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The Fluctuating Asymmetry of the Butterfly Wing Pattern Does Not Change along an Industrial Pollution Gradient

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Abstract: The rapid and selective responses to changes in habitat structure and climate have made butterflies valuable environmental indicators. In this study, we asked whether the decline in butterfly populations near the copper-nickel smelter in Monchegorsk in northwestern Russia is accompanied by phenotypic stress responses to toxic pollutants, expressed as a decrease in body size and an increase in fluctuating asymmetry. We measured the concentrations of nickel and copper, forewing length, and fluctuating asymmetry in two elements of wing patterns in *Boloria euphrosyne*, *Plebejus idas*, and *Agriades optilete* collected 1–65 km from Monchegorsk. Body metal concentrations increased toward the smelter, confirming the local origin of the collected butterflies. The wings of butterflies from the most polluted sites were 5–8% shorter than those in unpolluted localities, suggesting adverse effects of pollution on butterfly fitness due to larval feeding on contaminated plants. However, fluctuating asymmetry averaged across two hindwing spots did not change systematically with pollution, thereby questioning the use of fluctuating asymmetry as an indicator of habitat quality in butterfly conservation projects.

Keywords: copper-nickel smelter; fluctuating asymmetry; Kola Peninsula; Lepidoptera; phenotypic stress responses; wing length

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1. Introduction

Butterflies are valuable environmental indicators because they rapidly and selectively respond even to subtle changes in habitat structure and climate [1,2] (and references therein). Their colorful appearance and aesthetic appeal have attracted attention to these insects from both the general public and decision-makers [3] and have made butterflies focal objects of multiple national and international monitoring schemes [4,5], as well as of citizen science projects (e.g., www.iNaturalist.org (accessed on 20 March 2021)). Because of this attention, butterflies are one of the best studied groups of insects—the Global Biodiversity Information Facility at the beginning of 2019 included over 7.5 million complete and unique records of butterflies from around the globe [6].

Environmental pollution was recently identified as one of the five main drivers of biodiversity loss [7]. Along with direct (toxic) impacts on biota, pollution also changes habitat quality, which in turn affects biodiversity [8,9]. Therefore, pollution-induced changes in the diversity and abundance of butterflies are highly likely; however, the available evidence remains surprisingly scarce. Industrial pollution was hypothesized to be one reason for the declines in butterfly numbers in some regions [10,11], but mechanisms behind these declines have not been deciphered [12]. These mechanisms may be rather non-trivial; for example, the loss of thermophilous butterflies from the Netherlands was explained by microclimatic cooling due to the increased plant growth that arose due to a combined effect of increased nitrogen deposition and climate warming [13].

Counts of day-active moths and butterflies, conducted in 1991–1993 around the copper-nickel smelter in Monchegorsk in northwestern Russia, demonstrated that butterflies were most abundant in slightly polluted forests but were almost absent in the industrial

barrens (bleak open landscapes with small patches of vegetation surrounded by bare land) next to the smelter [14]. Although many insect populations decline with increases in pollution [12,15,16], thus making this pattern seem trivial, this decline may have emerged for various reasons. These include (but are not limited to) direct toxicity of pollutants for insects and/or multiple indirect effects, acting, e.g., through the disappearance of forest habitats and the resulting changes in microclimate.

In this study, we tested the hypothesis that the decline in butterfly populations near the smelter can be predicted based on phenotypic differences between the exposed and control populations. The concentrations of nickel and copper in plants growing near Monchegorsk in the 1990s were as high as 600–800 $\mu g\ g^{-1}$ (i.e., 50 times greater than the regional background: [17]). We therefore predicted that the metal concentrations in butterflies would increase with increasing proximity to the polluter, as the larvae nearer to the polluter would have fed on more highly contaminated plants. The subsequently increased body concentrations of toxic metals are likely to cause physiological stress, which can be detected by phenotypic changes [18], including decreases in insect size [16]. Finally, pollution has been repeatedly reported to disturb the developmental stability of different organisms [19–22]. These observations led us to predict a greater fluctuating asymmetry (FA) of the wing pattern in butterflies from polluted sites than from unpolluted sites. We verified our predictions by measuring concentrations of nickel and copper, forewing length, and FA of the two wing spots in three butterfly species collected at different distances from the polluter.

2. Materials and Methods

2.1. Study Region and Study Sites

The town of Monchegorsk (67°56′ N, 32°55′ E), the site of a large copper-nickel smelter, is located 150 km south of the northern tree line. A century ago, this area was covered by impenetrable forests of Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.).

The mean temperature at Monchegorsk is −13.8 °C in January and 14.1 °C in July, and the mean annual precipitation is 561 mm; the frost-free period ranges from 50 to 100 days. The smelter started its operations in 1939, and the peak annual values of its emissions amounted to 278,000 metric tonnes (t) of sulphur dioxide in 1983 and 13,150 t of non-ferrous metals in 1987; the current annual emissions are close to 40,000 t of sulphur dioxide and 1000 t of metals [9,23]. Along with these main pollutants, the smelter emits dozens of other potentially toxic substances [24].

The pollution has transformed over 250 km^2 of the previously forested areas around the smelter into industrial barrens. Both Scots pine and Norway spruce are practically absent in barren habitats, and low-stature (0.2–3 m tall) mature mountain birches (*Betula pubescens* var. *pumila* (L.) Govaerts) growing 5–15 m apart dominate the landscape. In the intermediate zone, the top canopy of sparse forests is formed by Norway spruce that show visible signs of damage (upper parts of crowns are dead, needle longevity is low) and mountain birches, whereas field-layer vegetation is sparse. Mountain birches are common also in Norway spruce forests that are visibly unaffected by pollution, where *Empetrum nigrum* ssp. *hermaphroditum* (Hagerup) Böcher, *Vaccinium myrtillus* L., and *V. vitis-idaea* L. dominate in the dense field layer. In dry pine forests, the field layer includes *Calluna vulgaris* (L.) Hull, whereas *V. uliginosum* L. and *Rhododendron tomentosum* Harmaja (=*Ledum palustre* L.) are common in wet microsites [9,23].

