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**Decline of *Eulia ministrana* (Lepidoptera: Tortricidae) in polluted habitats is not accompanied by phenotypic stress responses**

Running title: Moth decline in polluted habitats

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**Abstract** Environmental pollution is currently identified as one of the major drivers of rapid decline of insect populations, and this finding has revitalised interest in insect responses to pollution. We tested the hypothesis that the pollution-induced decline of insect populations can be predicted from phenotypic stress responses expressed as morphological differences between populations inhabiting polluted and unpolluted sites. We explored populations of the brassy tortrix *Eulia ministrana* in subarctic forests along an environmental disturbance gradient created by long-lasting severe impacts of aerial emissions of the copper-nickel smelter in Monchegorsk, northwestern Russia. We used pheromone traps to measure the population densities of this leafrolling moth and to collect specimens for assessment of three morphological stress indices: size, forewing melanisation and fluctuating asymmetry in wing venation. Wing length of *E. ministrana* increased by 10%, and neither forewing melanisation nor fluctuating asymmetry changed from the unpolluted forest to the heavily polluted industrial barren. However, the population density of *E. ministrana* decreased five- to ten-fold in the same pollution gradient. Thus, none of the studied potential morphological stress indicators signalled vulnerability of *E. ministrana* to environmental pollution and/or to pollution-induced environmental disturbance. We conclude that insect populations can decline without any visible signs of stress. The use of morphological proxies of insect fitness to predict the consequences of human impact on insect populations is therefore risky until causal relationships between these proxies and insect abundance are deciphered.

**Keywords** copper-nickel smelter; fluctuating asymmetry; industrial melanism; moth size; Kola Peninsula; pheromone trapping

## Introduction

Insects, ‘the little things that run the World’ (Wilson 1987), have currently attracted increasing attention from both scientists and lay people due to alarming reports regarding their rapid decline (Hallmann *et al.*, 2017; Seibold *et al.*, 2019; Wagner, 2020). This finding has revitalised interest in the impacts of chemical pollutants on insect populations in both natural and urban ecosystems (Fuentes *et al.*, 2016; Campbell & Vallano, 2018; Ghannem *et al.*, 2018; Skaldina & Sorvari, 2019), and a recent review identified environmental pollution as one of the major drivers of global insect decline (Cardoso *et al.*, 2020).

The responses of terrestrial biota, and of insects in particular, to pollution are far from being uniform (Zvereva & Kozlov, 2010). Long-term studies have revealed that insect abundance may increase, decrease or show no change in response to an increase in environmental contamination (Selikhovkin, 2013; Zvereva *et al.*, 2016). Moreover, the direction of density changes varies even among insects feeding on the same plant species in the same pollution gradient (Kozlov *et al.*, 2017). This variety of reactions calls for exploration of species or population characteristics which allow prediction of how insects will respond to pollution.

Moth species, whose larvae feed inside shelters made of rolled or tied plant leaves, generally benefit from pollution-induced environmental disturbance, as indicated by multiple reports on their increased densities near industrial polluters (Alstadt *et al.*, 1982; Kozlov, 1990; Jones *et al.*, 2008; Zvereva & Kozlov, 2010; Selikhovkin, 2013; Kozlov *et al.*, 2017). These effects arise from several factors, including an increase in the quality of their host plants due to pollutants (Riemer & Whittaker, 1989; Zvereva *et al.*, 2014), a pollution-induced decline in many of their natural enemies (Zvereva & Kozlov, 2000; Eeva *et al.*, 2012), accompanied by the protection afforded by the larval leaf shelters against the direct

effects of acid rain. The brassy tortrix *Eulia ministrana* (L.) belongs to this leaf-rolling/leaf-tying group of herbivorous insects; therefore, its abundance could be expected to positively correlate with pollution. However, in 1991, we surprisingly found that the density of this species declined more than tenfold in heavily polluted industrial barrens compared with unpolluted forests (Kozlov, 1997). This exceptional population behaviour of *E. ministrana* prompted us to explore whether its phenotypic responses to environmental stress could explain the observed population decline.

We used three morphological stress indices that presumably reflect the different aspects of fitness of *E. ministrana*: moth size, forewing melanisation and fluctuating asymmetry in wing venation (FA hereafter). Insect size generally decreases near industrial polluters (Zvereva & Kozlov, 2010), leading to decreases in fecundity (Du Merle & Cornic, 1991; Zvereva *et al.*, 1995) that, in turn, may cause population decline. An increase in melanisation may improve moth survival in polluted habitats by decreasing moth conspicuousness to visually hunting birds (Kettlewell, 1973) and/or by enhancing its immune defence (Mikkola & Rantala, 2010) or metal tolerance (Riley, 2013). FA (i.e. small, non-directional deviation from perfect symmetry in morphological traits) has repeatedly been found to increase with increases in pollution, reflecting a decrease in developmental stability (Zakharov, 1990; Clarke, 1992; Parsons, 1992). In male moths, an increase in FA was reported to decrease mating success (Tsubaki & Matsumoto, 1998; Koshio *et al.*, 2007).

