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Phenotypic Variability of *Aphantopus hyperantus* and *Coenonympha arcania* (Lepidoptera: Nymphalidae) in the Vicinity of the Middle Ural Copper Smelter. Part 1. Metal Content and Wing Length

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Abstract—We have tested the hypotheses that the satyrs *Aphantopus hyperantus* and *Coenonympha arcania* accumulate metals in higher concentrations near the Middle Ural Copper Smelter than in the background area and that metal accumulation in the body of adult individuals is negatively correlated with the wing length but positively correlated with the fluctuating asymmetry of the wing length. We measured the length of the forewing and individual concentrations of Zn, Cu, Pb, and Cd in the body of adults captured at different distances from the copper smelter (Revda city, Russia). The metal content reaches very high levels, with Zn concentrations being higher than Cu and Pb concentrations by an order of magnitude and Cd concentration by two orders of magnitude. In both species, metal accumulation is higher in males than in females. Maximum concentrations of Zn, Cu, and Cd have been recorded near the smelter. The wing length either did not differ between the sites or was higher near the smelter. The statistically significant negative relationship between Cu concentrations and the wing length has been recorded only for females of one of the species (*A. hyperantus*). In both species, the fluctuating asymmetry of the wing length did not differ between samples from different sites and did not depend on metal concentrations at the individual level.

Keywords: fluctuating asymmetry, body size, diurnal butterflies, air emissions, heavy metals, industrial pollution

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The negative consequences of pollution of terrestrial ecosystems with potentially toxic metals and metalloids are especially noticeable near large nonferrous smelters. Therefore, interest of researchers in such impact areas is quite understandable: they can be used as model situations for analyzing the mechanisms of biota resistance to stress factors and testing theoretical constructs [1, 2]. For insects, a decrease in the abundance and diversity is usually recorded with proximity to the source of emissions [3–7]; however, there are sometimes deviations from this pattern [8].

Phytophagous insects can accumulate metals supplied with food [9–11]. The physiological regulation of the content of nonessential elements, such as Cd and Pb, is limited: they accumulate throughout the life and can have a toxic effect even at low concentrations [12], while the content of essential microelements, e.g., Cu and Zn, can be regulated in the body of insects by excretion and through binding with metallothioneins and other compounds [10, 13, 14]. However, when the amount of input of these elements exceeds the physiological limits of regulation, they can also accumulate and cause physiological stress expressed in a decreased female fecundity [15], a decreased survival rate of caterpillars, an increased duration of preimaginal development, and a decreased growth rate and size of adults [14, 16, 17].

Of particular interest is the question of the effect of pollution on the size of organisms, both at the level of populations [18] and the entire community [19]. Similarly to many other insects, the reproductive success of lepidopterans directly depends on their size: large females lay more eggs and large males usually have an advantage in competing for living space or females [20–22]. Therefore, analysis of the variation in body size is important for predicting the fate of populations in contaminated sites.

One of the indicators of stress, including stress caused by metal pollution, is developmental instability, i.e., a decrease in the body ability to resist random deviations during the realization of an “ideal” or “target” phenotype in ontogeny [23–25]. Fluctuating asymmetry (FA) is widely used as a measure of developmental stability, which is understood as small nondirectional random deviations of a trait from ideal symmetry due to stochastic effects during the realization of the organism development program [25].

FA was taken as a leading indicator of negative changes in populations, which was confirmed by the results of meta-analysis: on the whole, FA can serve as a stress marker for insects [26]. However, metal pollution itself had no effect on the FA value more often than it had [26]. Hymenopterans [27] and lepidopterans [18, 28], living near nonferrous smelters had no correlation between the FA value and pollution levels.

As a rule, the influence of pollution on parameters of insect viability (body size, fecundity, mortality, development rate, digestion efficiency, etc.) and FA in natural conditions is assessed at the intergroup level. Samples taken at different distances from the point source of emissions are compared, assuming that the stressor has an equal action on all objects within a specific site, while the strength of its impact differs between different sites. However, almost in all cases, the sites also differ in the effect of other, unaccounted factors, which, nevertheless, may strongly and, possibly, multidirectionally influence the viability and FA. In well-flying insects, in particular, diurnal lepidopterans, the differences between the sites can additionally be mitigated as a result of the dispersal activity of adults, especially in situations when the physical extent of the pollution gradient is relatively small and individual sites are located close to each other. Therefore, it seems promising to analyze the effect of pollution on the viability of insects at the individual level, when the metal content in separate individuals is used as a measure of toxic load.

This study is devoted to two satyr species widespread in the Urals (Lepidoptera: Nymphalidae), *Aphantopus hyperantus* (Linnaeus, 1758) and *Coenonympha arcania* (Linnaeus, 1761), the local groups of which live under conditions of long-term environmental pollution with air emissions from the large copper smelter. Only these two species from the entire local diverse satyr fauna continue to live in the immediate vicinity of the smelter. Specific mechanisms of species tolerance to direct and indirect effects of pollution have not been studied. In particular, it is unknown whether these species accumulate metals in potentially toxic concentrations or have physiological mechanisms of their effective excretion. In addition, caterpillars feed on pseudometallophyte grasses near the smelter, which, unlike true metallophytes, have an effective root barrier that prevents the accumulation of metals in the leaves and stems [29].

It has been shown [10, 14] for a number of lepidopteran species that males and females can accumulate metals to varying degrees; however, there are no such data for *A. hyperantus* and *C. arcania*. To avoid the unification of groups potentially heterogeneous in metal contents into one sample, males and females were analyzed separately. Our approach is to assess the direct effect of pollution on phenotypic variability; therefore, we studied not only differences in metal accumulation between the sites, but also characterized the relationship of morphological traits with toxic load at the individual level.

The purpose of this study is to assess the effect of pollution on the variability (including FA) of three morphological traits of adults: wing length (as a characteristic of body size), wing shape, and wing pattern. The first parameter is considered in this article; the second communication will be devoted to the wing shape and eyespots of the wing pattern. We tested two hypotheses: (1) the studied species accumulate higher concentrations of metals near the smelter than in the background area; (2) metal concentrations in the body of adults are negatively correlated with the wing length but positively correlated with the wing length FA.

MATERIAL AND METHODS

The **study area** is in the vicinity of the Middle Ural Copper Smelter (MUCS), which is located near the Revda city, Sverdlovsk oblast. The smelter has been operating since 1940 and was one of the largest point sources of air pollution in Russia during the period of maximum emissions (1980s). Similarly to other factories of this type, the specificity of the toxic effect of its emissions is in the combined effect of polymetallic dust (which contains Cu, Pb, Cd, Zn, Fe, As, etc.) and soil-acidifying gaseous compounds (SO_2, NO_x, and HF). The total emission from the MUCS in 2003 (the year of material collection) was about 30000 tons. The dynamics of emissions from the smelter was documented earlier [30, 31].