2.2. Study Objects

We explored three of the most common butterfly species in the study region. *Boloria euphrosyne* (L.), an orange butterfly with black spots, has a wingspan of 38–46 mm and is on the wing during the first half of the summer. Its larvae feed on *Viola* sp., *R. tomentosum*, and *V. uliginosum*. The second species, *Plebejus idas* (L.), has a wingspan of 17–28 mm and flies during the second half of the summer. The males of this species have iridescent blue wings, whereas the females have brown wings with orange spots. The larvae of *P. idas* feed on *C. vulgaris*, *V. uliginosum*, *E. nigrum*, and on various Fabaceae species (most of which are absent from our study sites). The third species, *Agriades optilete* (Knoch), has a wingspan of 23–29 mm and flies simultaneously with *P. idas*; it also resembles *P. idas* in wing color and pattern, although the females of *A. optilete* may be almost as blue as the males. The larvae of *A. optilete* feed on *V. uliginosum*, *V. vitis-idaea*, *V. myrtillus*, *V. oxycoccos* L., *E. nigrum*, and *Andromeda polifolia* L. All these butterfly species hibernate as larvae.

We collected butterflies using standard insect collecting nets in 17 sites located 1–65 km from the smelter (Figure 1, Table 1) during the periodic assessment of insect diversity in the Monchegorsk pollution gradient from 1989–2001. Each year, we visited each site three to 10 times. During each visit, we collected all moths and butterflies seen during two hours. All collected butterflies (Supplementary Materials, Data S1) were pinned, and most of them were spread. We aimed at measuring 10–15 butterflies for each species-by-site combination; when the number of collected butterflies exceeded this desired value, we randomly selected individuals for this study from the available specimens. After measurements, the larger part of the collected butterflies was donated to the Zoological Museum, University of Helsinki, Finland.

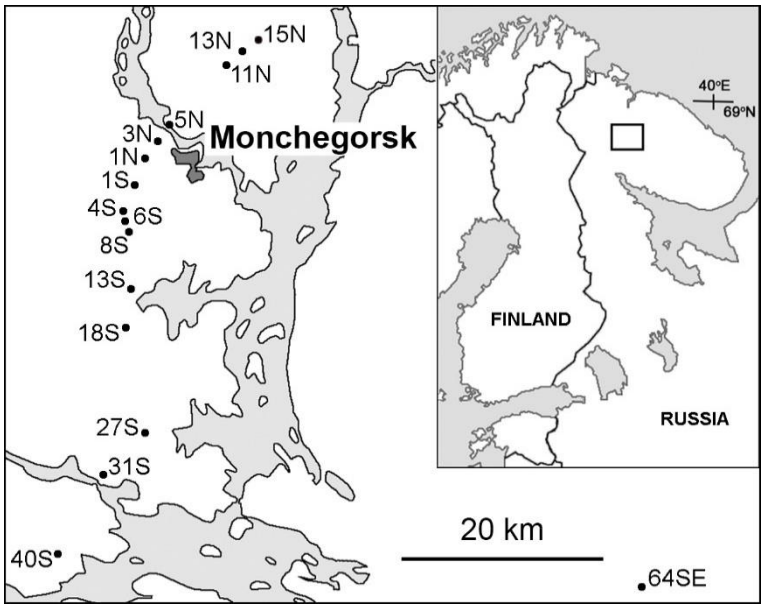


Figure 1. Position of the study area and distribution of study sites around the Monchegorsk nickel-copper smelter. For exact site coordinates, consult Table 1.

Table 1. The characteristics of the study sites.

Site †	Latitude, N	Longitude, E	Nickel in Birch Leaves ‡, μg g^{-1}		Stand Basal Area §, m^2 ha^{-1}	Cover of Field Layer §, %	Habitat Type ¶
			1991–1993	2008			
15N	68°03′	33°01′	40	26	5.3	40.5	DF
13N	68°02′	32°59′	30	-	-	-	SDF
11N	68°01′	32°57′	68	35	2.3	15.0	SDF
5N	67°58′	32°52′	138	66	0.3	0	IB
3N	67°57′	32°51′	208	100	0	0	IB
1N	67°56′	32°49′	338	223	0.3	0.4	IB
1S	67°55′	32°48′	168	-	0.2	-	BWC
4S	67°53′	32°47′	195	95	0	12.6	BWC
6S	67°52′	32°48′	186	119	0	1	IB
8S	67°51′	32°48′	153	92	0	0.7	IB
13S	67°48′	32°47′	90	59	2.0	16.6	SDF
18S	67°46′	32°48′	62	34	3.0	-	DF
27S	67°41′	32°50′	35	21	12.0	48.5	DF
31S	67°38′	32°45′	37	17	6.3	56.0	DF
40S	67°35′	32°33′	19	19	11.0	52.0	UF
64SE	67°32′	33°58′	10	8	18.7	29.0	UF

† The site codes indicate the approximate distance from the smelter in km and direction to the north, south or south-east of the smelter; ‡ Data for 1991−1993 from [17]; data for 2008: M. Kozlov, unpublished; § After [9], and unpublished; ¶ BWC, secondary birch- and willow-dominated community; DF, slightly damaged spruce forest; IB, industrial barren; SDF, severely damaged spruce forest; UF, undamaged spruce forest.

2.3. Measurements

We measured forewing length with a ruler (to the nearest 0.5 mm) as the distance between the base of the costal wing margin and the wing apex. This characteristic was only used to quantify the size of butterflies, because the locomotory traits are more buffered against developmental perturbations than non-locomotory traits are [25] and because precise measurements of wing size would require detaching the wings from the butterfly bodies. The FA was assessed in two spots on the underside of the hind wings (Figure 2). The size of the selected spots was measured (to the nearest 0.05 mm) on both the left and the right wings using a stereomicroscope micrometer. In *B. euphrosyne*, we measured the distance between the points at which the external margins of the selected spots approached the adjacent veins (Figure 2a), whereas in *P. idas* and in *A. optilete*, we measured the maximum distance between the opposite spot margins (Figure 2b,c). In 20 butterflies of each species, the measurements were repeated 12 months after the first measurement (Data S2). All measurements were performed by V.Z., blindly with respect to the collection site.

