting predator attention from vital body parts to wing margins), but only under specific lighting conditions [52] or with certain predator species [53, 54]. However, this effect was absent when tested with different lighting conditions and predator species [55, 56]. Nevertheless, the repeated independent evolution of eyespots across Lepidoptera lineages [15], along with their primary ventral placement on hindwings in Nymphalidae [16], may indirectly argue for their functional importance.

We found that pollution effects on spot size differed between species. In *A. hyperantus*, spot sizes showed no dependence on either metal concentrations in adult bodies or proximity to emission sources. For *C. arcania*, the pattern differed: both sexes showed inter-site variation, while females exhibited a negative correlation with Cu (but not Zn) concentrations. Notably, this latter effect was observed in only two of the five analyzed spots (P2 and G1).

Most likely, the observed inter-site differences reflect natural microgeographic variation unrelated to industrial pollution. Due to its low dispersal capacity, *C. arcania* tends to form localized groups that show

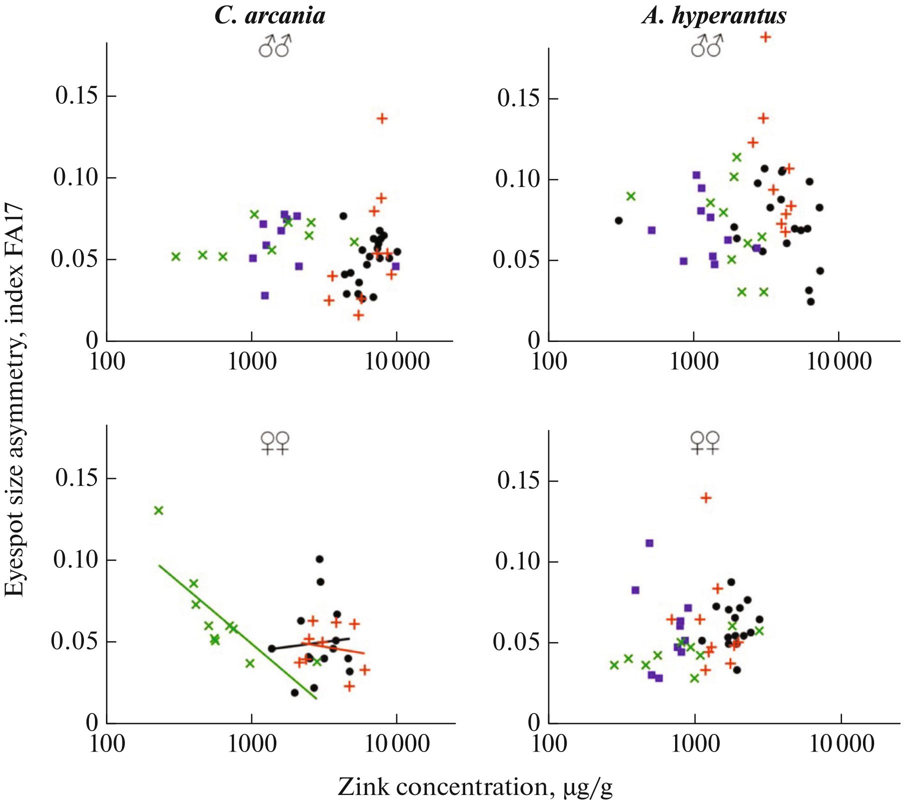


**Fig. 6.** Relationship between fluctuating asymmetry in eyespot size (FA17$_t$) and zinc concentration: green indicates the control site, blue indicates the background site, red indicates the buffer site, and black indicates the impact site.

some morphological differentiation, particularly in highly mosaic habitats [57]. The negative correlation between spot size and Cu concentrations appears unreliable due to the already mentioned small sample size. In any case, this pattern proves difficult to interpret and cannot be generalized across both species or specifically linked to pollution. The spot size FA results also provide negative evidence for our hypotheses. We detected no correlation between FA and the metal content in either species. Differences between the sites in spot size FA were found only in male *A. hyperantus*, with maximum FA levels at buffer rather than impact sites. Indirectly, this indicates the random nature of the differences, which are problematic to unambiguously interpret as a causal "dose–effect" relationship.

Among the three traits we examined, only eyespots – unlike wing size and shape—show no direct relationship to locomotion. On the one hand, recent experimental validations of the deflection hypothesis [52, 54] challenge the presumed neutrality of this trait. On the other hand, experiments on the satyrid butterfly *Bicyclus anynana* showed that when choosing a sexual partner, females assess eyespot size, but not their asymmetry [33]. There is also no evidence that ventral eyespot asymmetry in satyrid butterflies affects predation success under natural conditions. When at rest, these butterflies fold their wings vertically, exposing only one lateral surface to potential predators at any given time. Based on the above, theoretically, the symmetry of the eyespots we analyzed should be weakly controlled by selection. Notably, our hypothesis that metal accumulation would increase FA received no support, even for this theoretically most sensitive trait.

Literature reports on stress-mediated eyespot FA changes remain inconsistent. Thus, in *B. anynana*, identical developmental stages exposed to different temperature regimes showed divergent responses: cold shock increased eyespot size FA while heat shock did not [58, 59]. FA levels also vary significantly between wing surfaces, being markedly higher in dorsal spots (functioning as secondary sexual traits) compared to ventral ones [33, 59]. The only study known to us that examined the effects of industrial pollution on the FA of lepidopteran wing pattern spots [2] found no significant differences between polluted and uncontaminated sites.