The material was collected in the taiga zone from four sites with open-space vegetation (post-forest meadows and roadsides) (Fig. 1). The control site (no. 1) was 75 km southeast of the MUCS and background site (no. 2, regional level of pollution), buffer site (no. 3, medium pollution), and impact site (no. 4, heavy pollution) 14, 4–5, and 1–2 km west of the MUCS, respectively.

Site no. 1 is in the vicinity of the biological station of the Ural Federal University (UrFU), near the Iset and Sysert interfluve (environs of the Fomino village, Sysertsky district, Sverdlovsk oblast, 56°36′ N, 61°03′ E). The degree of pollution of the area is low and does not differ from other "environmentally safe areas" in the Ural region, which makes it possible to consider this site as a control plot in ecotoxicological studies [32]. Pine grass–shrub and grass forests and secondary birch and pine–birch grass forests currently prevail there. At the beginning of the 21st century, woodlands were exposed to significant anthropogenic loads that were not related to chemical pollution (recreation, berrying, mushroom picking, cattle grazing, and selective logging).

The site is an extended (about 1.5–2 km long) open zone along the power line, pine forest edges, and clearings penetrating 200–300 m deep into the forest. The southeastern end of the site abuts the bank of the

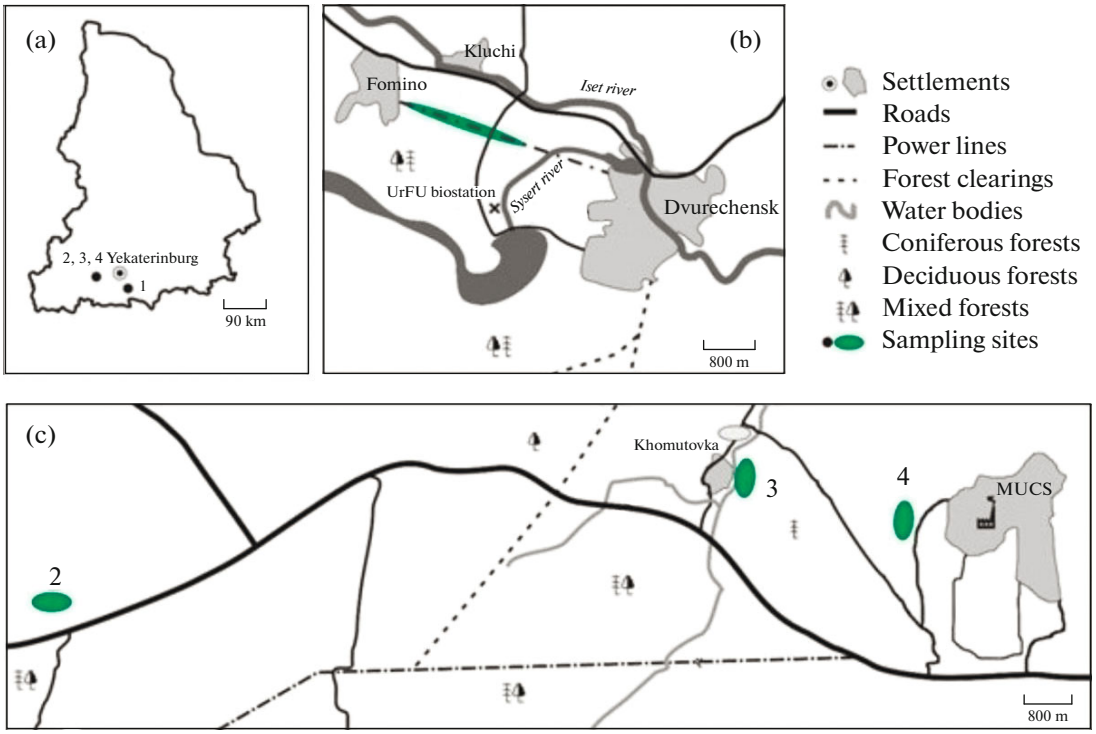

Fig. 1. Schematic map of the study area: (a) sampling sites in Sverdlovsk oblast; (b) zone of the biological station of the Ural Federal University (UrFU); (c) zone of the Middle Ural Copper Smelter (MUCS). Sites: (1) control, (2) background, (3) buffer, (4) impact.

Sysert River and represents a regularly mowed forb–grass floodplain meadow. The species composition of meadow communities is diverse and includes 23 grass and sedge species (Table S1). Grasses make up 20–50% of the total number of species in open meadow communities.

Site no. 2 (environs of the Ilmovka village, 56°49′ N, 59°37′ E) is irregularly shaped and includes a broad plain adjacent to a highway and individual forest glades united by a network of dirt roads and forest trails. In meadows, the diversity is represented by 63–69 species; herbage is multi-layered here and dominated by forbs (70–80% of biomass); grasses make up 20% and the proportion of sedges and legumes is insignificant, which is typical for post-forest meadows [33, 34]. In 2002–2003, 12 grass species grew in this site (see Table S1): during this period, meadows were regularly mowed.

Site no. 3 (near the Khomutovka village, 56°51′ N, 59°49′ E) includes forb–grass meadows on the bank of the Shaitanka River and roadside vegetation. In 2002–2003, eight grass species grew in this site (see Table S1). Meadows were also regularly mowed.

Site no. 4 (56°51′ N, 59°52′ E) owes its origin exclusively to technogenic impact. Spruce–fir forests that once surrounded the MUCS gradually died off and were exposed to fires; there was no self-recovery of forest vegetation in the burnt areas; instead, an industrial barren covered with explerent moss (*Pohlia nutans*) and patches of horsetail was formed there; subsequently, the moss was lined with grasses: colonial bentgrass *Agrostis tenuis* and tufted hair grass *Deschampsia caespitosa* (see Table S1). The site is an extended (about 1 km long) glade that is dominated by bentgrass and in which willows (*Salix* spp.) grow in the form of individual bushes and thickets.

As of 2003, the grass cover was closed, very low (15–20 cm), with heavy sod turning yellow by midsummer. This very simply organized meadow community remained unchanged and was not subject to mowing for a long time (at least from the mid-1990s). Heavy sod and the layer of nondecaying grass litter prevented the germination of not only herbaceous but also woody plants [33, 34].

Satyr taxocene. The composition of the taxocene was characterized based on materials from belt transect method in 2002–2003: in sites nos. 2–4, belt transects were carried out in the last decade of May–early June and then in the last decade of June–first decade of July for 7–10 days; in site no. 1, belt transects were regularly carried out from late May to mid-August. The abundance of *C. arcania* and *A. hyperantus* is indicated by the scale according to which the species is considered “abundant” if it is recorded more than 10 times per hour, “common” if it is recorded 9 to 1 times per hour, and “rare” if it is recorded less than 1 time per hour.