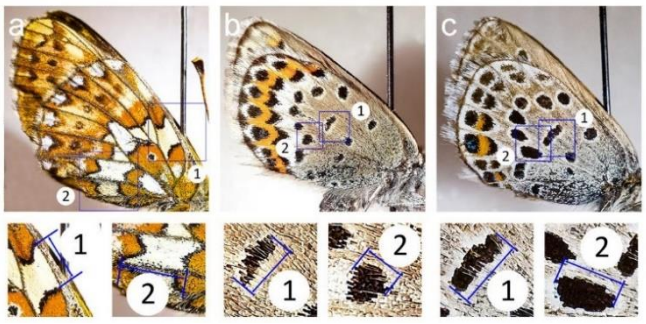


Figure 2. The measured elements of hind wing pattern (spots 1 and 2): (**a**) *Boloria euphrosyne*; (**b**) *Plebejus idas*; (**c**) *Agriades optilete*.

2.4. Chemical Analyses

Concentrations of nickel and copper were measured in *P. idas* and *A. optilete* specifically collected for this purpose 1–170 km from Monchegorsk in 2003–2004. The third

species, *B. euphrosyne*, was found too infrequently in the heavily polluted sites to obtain a representative sample of this species. The butterflies were handled and packed in paper envelopes with clean forceps to avoid cross-contamination. The randomly selected butterflies were digested in a 'Kjeldatherm' (Gerhardt, Germany) block digestion system with analytical-grade nitric acid (Merck). The residue was diluted to 5 mL with Milli-Q deionized water and filtered through paper (pore size 1.5–2 μm). Concentrations of Ni and Cu were measured by means of graphite furnace atomic absorption spectrometry using an AAnalyst 800 atomic absorption spectrometer (Perkin Elmer, Waltham, MA, USA) equipped with a THGA graphite furnace with Zeeman-effect background correlation and autosampler AS 800. The analyses were conducted at the Institute of North Industrial Ecology Problems, Apatity, Russia.

2.5. Data Analysis

The FA values were calculated as follows: FA = 2 × abs(WL − WR)/(WL + WR), where WL and WR refer to the spot size measured on the left and right wings of the same butterfly. The use of this index, labelled FA2 by Palmer and Strobeck [26], is justified by the significant positive correlation between the absolute difference in trait measurements between the left and right wings and an average trait size ($r = 0.27$, $n = 1452$ measurements, $p < 0.0001$).

We explored the data on 60 butterflies, which were measured twice, for the presence of FA and directional asymmetry (DA) relative to the measurement error by means of mixed-model ANOVA with the butterfly wing (right or left) considered as a fixed factor and the individual butterfly as a random factor. The reproducibility of the measurements was quantified by the index ME5 = (MSi − MSm)/(MSi + MSm), where MSi and MSm are the interaction and error mean squares from a side × individual ANOVA for two measurements of each trait in each individual [26]. This index expresses FA variation as a proportion of the total variation between sides, which includes variations due to both FA and measurement error. When mixed-model ANOVA revealed a significant difference between the right and left sides (i.e., the existence of DA), we compared the DA value with the FA4a index (FA4a = $0.798\sqrt{\text{var}}$ (R − L)), as suggested by Palmer and Strobeck [26].

Sources of variation in metal concentrations, wing length, and FA (averaged between two measured spots) were explored by means of a mixed-model ANCOVA (SAS GLIMMIX procedure, type 3 tests: [27]). We considered butterfly species and sex as fixed effects, the log-transformed distance from the smelter (a proxy of pollution load: Kozlov et al. [9]) as a covariate, and the site as a random effect. Following the practice commonly accepted in observational studies [28–30], we preferred to use the distance from the polluter rather than the concentration of one of the main pollutants (nickel or copper) in plant foliage or in a litter. This was done to avoid misinterpretation of our results, because we do not know which of the pollution-related factors (which all change with the distance from the smelter in a coordinated manner) may have affected the butterflies. In our study area, concentrations of metal pollutants in plants and in soil strongly correlate with log-transformed distance from the polluter [9,23]. To facilitate accurate *F*-tests of the fixed effects, we adjusted the standard errors and denominator degrees of freedom as described by Kenward and Roger [31]. The significance of a random factor was evaluated by testing the likelihood ratio against the Chi-squared distribution [32]. The associations between the study variables were explored by calculating the Pearson product-moment correlation coefficients (SAS CORR procedure [27]).

3. Results

The concentrations of nickel and copper did not differ between *P. idas* and *A. optilete*, and the concentrations increased by factors of 12 and 4, respectively, with increasing proximity to the smelter (Table 2, Figure 3).

Table 2. Sources of variation in concentrations of nickel and copper in butterflies (SAS GLIMMIX procedure, type 3 tests).

Source of Variation	Nickel		Copper	
	Test Statistics	***p***	**Test Statistics**	***p***
Species	$F_{1, 18.4} = 0.39$	0.54	$F_{1, 17.8} = 0.01$	0.92
Distance	$F_{1, 8.97} = 7.25$	0.02	$F_{1, 10.4} = 6.01$	0.03
Species × Distance	$F_{1, 19.0} = 0.46$	0.51	$F_{1, 17.3} = 0.11$	0.75
Site	$\chi^2_1 = 0.26$	0.61	$\chi^2_1 = 0.71$	0.40

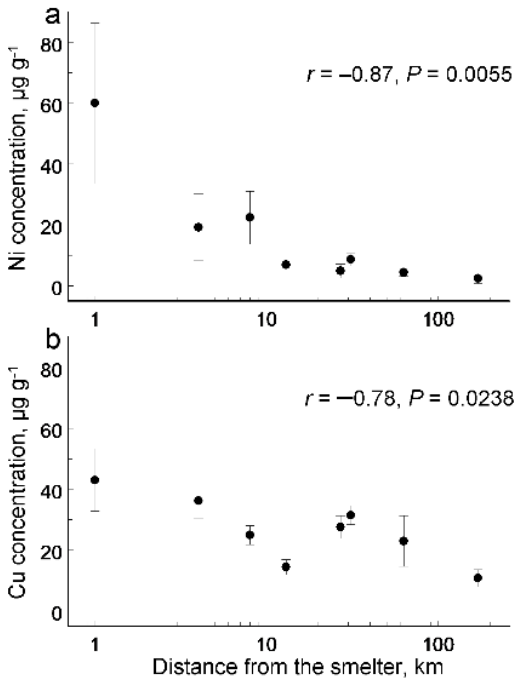


Figure 3. Concentrations (means ± S.E.) of the main metal pollutants ((**a**) nickel; (**b**) copper) in butterflies in relation to the distance from the Monchegorsk smelter.