### *Fluctuating Asymmetry as an Indicator of Stress*

The persistent research interest in FA likely stems from its presumed value as a stress biomarker. Stressors - including physical, chemical, and genetic factors - increase an organism's energetic demands, reducing resources available for growth, reproduction, and developmental stability. Chronic stress ultimately diminishes organismal fitness [60, 61].

For phytophagous insects, industrial emissions containing toxic metals and metalloids may represent significant stressors. In Lepidoptera, when these elements accumulate beyond physiological detoxification capacity, they can lead to increased larval mortality, reduced female fecundity, and decreased adult body size [62–65].

The most sensitive stress indicators include specific physiological or morphological modifications aimed at reducing stress, as well as a decrease in body size. Disruption of developmental stability is also recognized as a stress indicator [61]. However, FA-based assessment of developmental stability in bilateral traits demonstrates lower sensitivity and reliability. Moreover, this approach requires special methodological care, without which there is a high probability of erroneous conclusions [6, 66–68].

Following multiple studies reporting positive FA-stress relationships [69–72, etc.], an equal number of investigations failed to replicate these findings [45, 59, 73, 74]. With regard to the effect of industrial pollution on insects, examples of the former can be found in works [75–78], and of the latter, in [2, 51, 79, 80]. Probably, the sensitivity of FA to stressors varies across species, traits, and stressor types [51, 81, 82].

Identifying general patterns in the manifestation of the phenomenon under discussion, as well as the reasons for effect heterogeneity, is best achieved through meta-analyses that synthesize results from multiple individual studies. Meta-analysis is particularly valuable for examining weak effects and inconsistent findings [83], which is indeed the case for the impact of stressors on FA.

Several meta-analyses have examined the relationship between stress and FA across different animal groups [84, 85]. Their results indicate that, in general, the association between stress intensity and FA is weak [84], and the effect is less pronounced in natural insect populations compared to laboratory conditions [85]. These differences may arise from both the underestimation of numerous external factors influencing FA in natural settings and the reduced stress resistance of laboratory lines—potentially due to reduced genetic diversity [85, 86]. In the latter case, laboratory studies may overestimate the magnitude of the stressor's effect on FA. However, the impact of industrial pollution on FA has been studied on a limited set of taxa and indicators, highlighting the need continued work in natural populations with the inclusion of different pollution sources, species, and indicators.

Our study expands the existing data on FA in insects from contaminated areas by including rarely examined parameters such as wing shape and wing pattern. To our knowledge, only a few studies have investigated these traits: wing shape was studied in Hymenoptera [51], and wing pattern elements were studied in Lepidoptera of the families Nymphalidae and Lycaenidae [2]. Although these traits are theoretically preferable to traditionally used wing length as stress indicators because they are less fitness-related, the cited studies—like ours—reported negative results.

An important consideration should be noted: we cannot unequivocally conclude that individuals of the analyzed species inhabiting the impact site experience stress specifically caused by the uptake of potentially toxic metals. On the one hand, we observed extremely high concentrations of both essential (Cu, Zn) and toxic (Pb, Cd) elements in adult specimens from this site, with Zn reaching hyperaccumulation levels [1]. In Lepidoptera, metal exposure typically reduces body size [62, 64, 87], which is generally interpreted as a stress response [61]. On the other hand, we found a negative correlation between wing length and metal concentrations only in female *A. hyperantus*, suggesting this response is not general [1]. In addition, since out of a dozen species of satyrid inhabiting the background territory, only the considered *A. hyperantus* and *C. arcania* persist near the copper smelter [1], this may indicate their adaptation to polluted conditions. This uncertainty unfortunately complicates the interpretation of our results when discussing FA as a potential stress indicator.

Several hypothetical explanations for our negative results can be proposed. First, metal accumulation, even at high concentrations, may not act as a stressor in the studied species due to their potential adaptation to polluted environments. Second, the functional importance and direct link to fitness of the analyzed traits may render their FA a poor indicator of stress. Third, the small sample size may have limited the statistical power of our analysis, particularly given the typically weak relationship between FA and stressors in natural populations. Finally, fourth, there is indeed no causal relationship between FA and stress.

At this stage, we have no grounds to discuss the validity of each of these explanations. But whatever the reason for our negative result, it adds arguments to skeptics who declare the need for caution when using FA as a stress indicator in natural insect populations.

## CONCLUSIONS

The hypotheses tested in our study regarding the effects of metal pollution on wing shape, eyespot patterns, and their fluctuating asymmetry were not unequivocally supported. Although some effects were detected at both the group (differences between polluted and uncontaminated sites) and individual level (correlation with metal concentrations in adult body), they were very weak, specific in relation to the trait, species and sex, and therefore, most likely, random.

Our findings for wing shape and eyespot size mirror previous results for wing length [1], providing insufficient evidence to support FA as a reliable indicator of industrial pollution-induced stress. Contrary to theoretical expectations, the studied traits—despite being less fitness-related and having multidimensional nature, which theoretically should increase sensitivity compared to body size—did not demonstrate greater resolution as stress markers. The current scarcity of data on developmental stability in insect populations exposed to industrial pollution prevents broader generalization of our findings. Nevertheless, this knowledge gap underscores the importance of further research incorporating wider taxonomic sampling and additional morphological traits.

## SUPPLEMENTARY INFORMATION

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## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Butterfly collection and analysis were conducted with approval from the Bioethics Commission of the Institute of Plant and Animal Ecology, Ural Branch of the Russian Academy of Sciences (protocol no. 13, November 1, 2022).

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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