Table 1. Sample size and structure

Site	*C. arcania*				*A. hyperantus*			
	males		females		males		females	
	thorax	abdomen	thorax	abdomen	thorax	abdomen	thorax	abdomen
Control	2	10	3	10	3	10	3	10
Background	4	10	2	3*	3	10	2	10
Buffer	2	10	3	10	3	10	4	10
Impact	7	20	7	15	6	20	6	15

*Samples were used only when comparing metal concentrations in the thorax and abdomen.

In site no. 1, the satyr taxocene is represented by 13 species from eight genera, which are typical for the pre-forest steppe pine–birch forests of the Middle Urals (Table S2); among them, *A. hyperantus* is an abundant species and *C. arcania* is a common species. The species composition of satyrs from sites nos. 2 and 3 is the same—16 species from nine genera (see Table S2); the abundance of *A. hyperanthus* (abundant species) is higher than that of *C. arcania* (common species) in both sites. In site no. 4, we encountered only two species, *A. hyperanthus* and *C. arcania*, which can be classified here as common. *Lopinga achine*, *Lasiommata maera*, *Coenonympha hero*, and *C. glycerion* occasionally flew to the meadows in site no. 4 from neighboring forest communities; however, they were not constantly present in this area.

Characteristics of satyr species. *Aphantopus hyperantus* is a widespread meadow–forest transpalearctic species [35–37]. The range of preferred biotopes is very wide: open areas (dry, mixed-grass, grass, wet, and other types of meadows), glades, and edges of deciduous and mixed forests, swamp edges, etc. The species is tolerant to anthropogenic load: it inhabits agricultural landscapes, forest belts, artificial plantings, parks, field sides, and roadsides [35, 38, 39]; however, it prefers natural meadows [40]. According to the literature data and our observations, *A. hyperantus* is a sedentary species that exists in small, easily differentiated populations [41–43]. It is strictly univoltine throughout its range; caterpillars develop on different grasses and overwinter in the third or fourth instar [37, 44].

Coenonympha arcania is a West Eurasian species that is widespread (except the extreme northern regions of Europe) east to the Urals. Adults in different areas fly from early June to late July in one generation, occasionally in two generations [37, 45, our observations]. Different grass genera (*Agrostis*, *Brachypodium*, *Bromus*, *Cynosurus*, *Danthonia*, *Festuca*, *Holcus*, *Melica*, and *Poa*) and sedge genera (*Carex*) are indicated as food plants [37, 45].

In natural communities of southern taiga, adults fly along forest edges of birch, pine, and mixed forests, as well as along the sides of country roads and clearings. The preferred habitats of the species in the Middle Urals are different types of grass and forb–grass meadows. In the steppe and forest-steppe zones, butterflies fly along the slopes of hills and stay close to outliers, willow thickets, and other shrubs. According to the literature data and our observations, the range of habitats *of C. arcania* is quite wide. The occurrence of the species in a certain habitat depends on two factors: (1) the presence of large (with a radius of not less than 100 m) open meadow areas with food plants from the grass and sedge families and (2) the presence of shrubs in meadows where butterflies rest and bask. A habitat where at least one of these conditions is absent is not used by this species [47–49].

Sample collection. Using an entomological net, we caught 225 adults of *C. arcania* and 559 adults of *A. hyperantus* from all sites from June 29 to July 7, 2003. All adults were dried and stored on cottonwool layers. For further analysis, ten to 20 individuals were randomly selected (using a random number generator) from each site (Table 1).

Metal concentrations. We estimated the individual level of accumulation of four metals—Cd, Pb, Cu, and Zn—in adults. Since metals can be differently deposited in different tissues, we preliminarily estimated differences in concentrations in two body parts using a small subsample. For this purpose, the thorax (without wings and legs) and abdomen were separated from the dried adults. Since the concentrations proved to be much higher in the abdomen than in the thorax (see below), the bulk of the analyzes were performed only for the abdomen.

Samples were weighted on a KERN-770 analytical balance (accurate to 0.0001 g) and placed in teflon vessels with addition of 7 mL of concentrated HNO_3 (special purity grade) and 1 mL of deionized H_2O, where they were kept for 30 min and then digested in an MWS-2 microwave oven (Berghof, Germany) in accordance with manufacturer's instructions. After digesting, the sample volume was diluted to 10 mL with deionized H_2O. The metal concentration (μg/g dry weight) was measured by atomic absorption on a ContrAA 700 spectrometer (Analytik Jena, Germany) using electrothermal atomization. Detection limit,

μg/mL: 0.013 for Cu, 0.005 for Zn, 0.001 for Cd, and 0.013 for Pb. The quality of measurements was controlled by the CRM 185R international standard sample. The recovery was 93.2% for Cu, 99.8% for Zn, 114.2% for Cd, and 94.4% for Pb.

Unfortunately, we failed to correctly measure the concentrations of Cd and Pb in several samples due to very low values (19 and 22 of the 183 values, respectively). During data analysis, they were replaced by the minimum values for the site.

Wing length. The wings were separated from the thorax and photographed from the ventral side using a Canon Eos 600D camera; its height and angle were fixed with a tripod. The length of the forewing was measured as the distance between the base of the wing (the base of the veins forming the medial cell) and end of the R_4 vein. It should be emphasized that the measured parameter does not always coincide with the maximum length of the wing, i.e., with the distance from its base to its apex, since the position of the end of the R_4 vein can slightly vary. Nevertheless, unlike Microlepidoptera [50], the variability in the position of the veins is low in diurnal butterflies. This can be indirectly judged from the fact that the pattern of venation is used as a diagnostic trait during species identification. The points limiting the measurement are clearly visible in photographs of the wings and are homologous (i.e., clearly the same) for all objects in the sample. This reduces subjectivity compared to using the distance to the apex and guarantees that the same morphometric feature was measured in all cases, which is methodologically very important. Measurements (with an accuracy of 0.05 mm) were carried out using the images of wings in ImageJ 1.48v [51] twice on each side of the individual. Repeated measurements were time-separated over 14 days. To avoid the confirmation bias, which is especially critical during FA analysis [52], measurements were performed by an operator who was not informed about the collection sites of butterflies. In total, the wing length was measured in 183 individuals.

Data analysis. The significance of differences in metal concentrations between different body regions (thorax and abdomen) and between the sites was compared using three-way MANOVAs. The factors were the species, sex, and body part in the former case and species, sex, and site in the latter. Concentrations were log-transformed. The strength of influence of the factors in the model was compared using partial eta-square as a measure of effect size: $\eta^2 = SS_{effect}/(SS_{effect} + SS_{error})$, where SS_{effect} is the sum of squares explained by the factor and SS_{error} is the sum of squares for error.