The forewing length in the three butterfly species similarly decreased toward the smelter (Table 3), being 5–8% smaller in the most polluted sites than in the unpolluted sites (Figure 4). However, the species-specific correlations between forewing length and distance from the smelter were significant only in *B. euphrosyne* and *P. idas* (Figure 4a,b).

Table 3. Sources of variation in forewing length and fluctuating asymmetry of butterflies (SAS GLIMMIX procedure, type 3 tests).

Source of Variation	Forewing Length		Fluctuating Asymmetry	
	Test Statistics	***p***	**Test Statistics**	***p***
Species	$F_{2, 199.9} = 66.3$	<0.0001	$F_{2, 378.4} = 5.00$	0.0072
Sex	$F_{1, 704.6} = 2.92$	0.09	$F_{1, 667.5} = 1.95$	0.16
Species × Sex	$F_{2, 705.3} = 2.31$	0.10	$F_{2, 670.5} = 0.58$	0.56
Distance	$F_{1, 37.6} = 33.4$	<0.0001	$F_{1, 37.4} = 1.20$	0.28
Sex × Distance	$F_{1, 704.9} = 0.04$	0.83	$F_{1, 667.7} = 1.63$	0.20
Species × Distance	$F_{2, 250.5} = 1.83$	0.16	$F_{2, 401.5} = 1.47$	0.23
Species × Sex × Distance	$F_{2, 704.8} = 2.43$	0.09	$F_{2, 671.4} = 0.80$	0.45
Site	$\chi^2_1 = 0.20$	0.65	$\chi^2_1 = 3.78$	0.05

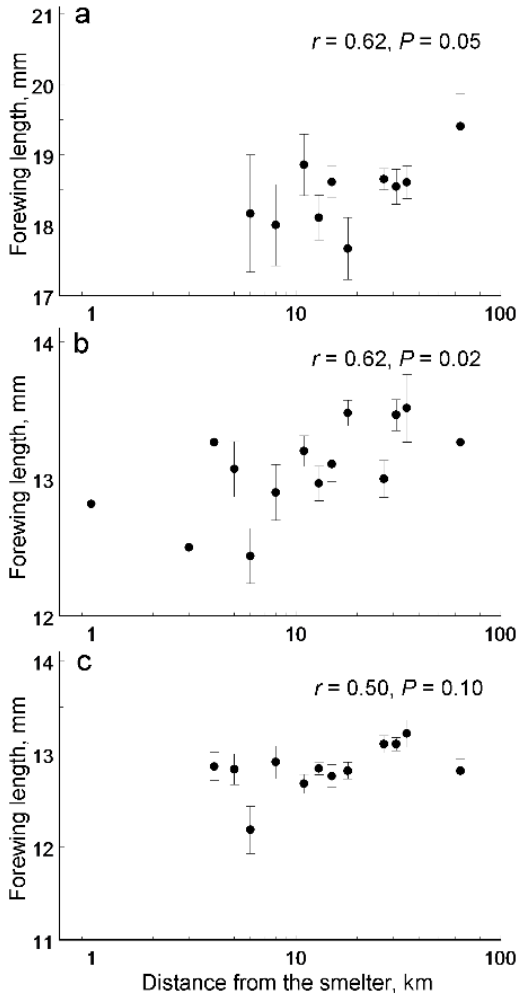


Figure 4. Forewing length (means ± S.E.) of butterflies ((**a**), *Boloria euphrosyne*; (**b**), *Plebejus idas*; (**c**), *Agriades optilete*) in relation to the distance from the Monchegorsk smelter.

The side × individual interaction was significant for both measured spots in all butterfly species (Table 4), thereby confirming the existence of FA in spot size and our ability to quantify it using measurements of the given accuracy. The DA was significant in the first spot in *B. euphrosyne* and *P. idas*, but not in *A. optilete* (Table 4). However, the DA in both *B. euphrosyne* and *P. idas* was smaller than the FA4a index, suggesting that DA's contribution to the total variation in size of the first spot was small and can therefore be neglected.

The FA of the two spots did not correlate either with each other (*B. euphrosyne*: $r = 0.05$, $n = 84$, $p = 0.65$; *P. idas*: $r = 0.08$, $n = 312$, $p = 0.15$; *A. optilete*: $r = 0.10$, $n = 298$, $p = 0.09$) or with forewing length ($r = -0.10 \ldots 0.17$, $n = 86 \ldots 314$, $p = 0.08 \ldots 0.96$). The FA averaged across the two spots varied among the study sites, but this variation was not explained by the distance from the smelter (Table 3) or by nickel concentrations in birch leaves (results not shown). The spot-specific correlations with distance were generally non-significant (Figure 5a–e), with the exception of the second spot in *A. optilete*, which showed a decreasing FA with increasing proximity to the smelter (Figure 5f).

Table 4. Basic statistics on measurements of differences in size of two spots between the left and right wings of three butterfly species.

Species	Spot †	DA ‡, mm	Source of Variation: Side		FA §	Source of Variation: Side × Individual		Reproducibility (ME5)
			$F_{1, 19}$	p		$F_{19, 38}$	p	
Boloria euphrosyne	1	−0.072	6.74	0.02	0.068	2.08	0.02	0.350
	2	0.008	1.01	0.33	0.044	8.04	<0.0001	0.779
Plebejus idas	1	0.041	5.71	0.03	0.078	2.20	0.02	0.375
	2	0.024	1.64	0.22	0.100	13.57	<0.0001	0.860
Agriades optilete	1	−0.004	0.05	0.83	0.051	2.09	0.02	0.350
	2	−0.006	0.32	0.58	0.083	7.19	<0.0001	0.754

† For explanations, consult Figure 1; ‡ DA, average directional asymmetry; § FA, average fluctuating asymmetry.