The wing length asymmetry was analyzed according to the protocol [25]. At the first step, the statistical significance of directional and fluctuating asymmetry was estimated using a mixed model of two-way ANOVA, in which the side of an individual (right or left wing) was considered as a fixed factor and the individual as a random factor. The conclusion about the presence of directional asymmetry (DA) was made based on the statistical significance of the "side" factor and the presence or absence of FA were established by the statistical significance of the "individual × side" interaction. If a statistically significant DA was found, the DA value (average difference between the length of the right and left wings) was compared with the FA4a index according to the recommendations from [25]; the FA4a index is equal to $0.798\sqrt{(\mathrm{var}(R - L)}$, where var is the variance, R is the length of the right wing, and L is the length of the left wing. If DA ≤ FA4a, directional asymmetry can be neglected. The reproducibility of wing length measurements was estimated using the *ME5* index for two repeated measurements: $ME5 = (MS_i - MS_m)/(MS_i + MS_m)$, where MS_i is the mean square of the "individual × side" interaction and MS_m is the mean squared error in the mixed model of two-way ANOVA [25].

At the second step (if the FA value significantly exceeded the measurement error, i.e., was statistically significant), a measure of individual asymmetry, namely, the absolute difference between the length of the right and left wings (which corresponds to the FA1 index in [25]), was calculated for each individual. The use of the FA1 index is considered correct only if there is no relationship between the value of individual asymmetry and the size of the trait [25]. A preliminary check showed the absence of this dependence (Pearson correlation coefficients ranged from −0.01 to −0.25 and p was more than 0.05 in all cases). We additionally calculated the FA2 index, i.e., the ratio of the absolute difference between the lengths of the right and left wings to half the sum of their lengths [25]. Since the FA2 index is a relative value, its use makes it possible to compare FA in objects significantly differing in size.

The effect of individual toxic load on the wing length and its FA was estimated using an analysis of covariance (ANCOVA). The model design was the same in both cases. It was based on two covariates: the decimal logarithm of copper concentration ($\log_{10}$ Cu) and zinc concentration ($\log_{10}$ Zn) in the abdomen; the categorial factor was the site. A preliminary check showed that the logarithms of Cu and Zn concentrations did not correlate with each other (Pearson correlation coefficients were insignificant in all cases, $p > 0.05$). Cd and Pb were not included in this model due to the lower reliability of its concentration estimates and *C. arcania* females from the background site were excluded from the analysis due to their small sample size (three specimens).

Data analysis were performed in Statistica 10.0 (Statsoft, inc.) and Past [53].

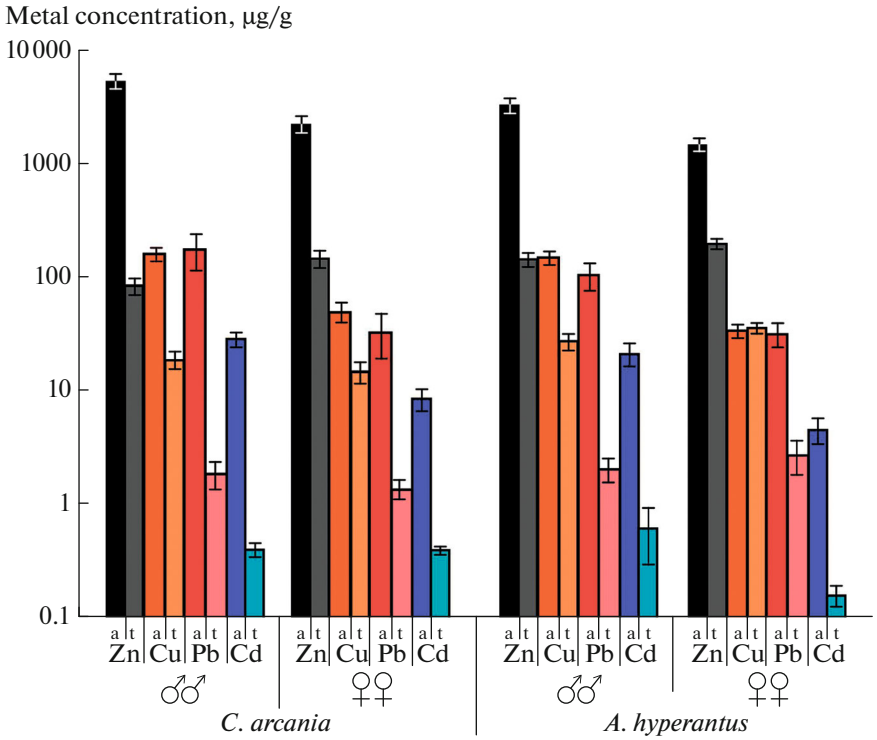

Fig. 2. Concentration of metals (mean ± error) in different body parts of the two species (averaged over all sites): (a) abdomen, (t), thorax.

RESULTS

Metal concentrations. The concentrations of all metals proved to be much higher in the abdomen than in the thorax (Fig. 2); the differences were statistically significant (Table 2). This pattern is also true for both males and females of both species. Since the estimates of concentrations in the abdomen are more reliable in terms of analytical measurement errors, they were used in further analysis.

Zn concentrations proved to be an order of magnitude higher than Cu and Pb concentrations and two orders of magnitude higher than Cd concentrations. Interspecific and sex differences were identified: *C. arcania* accumulated higher metal concentrations than *A. hyperanthus*; in both species, males accumulated a greater amount of metals than females (Table 3, Fig. 3).

The content of metals significantly differed between the sites: the maximum concentrations of Cu, Zn, and Cd were recorded in the impact site, while their minimum concentrations in the control and background sites. The pattern was different for Pb: in *A. hyperantus,* the maximum concentrations were recorded in the buffer rather than impact site (see Fig. 3).

Wing length. Statistically significant differences between the sites were found in *C. arcania* males and *A. hyperantus* females (Table 4). The largest *C. arcania* males lived in the control site (Fig. 4). In *A. hyperantus* females, significant differences were recorded only

Table 2. MANOVA results for differences in concentrations of the four metals in the body parts of the two satyr species

Source of variability	Partial η^2	Wilks Λ	*F*	*df* 1	*df* 2	*p*
Species	0.14	0.86	4.3	4	109	<0.001
Sex	0.29	0.71	11.0	4	109	<0.001
Body part	0.87	0.13	175.0	4	109	<0.001
Species × sex	0.03	0.97	0.9	4	109	0.488
Species × body part	0.17	0.83	5.6	4	109	<0.001
Sex × body part	0.25	0.75	9.0	4	109	<0.001
Species × sex × body part	0.07	0.93	1.9	4	109	0.108

Table 3. MANOVA results for differences in concentrations of the four metals in the abdomen of the two satyr species from different sites