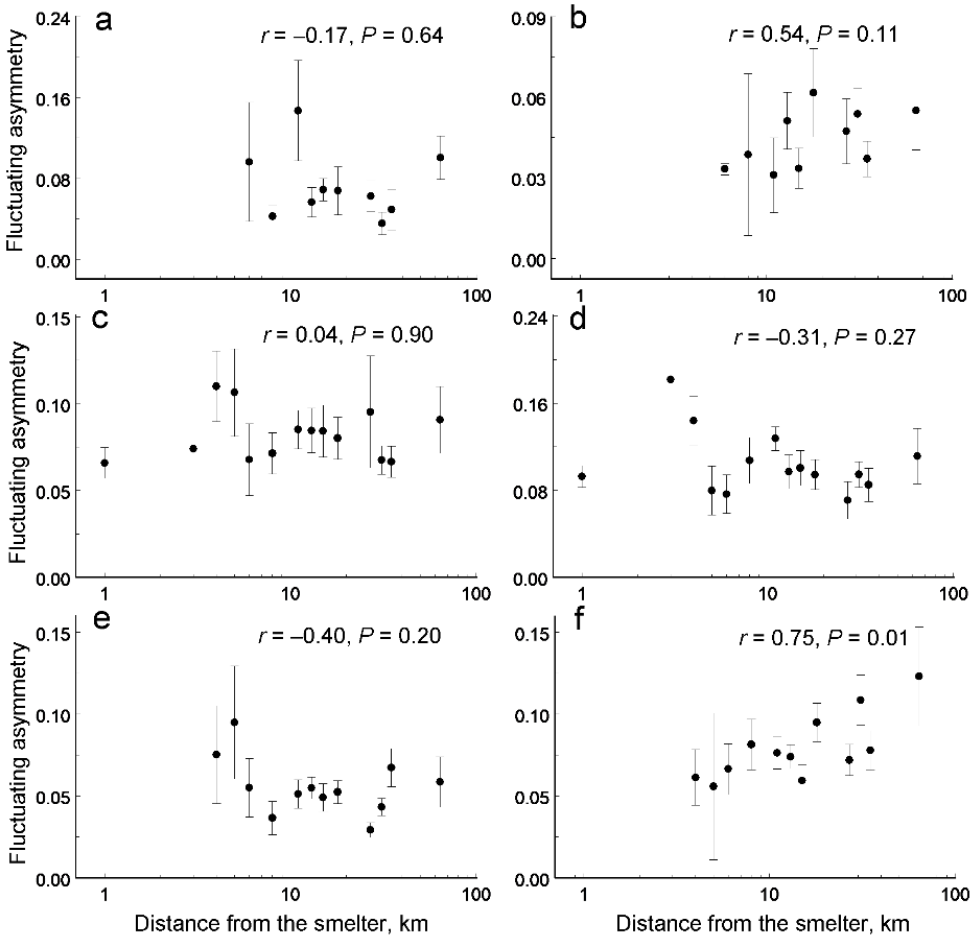


Figure 5. Fluctuating asymmetry (means ± S.E.) of two spots ((**a**,**c**,**e**) spot 1; (**b**,**d**,**f**) spot 2) of butterflies ((**a**,**b**) *Boloria euphrosyne*; (**c**,**d**) *Plebejus idas*; (**e**,**f**) *Agriades optilete*) in relation to the distance from the Monchegorsk smelter. For the identity of measured spots consult Figure 1.

4. Discussion

Insects living in polluted habitats often accumulate heavy metals, including nickel and copper [12]. The concentrations of nickel in butterflies were of the same range as in other plant-feeding insects collected from the same sites [33,34], whereas the concentrations of copper amounted to one-fifth of those in other insects. Importantly, the metal concentrations in the butterflies were much smaller than those reported in plants [17,35]. This result is in line with the earlier finding that larvae of *Eriocrania semipurpurella* excrete 90–95% of the nickel and 50–80% of the copper consumed from contaminated birch leaves [33]. The

metals are excreted with feces [33], larval exuviae, and pupal shells [36,37]. More generally, our findings confirm an opinion by Laskowski [38] that the accumulation of heavy metals in food webs (biomagnification) is an exception rather than the general rule.

The increased concentrations of nickel and copper in butterflies collected near Monchegorsk indicate their local origin, because their larvae have consumed polluted food. The concentrations of nickel and copper in leaves of different plants growing in the most polluted sites near the Monchegorck smelter reached 500–800 μg g^{-1} [17,35]. The earlier experiments demonstrated that 50–200 μg g^{-1} of copper added to the larval diet decreased the pupal weight of the oriental leafworm moth, *Spodoptera litura* (F.), to 80% of the control value [39], whereas 100 μg g^{-1} of nickel did not affect wing size in the cabbage white butterfly, *Pieris rapae* (L.) [40]. At the same time, the wing length of the autumnal moth *Epirrita autumnata* (Bkh.) did not change along the Monchegorsk pollution gradient [41], and the wing length of the brassy tortrix *Eulia ministrana* (L.) increased by 10% [30]. Thus, although metal toxicity is a likely reason for the recorded decrease in the size of butterflies accompanying the increase in pollution, experimental data are required to uncover the immediate reason behind the observed effect.

The abundance of butterflies near the smelter was reduced to 10% relative to unpolluted and slightly polluted sites [14]. Similarly, the biomass of dwarf shrubs, which include food plants of *B. euphrosyne*, *P. idas*, and *A. optilete*, in the most polluted sites was reduced to 0–20% of unpolluted controls [23]. Thus, the abundance of butterflies decreased proportionally to the decline in larval food resources. Nevertheless, the densities of butterflies in polluted sites were so small (0.3 butterflies seen during an hour [14]), that we do not consider larval starvation to be a possible reason for the small size of the butterflies. Instead, we suggest that this reduction in body size results from the toxicity of metal pollutants accumulated in larval host plants [42,43], combined with the physiological costs of metal detoxification and excretion. Metal toxicity could increase larval mortality [43], whereas a decrease in body size reduces the fecundity of female butterflies [44,45].

The decline in the butterflies in a Dutch nature reserve during the 1990s was attributed to the adverse effects of heavy metals on the nectar plants [46]. However, the proportion of flowering patches of *V. myrtillus* and *V. vitis-idaea* near Monchegorsk was higher, and these patches had more flowers compared to the unpolluted localities [47]. This means that nectar availability to adult butterflies decreases with an increase in pollution at a smaller rate than food availability to the larvae. Therefore, we doubt that a shortage of nectar could be the primary reason for the decline in butterflies near the Monchegorsk smelter.

Based on the increased body content of metals and the significant reduction in wing length, we conclude that butterflies living near Monchegorsk suffer from adverse environmental conditions. Following the arguments of Waddington [48], these adverse conditions are likely to disturb developmental stability, resulting in an increase in FA. Due to its seeming simplicity, this concept gave rise to a wealth of studies that explored the impacts of environmental stress on FA in different organisms [20,49,50].