Source of variability	Partial η^2	Wilks Λ	*F*	*df* 1	*df* 2	*p*
Species	0.09	0.91	4.1	4	162.0	0.004
Sex	0.62	0.38	64.8	4	162.0	<0.001
Site	0.37	0.30	20.8	12	428.9	<0.001
Species × sex	0.05	0.95	2.2	4	162.0	0.067
Species × site	0.08	0.80	3.2	12	428.9	<0.001
Sex × site	0.02	0.96	0.6	12	428.9	0.859
Species × sex × site	0.02	0.96	0.8	8	324.0	0.642

Table 4. ANCOVA results for the wing length

Predictor	SS	*df*	*F*	*p*	Slope coefficient (± error)
C. arcania females. $R^2 = 0.38$, $F(5, 44) = 5.3$, $p < 0.01$					
Site	8.88	3	6.3	<0.01	
$Log_{10}Cu$	0.27	1	0.6	0.46	0.24 ± 0.31
$Log_{10}Zn$	0.90	1	1.9	0.17	–0.50 ± 0.36
C. arcania females. $R^2 = 0.23$, $F(4, 30) = 2.2$, $p = 0.09$					
Site	0.49	2	0.6	0.58	
$Log_{10}Cu$	1.62	1	3.6	0.07	–1.09 ± 0.57
$Log_{10}Zn$	0.41	1	0.9	0.34	–0.55 ± 0.57
A. hyperantus males. $R^2 = 0.12$, $F(5, 44) = 1.2$, $p = 0.31$					
Site	1.42	3	1.9	0.15	
$Log_{10}Cu$	0.01	1	<0.1	0.98	0.01 ± 0.45
$Log_{10}Zn$	0.01	1	<0.1	0.84	–0.07 ± 0.32
A. hyperantus females. $R^2 = 0.26$, $F(5, 38) = 2.7$, $p = 0.03$					
Site	3.68	3	2.9	<0.05	
$Log_{10}Cu$	2.11	1	5.0	0.03	–0.85 ± 0.38
$Log_{10}Zn$	0.10	1	0.2	0.63	–0.27 ± 0.57

between samples from the impact and control sites, with the wing size being larger in samples from the impact site.

Zn concentrations were not correlated with the wing size. Cu concentrations in all sites had a statistically significant negative correlation with the wing size, but only in one case, namely, in *A. hyperanthus* females (Table 4, Fig. 5). The Site × $\log_{10}$ Cu interaction proved to be statistically insignificant ($F(3, 38) = 0.35$, $p = 0.79$); i.e., regression slope coefficients did not differ between the sites. The influence of the site on the variability in the wing length proved to be comparable ($\eta^2 = 0.18$) with the influence of Cu concentration ($\eta^2 = 0.11$).

Wing length asymmetry. A statistically significant FA of the wing length was recorded in all samples (Table 5). In other words, the measurement accuracy proved to be sufficient to analyze the influence of the factors on FA. The reproducibility of wing length measurements (i.e., *ME*5) varied from 0.44 to 0.71 (see Table 5), which indicates an acceptable reliability of the results. Samples of *C. arcania* and *A. hyperantus* females had a statistically significant DA of the wing length (see Table 5): DA = –0.09 mm and FA4a = 0.16 for *C. arcania* females and DA = –0.09 mm and FA4a = 0.20 for *A. hyperantus* females. Therefore, the influence of directional asymmetry on the FA estimate can be neglected.

In each site, the wing length FA1 does not depend on the concentration of Cu or Zn, and there are no statistically significant differences between the sites (Table 6). In different sites, FA varied from 0.1 to 0.2 mm in absolute values (FA1) or from 0.005 to 0.013 in relative (FA2) values at a wing size of 16–18 mm in *C. arcania* and 19–21 mm in *A. hyperantus* (Table 7).

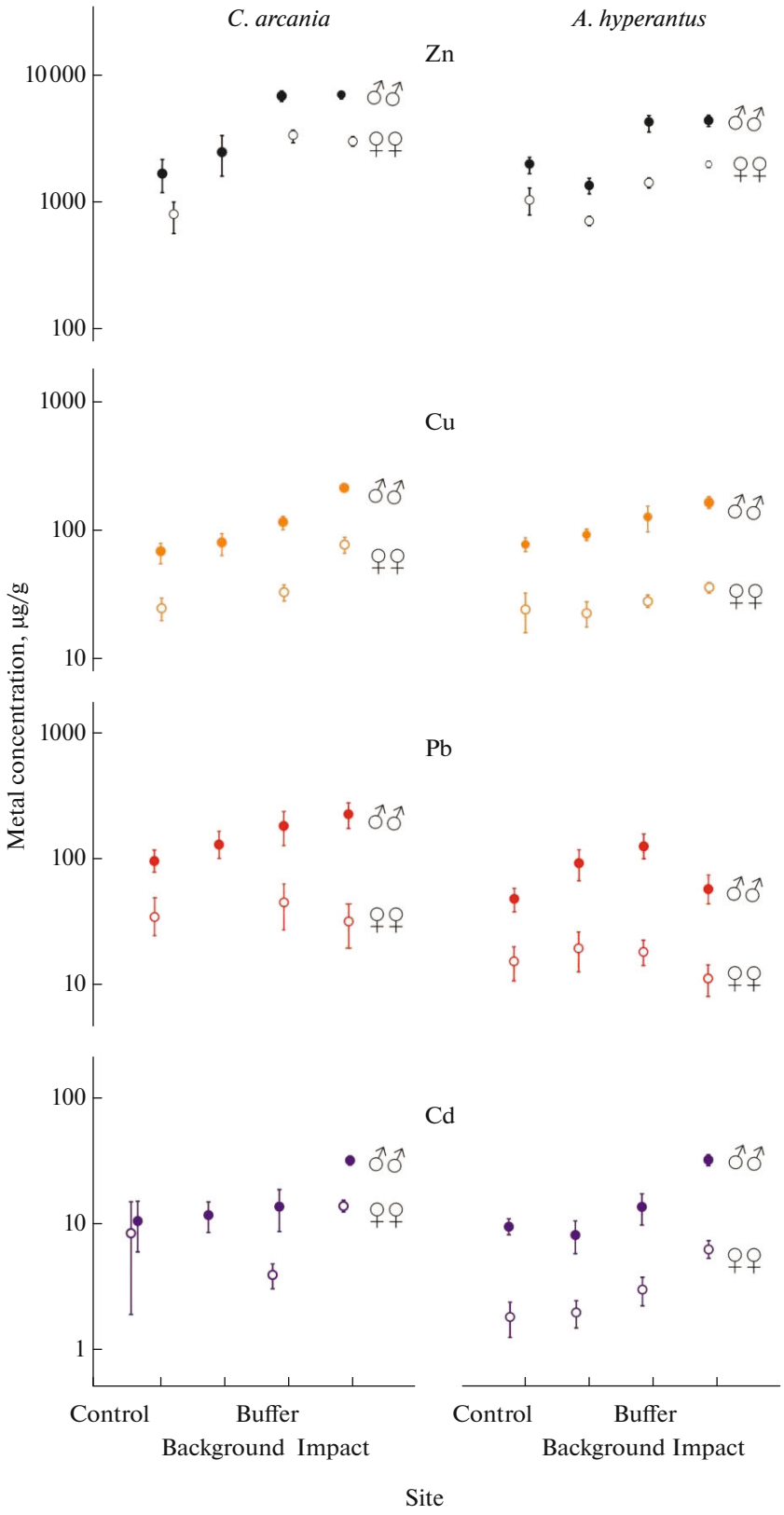

Fig. 3. Metal concentrations (mean ± error) in the abdomen of males and females of the two species from different sites.