Contrary to optimistic expectations [51–53], the accumulated evidence does not confirm that FA consistently increases in living beings facing unfavourable conditions during their development [22,54]. The studies of insects detected both positive associations between pollution and FA [55] and absences of correlation between these variables [30,56]. We estimate that, across organisms and traits, the support for this hypothesis was found in no more than half of the examined data sets [57]. The present study further confirms that life in extreme environments does not necessarily result in an increase in FA.

Only about a half of the previous studies measured FA in more than one trait per individual, and only a small fraction of these studies statistically combined information across traits [50] or explored among-trait correlations [25]. The single-trait approach reflects an implicit assumption of evolutionary models that any trait showing FA is suitable for the analysis of environmental impacts on developmental stability. However, we found that the FA values in the two wing spots were not related to each other. This finding, which is in line with earlier studies of FA in multiple traits of butterflies and moths [30,58,59],

seemingly contradicts the outcomes of meta-analysis, which confirmed the existence of the organism-wide response in FA [25]. However, the among-trait correlation in FA is so small (an average effect size of 0.05 [25]) that it can hardly be detected in an individual study, keeping in mind the obvious constraints of sample size.

Although the FA of different traits is practically uncorrelated, we are not aware of any theoretical model that predicts whether the FA in a particular trait of a given species will change in response to a specific environmental factor [57]. Nevertheless, the popularity of the theory linking environmental stress with a decrease in developmental stability and an increase in FA is so high that researchers exploring FA are forced to provide explanations for each 'negative' result, erecting hypotheses as to why their results do not fit the theoretical predictions (e.g., [60,61]). At the same time, the confirmatory evidence has long been published without deep examination of the research methodology used to arrive at these 'positive' results.

Two decades ago, when discussing the criticism directed toward the theory outlined above, Gangestad and Thornhill [62] (p. 414) wrote: " . . . bodies of evidence could have turned out quite differently and, hence, falsified prevailing notions about developmental imprecision and asymmetry. That that they did not but, rather, fit nicely with theory is a strange coincidence if those notions are entirely mistaken". We now know that this coincidence may have resulted from confirmation bias, defined as the tendency of humans to seek out evidence in a manner that confirms their beliefs and hypotheses. We experimentally demonstrated that when scientists expected to find high FA in some samples, the results of their measurements confirmed their expectations [63]. False discoveries of this type can be avoided by blinding [64], which we regard as obligatory in future studies of FA.

We suggest that authors and reviewers always ensure that (1) samples are collected either randomly or blindly with respect to the expected result; (2) the measurements of FA are conducted blindly (i.e., the measurer is not aware of the object's origin or of the hypothesis tested); and (3) at least a portion of the objects are remeasured and the FA is tested against the measurement error. We recommend that studies violating these criteria not be published or, if published, their outcomes not be included in future meta-analyses exploring the sources of variation in the FA of living beings. We also encourage the authors to publish their 'negative' and inconclusive results when they were obtained using adequate methodology, and to open up their file drawers, as Lane et al. [65] did. This is the only way to make the publication portfolio more representative of the actual findings.

In conclusion, the increased concentrations of nickel and copper in butterflies collected from polluted sites suggest that these butterflies are of local origin. The decline in abundance of butterflies with an increase in pollution, as observed near the Monchegorsk smelter, is likely driven by a combination of the pollution-induced decrease in biomass of the larval host plants and the toxicity of the metal pollutants accumulated in these plants. However, we did not detect any increase in the FA of the wing spots in the butterflies persisting in the polluted sites. This finding adds to a growing body of 'negative' results (in terms of the hypothesis predicting an increase in FA in response to environmental stress) and questions the use of FA as an indicator of habitat quality in butterfly conservation projects.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/sym13040626/s1, Data S1: Results of the first measurement, Data S2: Results of the second measurement.