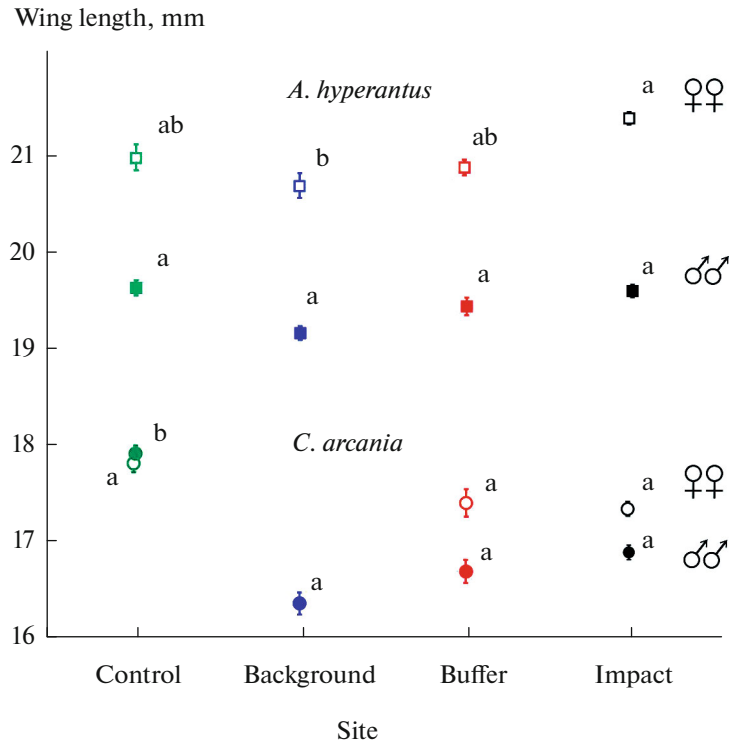

Fig. 4. Wing length (mean ± error) in the two species from different sites: the same letters indicate the absence of significant differences according to Tukey's test ($p < 0.05$).

DISCUSSION

Changes in the composition of both plant communities and the satyr taxocene with proximity to the source of emissions fit into the traditional assumptions about the negative impact of industrial pollution on species diversity [3–6]. The forb–grass meadows (typical for the southern taiga of the Middle Urals) in the control, background, and buffer sites, usually consisting of no less than ten grass and sedge species, were transformed into very unique monodominant bentgrass meadows in the impact area. The species composition of satyrs on these meadows decreased from 16 to two species considered in this study, *Aphantopus hyperanthus* and *Coenonympha arcania*.

We believe that the persistence of these two species is determined by a number of their biological features. *A. hyperantus* inhabits very diverse open areas throughout the range, which makes it possible to consider it as generalist species tolerant to anthropogenic transformation of plant communities [37, 38, 40, 43, etc.]. For *C. arcania*, bentgrass meadows are suitable owing to the growth of willow shrubs, the absence of which limits the colonization of open space by this species [46–48].

It should be emphasized that the occurrence of these two species from the entire multispecific satyr taxocene in impact meadows indicates their high tolerance not only to plant contamination with metals but also their forced transition to narrow oligophagy, i.e., to feeding exclusively on two grass species, *Agrostis tenuis* and *Deschampsia cespitosa*.

Metal accumulation. In the organism of phytophagous insects, metals are predominantly deposited in the fat body, midgut, excretory organs, gonads, and sex gamets [15, 54, 55]. Since up to 95% of the weight of the thorax in insects is formed by the wings and legs muscles [56], the detected higher concentrations of metals in the abdomen than in the thorax are quite expected. There is little information about the concentrations of metals in diurnal lepidopteran adults from natural populations living in industrial polluted areas [28]. This contrasts with numerous data on other insect groups, e.g., orthopterans [57], ground beetles [58], water-scavenger beetles [59], heterocerans (Lepidoptera) [10, 60], and other taxa [61].

The results of our research indicate that metals (in particular, Zn) can accumulate in the body of adult *A. hyperanthus* and *C. arcania* in very high concentrations. The maximum recorded concentrations of Zn in the abdomen of adults (9964 μg/g in *C. arcania* and 8021 μg/g in *A. hyperantus*) significantly exceed the values reported by other authors, e.g., 2200 μg/g in [10] and 4650 μg/kg in [55]. The recorded Cd concentrations are comparable (the maximum value is 65 μg/g) with the results of some authors, e.g., 230 μg/g in [10], but exceed the values reported by other authors, e.g., 2.9 μg/g in [60]. The Pb concen-

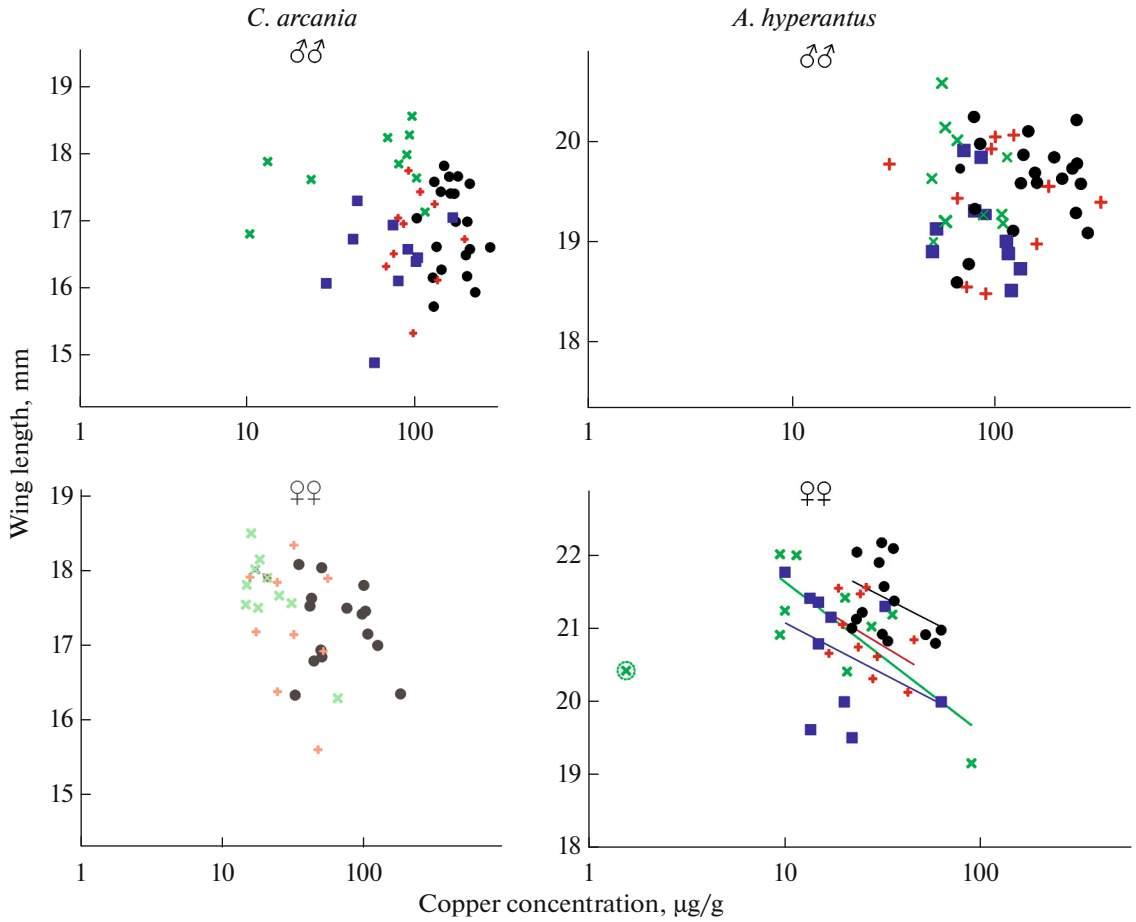

Fig. 5. Dependence of wing length on copper concentration in the abdomen: green color indicates the control site, blue color indicates the background site, red color indicates the buffer site, and black color indicates the impact site; the dotted line indicates a sharply deviating copper concentration (excluded from the statistical analysis).

trations reported in [10] (about 8 μg/g) are significantly lower than those recorded by us (764 and 324 μg/g); however, the authors specially emphasized that their model object (*Lymantria dispar*) barely assimilated Pb from feed.

Metals are removed from the body together with exuvium and meconium during molting, pupation, and the emergence of adults [10, 14, 55]. Therefore, it can be assumed that the concentrations may be even higher at the larval stage than at the adult stage. However, even the values presented by us can be characterized as extremely high. Plants accumulating Zn in concentrations above 10 000 μg/g are classified as hyperaccumulators [62]. As far as we know, such terminology is not used for insects. By analogy with nickel accumulation [62], *A. hyperanthus* and *C. arcania* can be considered high-Zn species. The specific mechanisms of this phenomenon require special study.

The results of the study of differences between females and males in metal accumulation are contradicting. In some lepidopterans, females can accumulate more Zn than males (e.g., females of *Spodoptera litura*) [14]. In other species, such as *Lymantria dispar* [10], males accumulate more Cu and Zn than females. Our results indicate a more intensive metal accumulation by males of *A. hyperanthus* and *C. arcania* than by females. Possibly, females more efficiently remove metals from the body, as shown for *S. litura* [14]. However, it cannot be excluded that males are tolerant to the toxic effect of Cu and Zn (or other elements), while females are eliminated from the population after the content of these elements exceeds a certain concentration threshold. This situation can be clarified only direct experiments.

Significant differences in metal accumulation in adults of both species from different sites can be considered as indirect evidence of their low migratory activity. This means that micropopulations at a distance of 5–10 km can be significantly isolated from each other when there are natural barriers (continuous woodlands with a small number of roads and clearings). A similar conclusion was previously made based on a comparison of morphological parameters of

Table 5. Two-way mixed ANOVA results for the asymmetry of wing length

Sample	Factor	SS	df	MS	*F*	*p*	*ME5*
C. arcania males	Individual	132.23	49	2.70	67.3	<0.01	0.71
	Side	0.05	1	0.05	1.2	0.28	
	Individual × side	1.96	49	0.04	5.9	<0.01	
	Error	0.68	100	0.01			
C. arcania females	Individual	76.14	37	2.06	51.8	<0.01	0.65
	Side	0.31	1	0.31	7.8	<0.01	
	Individual × side	1.47	37	0.04	4.8	<0.01	
	Error	0.63	76	0.01			
A. hyperantus males	Individual	45.58	49	0.93	20.6	<0.01	0.44
	Side	0.02	1	0.02	0.4	0.52	
	Individual × side	2.22	49	0.05	2.5	<0.01	
	Error	1.42	80	0.02			
A. hyperantus females	Individual	86.82	43	2.02	32.8	<0.01	0.60
	Side	0.35	1	0.35	5.8	0.02	
	Individual × side	2.65	43	0.06	4.0	<0.01	
	Error	1.37	88	0.02			

Table 6. ANCOVA results for the fluctuating asymmetry (FA1) of wing length

Predictor	SS	df	*F*	*p*	Slope coefficient (± error)
C. arcania males. $R^2 = 0.05$, $F(5, 44) = 0.5$, $p = 0.77$					
Site	0.03	3	0.7	0.58	
$Log_{10}Cu$	0.01	1	0.1	0.83	0.02 ± 0.08
$Log_{10}Zn$	0.01	1	<0.1	0.89	–0.01 ± 0.08
C. arcania females. $R^2 = 0.18$, $F(4, 30) = 1.6$, $p = 0.19$					
Site	0.09	2	3.1	0.06	
$Log_{10}Cu$	0.02	1	1.2	0.29	–0.12 ± 0.11
$Log_{10}Zn$	0.03	1	1.9	0.18	0.15 ± 0.11
A. hyperantus males. $R^2 = 0.06$, $F(5, 44) = 0.5$, $p = 0.76$					
Site	0.03	3	0.5	0.68	
$Log_{10}Cu$	0.01	1	0.2	0.68	0.05 ± 0.13
$Log_{10}Zn$	0.01	1	0.4	0.55	–0.05 ± 0.09
A. hyperantus females. $R^2 = 0.02$, $F(5, 38) = 0.2$, $p = 0.97$					
Site	0.01	3	0.1	0.99	
$Log_{10}Cu$	0.01	1	<0.1	0.85	–0.02 ± 0.13
$Log_{10}Zn$	0.01	1	0.4	0.53	–0.10 ± 0.16

micropopulations of satyrs *A. hyperanthus* and *Erebia ligea*, separated by short distances [43, 63].