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References

1. New, T.R. Are Lepidoptera an effective 'umbrella group' for biodiversity conservation? *J. Insect Conserv.* **1997**, *1*, 5–12. [CrossRef]
2. Brereton, T.; van Swaay, C.; van Strien, A. Developing a butterfly indicator to assess changes in Europe's biodiversity. *Avocetta* **2009**, *33*, 19–27.
3. Kühn, E.; Feldmann, R.; Harpke, A.; Hirneisen, N.; Musche, M.; Leopold, P.; Settele, J. Getting the public involved in butterfly conservation: Lessons learned from a new monitoring scheme in Germany. *Isr. J. Ecol. Evol.* **2008**, *54*, 89–103. [CrossRef]
4. Pollard, E.; Yates, T.J. *Monitoring Butterflies for Ecology and Conservation: The British Butterfly Monitoring Scheme*; Chapman and Hall: London, UK, 1993.
5. Lee, M.S.; Comas, J.; Stefanescu, C.; Albajes, R. The Catalan butterfly monitoring scheme has the capacity to detect effects of modifying agricultural practices. *Ecosphere* **2020**, *11*, e03004. [CrossRef]
6. Girardello, M.; Chapman, A.; Dennis, R.; Kaila, L.; Borges, P.A.; Santangeli, A. Gaps in butterfly inventory data: A global analysis. *Biol. Conserv.* **2019**, *236*, 289–295. [CrossRef]
7. IPBES. *Summary for Policymakers of the Global Assessment Report on Biodiversity and Ecosystem Services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services*; IPBES Secretariat: Bonn, Germany, 2019.
8. Barker, J.R.; Tingey, D.T. (Eds.) *Air Pollution Effects on Biodiversity*; Van Nostrand Reinhold: New York, NY, USA, 1992.
9. Kozlov, M.V.; Zvereva, E.L.; Zverev, V.E. *Impacts of Point Polluters on Terrestrial Biota: Comparative Analysis of 18 Contaminated Areas*; Springer: Dordrecht, The Netherlands, 2009.
10. Collins, N.M.; Morris, M.G. *Threatened Swallowtail Butterflies of the World. The IUCN Red Data Book*; IUCN: Gland, Switzerland, 1985.
11. Kudrna, O. *Aspects of the Conservation of Butterflies in Europe. Butterflies of Europe*; Aula: Wiesbaden, Germany, 1986; Volume 8.
12. Heliövaara, K.; Väisänen, R. *Insects and Pollution*; CRC Press: Boca Raton, FL, USA, 1993.
13. deVries, M.W.; van Swaay, C. Global warming and excess nitrogen may induce butterfly decline by microclimatic cooling. *Glob. Chang. Biol.* **2006**, *12*, 1620–1626.
14. Kozlov, M.V.; Lvovsky, A.; Mikkola, K. Abundance of day-flying Lepidoptera along an air pollution gradient in the northern boreal forest zone. *Èntomol. Fenn.* **1996**, *7*, 137–144. [CrossRef]
15. Alstad, D.N.; Edmunds, G.F.; Weinstein, L.H. Effects of air pollutants on insect populations. *Annu. Rev. Èntomol.* **1982**, *27*, 369–384. [CrossRef]
16. Zvereva, E.L.; Kozlov, M.V. Responses of terrestrial arthropods to air pollution: A meta-analysis. *Environ. Sci. Pollut. Res.* **2010**, *17*, 297–311. [CrossRef]
17. Kozlov, M.V.; Haukioja, E.; Bakhtiarov, A.V.; Stroganov, D.N. Heavy metals in birch leaves around a nickel-copper smelter at Monchegorsk, Northwestern Russia. *Environ. Pollut.* **1995**, *90*, 291–299. [CrossRef]
18. Odum, E.P. Trends expected in stressed ecosystems. *BioScience* **1985**, *35*, 419–422. [CrossRef]
19. Palmer, A.R.; Strobeck, C. Fluctuating asymmetry: Measurement, analysis, patterns. *Annu. Rev. Ecol. Syst.* **1986**, *17*, 391–421. [CrossRef]
20. Møller, A.P.; Swaddle, J.P. *Asymmetry, Developmental Stability, and Evolution*; Oxford University Press: Oxford, UK, 1997.
21. Leamy, L.J.; Klingenberg, C.P. The genetics and evolution of fluctuating asymmetry. *Annu. Rev. Ecol. Evol. Syst.* **2005**, *36*, 1–21. [CrossRef]
22. Graham, J.H.; Raz, S.; Hel-Or, H.; Nevo, E. Fluctuating asymmetry: Methods, theory, and applications. *Symmetry* **2010**, *2*, 466–540. [CrossRef]
23. Manninen, S.; Zverev, V.; Bergman, I.; Kozlov, M.V. Consequences of long-term severe industrial pollution for aboveground carbon and nitrogen pools in northern taiga forests at local and regional scales. *Sci. Total Environ.* **2015**, *536*, 616–624. [CrossRef]
24. Barcan, V.S. Nature and origin of multicomponent aerial emissions of the copper–nickel smelter complex. *Environ. Int.* **2002**, *28*, 451–456. [CrossRef]
25. Polak, M.; Møller, A.P.; Gangestad, S.W.; Kroeger, D.E.; Manning, J.T.; Thornhill, R. Does an individual asymmetry parameter exist? A meta-analysis. In *Developmental Instability: Causes and Consequences*; Polak, M., Ed.; Oxford University Press: New York, NY, USA, 2003; pp. 81–96.
26. Palmer, A.R.; Strobeck, C. Fluctuating asymmetry analyses revisited. In *Developmental Instability: Causes and Consequences*; Polak, M., Ed.; Oxford University Press: New York, NY, USA, 2003; pp. 279–319.
27. SAS Institute. *SAS/Stat. User's Guide, Version 9.2*; SAS Institute: Cary, NC, USA, 2009.
28. Freedman, B.; Hutchinson, T.C. Long-term effects of smelter pollution at Sudbury, Ontario, on forest community composition. *Can. J. Bot.* **1980**, *58*, 2123–2140. [CrossRef]

29. Brändle, M.; Amarell, U.; Auge, H.; Klotz, S.; Brandl, R. Plant and insect diversity along a pollution gradient: Understanding species richness across trophic levels. *Biodivers. Conserv.* **2001**, *10*, 1497–1511. [CrossRef]
30. Zverev, V.; Kozlov, M.V. Decline of *Eulia ministrana* (Lepidoptera: Tortricidae) in polluted habitats is not accompanied by phenotypic stress responses. *Insect Sci.* **2021**. [CrossRef]
31. Kenward, M.G.; Roger, J.H. An improved approximation to the precision of fixed effects from restricted maximum likelihood. *Comput. Stat. Data Anal.* **2009**, *53*, 2583–2595. [CrossRef]
32. Littell, R.C.; Milliken, G.A.; Stroup, W.W.; Wolfinger, R.D.; Schabenberger, O. *SAS for Mixed Models*, 2nd ed.; SAS Institute: Cary, NC, USA, 2006.
33. Kozlov, M.V.; Haukioja, E.; Kovnatsky, E.F. Uptake and excretion of nickel and copper by leaf-mining larvae of *Eriocrania semipurpurella* (Lepidoptera: Eriocraniidae) feeding on contaminated birch foliage. *Environ. Pollut.* **2000**, *108*, 303–310. [CrossRef]
34. Zvereva, E.; Serebrov, V.; Glupov, V.; Dubovskiy, I. Activity and heavy metal resistance of non-specific esterases in leaf beetle *Chrysomela lapponica* from polluted and unpolluted habitats. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2003**, *135*, 383–391. [CrossRef]
35. Isaeva, L.G.; Sukhareva, T. Elemental composition of wild small shrubs in the area of influence of "Severonickel" combine: Data of long-term monitoring. *Tsvetnye Met.* **2013**, *10*, 87–92. (In Russian)
36. Zhulidov, A.V. Excretion of heavy metals from organisms of invertebrates. In *Ecotoxicology and Nature Protection*; Krivolutsky, D.A., Bocharov, V.F., Eds.; Nauka: Moscow, Russia, 1988; pp. 170–176. (In Russian)
37. Andrzejewska, L.; Czarnowska, K.; Matel, B. Distribution of heavy metal pollution in plants and herbivorous *Spodoptera littoralis* L. (Lepidoptera). *Ekol. Pol.* **1990**, *38*, 185–199.
38. Laskowski, R. Are the top carnivores endangered by heavy metal biomagnification? *Oikos* **1991**, *60*, 387–390. [CrossRef]
39. Huang, D.; Kong, J.; Seng, Y. Effects of the heavy metal Cu^{2+} on growth, development, and population dynamics of *Spodoptera litura* (Lepidoptera: Noctuidae). *J. Econ. Èntomol.* **2012**, *105*, 288–294. [CrossRef]
40. Kobieva, M.E.; Snell-Rood, E.C. Nickel exposure has complex transgenerational effects in a butterfly. *Integr. Comp. Biol.* **2018**, *58*, 1008–1017.
41. Ruohomäki, K.; Kaitaniemi, P.; Kozlov, M.V.; Tammaru, T.; Haukioja, E. Density and performance of *Epirrita autumnata* (Lepidoptera: Geometridae) along three air pollution gradients in Northern Europe. *J. Appl. Ecol.* **1996**, *33*, 773–785. [CrossRef]
42. Boyd, R.S.; Martens, S.N. Nickel hyperaccumulated by *Thlaspi montanum* var. *montanum* is acutely toxic to an insect herbivore. *Oikos* **1994**, *70*, 21–25.
43. Jhee, E.M.; Boyd, R.S.; Eubanks, M.D.; Davis, M.A. Nickel hyperaccumulation by *Streptanthus polygaloides* protects against the folivore *Plutella xylostella* (Lepidoptera: Plutellidae). *Plant Ecol.* **2005**, *183*, 91–104. [CrossRef]
44. Hughes, L.; Chang, B.S.-W.; Wagner, D.; Pierce, N.E. Effects of mating history on ejaculate size, fecundity, longevity, and copulation duration in the ant-tended lycaenid butterfly, *Jalmenus evagoras*. *Behav. Ecol. Sociobiol.* **2000**, *47*, 119–128. [CrossRef]
45. Bauerfeind, S.S.; Fischer, K. Maternal body size as a morphological constraint on egg size and fecundity in butterflies. *Basic Appl. Ecol.* **2008**, *9*, 443–451. [CrossRef]
46. Mulder, C.; Aldenberg, T.; De Zwart, D.; Van Wijnen, H.J.; Breure, A.M. Evaluating the impact of pollution on plant-Lepidoptera relationships. *Environmetrics* **2005**, *16*, 357–373. [CrossRef]
47. Zvereva, E.L.; Kozlov, M.V. Growth and reproduction of dwarf shrubs, *Vaccinium myrtillus* and *V. vitis-idaea*, in a severely polluted area. *Basic Appl. Ecol.* **2005**, *6*, 261–274. [CrossRef]
48. Waddington, C.H. Canalization of development and the inheritance of acquired characters. *Nature* **1942**, *150*, 563–565. [CrossRef]
49. Lens, L.; Eggermont, H. Fluctuating asymmetry as a putative marker of human-induced stress in avian conservation. *Bird Conserv. Int.* **2008**, *18*, S125–S143. [CrossRef]
50. Beasley, D.A.E.; Bonisoli-Alquati, A.; Mousseau, T.A. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: A meta-analysis. *Ecol. Indic.* **2013**, *30*, 218–226. [CrossRef]
51. Zakharov, V.M. Analysis of fluctuating asymmetry as a method of biomonitoring at the population level. In *Bioindications of Chemical and Radioactive Pollution*; Krivolutsky, D.A., Ed.; Mir Publishers: Moscow, Russia, 1990; pp. 187–198.
52. Clarke, G.M. Fluctuating asymmetry: A technique for measuring developmental stress of genetic and environmental origin. *Acta Zool. Fenn.* **1992**, *191*, 31–35.
53. Parsons, P.A. Fluctuating asymmetry—A biological monitor of environmental and genomic stress. *Heredity* **1992**, *68*, 361–364. [CrossRef] [PubMed]
54. Kozlov, M.V. Plant studies on fluctuating asymmetry in Russia: Mythology and methodology. *Russ. J. Ecol.* **2017**, *48*, 1–9. [CrossRef]
55. Bonada, N.V.; Rieradevall, M.; Prat, N. Relationship between pollution and fluctuating asymmetry in the pollution-tolerant caddisfly *Hydropsyche exocellata* (Trichoptera, Insecta). *Arch. Hydrobiol.* **2005**, *162*, 167–185. [CrossRef]
56. Rabitsch, W.B. Levels of asymmetry in *Formica pratensis* Retz. (Hymenoptera, Insecta) from a chronic metal-contaminated site. *Environ. Toxicol. Chem.* **1997**, *16*, 1433–1440. [CrossRef]
57. Zverev, V.; Kozlov, M.V. Variation in leaf size and fluctuating asymmetry of mountain birch (*Betula pubescens* var. *pumila*) in space and time: Implications for global change research. *Symmetry* **2020**, *12*, 1703. [CrossRef]
58. Brookes, M.I.; Graneau, Y.A.; King, P.; Rose, O.C.; Thomas, C.D.; Mallet, J.L.B. Genetic analysis of founder bottlenecks in the rare British butterfly *Plebejus argus*. *Conserv. Biol.* **1997**, *11*, 648–669. [CrossRef]

59. Torres, M.A.J.; Sarte, C.E.; Demayo, C.G. Developmental independence of the eyespots in the wings of the nymphalid butterfly *Ypthima lisandra* Cramer 1782. *Asia Life Sci.* **2008**, *17*, 91–107.
60. Kark, S.; Lens, L.; Van Dongen, S.; Schmidt, E. Asymmetry patterns across the distribution range: Does the species matter? *Biol. J. Linn. Soc.* **2004**, *81*, 313–324. [CrossRef]
61. Campero, M.; De Block, M.; Ollevier, F.; Stoks, R. Metamorphosis offsets the link between larval stress, adult asymmetry and individual quality. *Funct. Ecol.* **2008**, *22*, 271–277. [CrossRef]
62. Gangestad, S.W.; Thornhill, R. Individual differences in developmental precision and fluctuating asymmetry: A model and its implications. *J. Evol. Biol.* **1999**, *12*, 402–416. [CrossRef]
63. Kozlov, M.V.; Zvereva, E.L. Confirmation bias in studies of fluctuating asymmetry. *Ecol. Indic.* **2015**, *57*, 293–297. [CrossRef]
64. Forstmeier, W.; Wagenmakers, E.J.; Parker, T.H. Detecting and avoiding likely false-positive findings—A practical guide. *Biol. Rev.* **2017**, *92*, 1941–1968. [CrossRef]
65. Lane, A.; Luminet, O.; Nave, G.; Mikolajczak, M. Is there a publication bias in behavioural intranasal oxytocin research on humans? Opening the file drawer of one laboratory. *J. Neuroendocr.* **2016**, *28*. [CrossRef] [PubMed]