Wing size. The size of adult insects is plastic and determined by the influence of many environmental factors. The slowdown of growth rates and the decrease in the size of adults are well-known and common responses of lepidopterans to the intake of metals [14, 16, 17]. Our results proved to be contradicting: Zn accumulation did not have a statistically significant effect on the wing size in both species; however, Cu concentration was negatively correlated with the size in females of one of the species, *A. hyperantus*. The absence of the decrease in wing size in males (in par-

Table 7. Indices of fluctuating asymmetry of wing length (FA1 index ± error (mm) in the numerator and FA2 index ± error [25] in the denominator).

Sex and species	Site			
	control	background	buffer	impact
C. arcania males	0.11 ± 0.04/ 0.006 ± 0.002	0.18 ± 0.04/ 0.011 ± 0.002	0.19 ± 0.04/ 0.011 ± 0.002	0.16 ± 0.03/ 0.010 ± 0.002
C. arcania females	0.18 ± 0.04/ 0.010 ± 0.002	–*	0.12 ± 0.04/ 0.007 ± 0.002	0.23 ± 0.04/ 0.013 ± 0.002
A. hyperantus males	0.15 ± 0.04/ 0.008 ± 0.002	0.17 ± 0.04/ 0.009 ± 0.002	0.09 ± 0.04/ 0.005 ± 0.002	0.17 ± 0.03/ 0.009 ± 0.002
A. hyperantus females	0.22 ± 0.04/ 0.011 ± 0.002	0.21 ± 0.04/ 0.010 ± 0.002	0.20 ± 0.04/ 0.010 ± 0.002	0.20 ± 0.04/ 0.009 ± 0.002

* Indicators were not calculated due to a small sample size.

ticular, taking into account that the concentrations of Cu and Zn are two times higher than in female) can also be interpreted as a greater tolerance to pollution in males than in females.

Although the Cu concentration in the body is negatively correlated with the female size in *A. hyperantus*, we did not find a statistically significant decrease in the size of adults in the most contaminated site. On the contrary, females captured in the impact site were somewhat larger than those from other sites. The body size also did not decrease in *C. arcania* and males of *A. hyperantus* from the impact site.

The body size of insects usually decreases with their proximity to the point source of pollution [8]. However, there are some exceptions: for instance, leaf roller *Eulia ministrana* became larger with proximity to the copper–nickel plant on the Kola Peninsula [18]. The reasons for this contradiction are not entirely clear.

It can be assumed that the habitats near the emission source may be favorable for the studied species, which compensates for the direct effect of the toxic action of metals on the body size. Thus, many phytophagous insects are characterized by an increase in numbers near sources of pollution [8], including the area near the MUCS [64]. Two main hypotheses were put forward to explain this phenomenon: improved food quality and decreased predation pressure [65]. Although the hypotheses were proposed to explain the abundance growth, they may also explain the increase in the size of phytophages.

According to the first hypothesis, the availability of nutrients increases, the concentration of protective compounds decreases, and/or the ratio of concentrations of nutrients and protective substances changes in food plants exposed to stress [66]. As a rule, phytophagous insects respond to improved food quality by increasing their size and fecundity [67, 68]. According to the second hypothesis, the species diversity and abundance of predators decrease in contaminated areas, which may favor the activity of phytophages. After reaching a critical size [69], the caterpillar either begins pupation or continues feeding and growth for some time. In the latter case, it acquires additional advantages (an increased fecundity as a result of increased body weight), which, however, involves the risk of death at the preimaginal stage [22, 70]. Under conditions of relaxed predation pressure, the extension of the growth period may prove to be a winning strategy.

In addition to the above hypotheses, the increase in size may also be associated with microclimate changes in the impact site, where the average air temperature during the growing season is 0.7–1.0°C higher than that in the background area [71]. Similarly to many other ectothermic organisms, the growth and development of insects are subject to the temperature–size rule, which postulates that the size of adult individuals is inversely proportional to the ambient temperature during ontogeny [72]. This is determined by the fact that ectotherms developing at lower temperatures grow to the adult stage much longer (although they accumulate their weight at a lower rate), thereby reaching a larger size than individuals developing at higher temperatures. However, there are numerous exceptions to this rule: univoltine terrestrial arthropods, including lepidopterans, can become larger with increase in environmental temperature [73, 74]. We do not know direct observations on the correlation between temperature and the size of adults for the univoltine satyrs *C. arcania* and *A. hyperantus*; therefore, we cannot unambiguously associate the differences in size with the temperature effect.

Fluctuating asymmetry. In insects, FA is often positively correlated with the intensity of a potential stressor. However, this effect is less pronounced in natural conditions than in laboratory conditions, most likely due to the possible effect of many unaccounted factors. Also, the relationship between FA and the intensity of impact is stronger in cases when the negative effect of the factor on viability is clearly pronounced [26].

Our results concern natural populations. No negative effect of metals on the size was found at least for males of the studied species. Therefore, the negative result is not surprising in our case: the FA value of the wing size does not depend on metal accumulation in the body and does not differ between clean and contaminated areas.

It was previously shown [75] that FA in lepidopterans was weakly correlated with the level of stress, including that caused by industrial metal pollution [18, 28]. The absence of the effect of metal pollution on FA was also described for the ant *Formica pratensis* [76] and bee *Osmia bicornis* [27]. Most likely, the change in FA under stress is differently manifested in different species and traits and is specific to the acting factor [27]. Without preliminary laboratory studies demonstrating that a certain factor really increases FA in a specific species, the use of this indicator as a universal stress marker in natural populations is questionable. Our results add arguments in favor of this assertion.

CONCLUSIONS

The hypothesis that the satyrs *C. arcania* and *A. hyperantus* accumulate metals in higher concentrations near the smelter than in the background area was confirmed. Very high concentrations of metals, in particular, Zn, were recorded in the abdomen of adult individuals of these species from the impact site. Since only these two species from the entire multispecific satyr taxocene have persisted in the highly polluted area, it can be concluded that they are tolerant both to the direct toxic effects of metals and gaseous emissions from the smelter as well as to habitat changes caused by industrial emissions.

The hypothesis that pollution negatively affects the size of wings and increases their fluctuating asymmetry was not confirmed. Contrary to the expectation, individuals living near the smelter were not the smallest. At the individual level, the statistically significant dependence of size on metal concentrations was recorded only for Cu and only for females of one of the species (*A. hyperantus*); i.e., it cannot be considered common. There is no effect of pollution on FA both at the group level (there are no differences between the sites) and individual level (there is no dependence on metal concentrations). The negative results may be partly determined by the choice of objects. It is quite possible that the hypothesis will be confirmed for species more sensitive to metals, the distribution of which is limited to areas with intermediate levels of pollution. For further research, it is also promising to select species for which the negative impact of pollution on viability parameters (in particular, body size) was clearly established in laboratory conditions and study them along a pollution gradient in natural conditions.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The collection and analysis of invertebrates were carried out with the approval of the Bioethics Commission of the Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences (Protocol No. 13, dated November 1, 2022).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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