In the present study, we use *E. ministrana* as a model object to test the hypothesis that the pollution-induced decline in insect populations can be predicted from phenotypic differences between the exposed and control populations. Our ultimate goal was to identify morphological indicator(s) that could be used to signal problems portending the extinction of insect populations before these problems become apparent.

## Materials and methods

### *Study species*

*Eulia ministrana* (Lepidoptera: Tortricidae) is a medium-sized (wingspan 18–26 mm), easily recognisable moth species that is widely distributed in the Palaearctic and Nearctic. Its forewing colour varies from pale ochreous brown to dark reddish brown. In our study region, the moths are on wing from mid-June to late July. The larva feeds inside leaf rolls on a variety of deciduous shrubs and trees, including birch, alder, willows, rowan, and bilberry, which are relatively common in our study region. The species is univoltine; the larva hibernates and pupates in spring or in early summer.

### *Study area and study sites*

The study was conducted on the Kola Peninsula in northwestern Russia, north of the Arctic Circle. The sites (20 in total; Table S1) were selected 1–40 km from the copper-nickel smelter at Monchegorsk (67°56'N, 32°49'E), which started its operations in 1939. All selected sites were originally covered by sparse subarctic forest (10–15 m high) formed by Norway spruce [*Picea abies* (L.) Karst.] and mountain birch [*Betula pubescens* var. *pumila* (L.) Govaerts]. A dense field layer vegetation (cover 30–60%) is dominated by crowberry, *Empetrum nigrum* ssp. *hermaphroditum* (Hagerup) Böcher, and bilberry, *Vaccinium myrtillus* L.

The smelter at Monchegorsk imposes adverse impacts on the surroundings habitats, primarily due to emissions of sulphur dioxide and non-ferrous metals into an ambient air. The annual sulphur dioxide emissions reached a maximum of 278,000 metric tons (t) in 1983,

steadily declined to about 100,000 t by the mid-1990s, dropped to 45,000 t in 1999, and remained at about 33–37,000 t since then. Metal emissions during the 1980s–1990s amounted to 3–8,000 t of Ni and 1–6,000 t of Cu annually, but decreased to 300–1,000 t of each of these metals in the 2000s.

The long-term severe impacts of aerial emissions, combined with logging and fires, has created steep environmental gradients around the polluter. The forests near Monchegorsk were transformed into an industrial barren, which covers 200–250 km<sup>2</sup>. The upper canopy trees are absent in this specific habitat, and woody plants are represented by low-stature (0.3–1.5 m high) shrubby mountain birch trees growing 5–15 m apart. The field layer vegetation is nearly missing (cover <1%), and small patches of vegetation are surrounded by bare land with the illuvial horizon or even the rock exposed due to intensive soil erosion. This barren zone is surrounded by a secondary birch- and willow-dominated community, with no top-canopy trees and very sparse field layer vegetation scattered over bare ground. In the severely damaged forest, over 50% of the top-canopy trees have died due to pollution, and over 25% of the living spruce trees have dead upper canopies; the field layer vegetation is patchy, with gaps of bare ground. The slightly damaged forest includes 5–25% spruce trees with dead upper canopies; the field layer vegetation is dense.

### *Moth trapping*

The densities of *E. ministrana* were evaluated using cardboard delta traps with a sticky insert, into which we placed a piece of rubber pipe containing 0.9 mg of (z)-11-tetradecenyl acetate and 0.1 mg of (z)-9-tetradecenyl acetate. This synthetic sex attractant, called PH-91, was produced by Flora Inc. (Tartu, Estonia) for monitoring of dark fruit-tree tortrix, *Pandemis heparana* (Den. & Schiff.). The traps (five per site) were exposed at 12 sites from 18 June to

7 July 1992 and at 18 sites from 20 June to 24 July 1993. Captured moths were counted at one-week intervals.

Males for investigation of wing melanisation and FA were collected in 1992 at five sites using non-sticky Multi-Pher I traps (Services Bio-Contrôle, Ste-Foy, Quebec, Canada) with the same (PH-91) attractant. Traps (five per site) were exposed at five sites from 18 June to 7 July and emptied once a week; all collected moths were pinned.

### *Morphological investigations*

We measured forewing length (to the nearest 0.1 mm, using a stereomicroscope micrometer) for 357 of 530 males collected with sticky traps in 1993. We measured only intact specimens, up to 25 males per site. Due to the low moth abundance, we failed to obtain representative samples from two heavily polluted sites, and this influenced the number of degrees of freedom in the analysis of wing length.

The level of forewing melanisation was assessed visually in all 394 pinned males collected with the non-sticky traps in 1992. The moths were sorted by colour from lightest to darkest, and then arbitrarily divided into five melanisation classes. The observer who performed this classification was blinded regarding the sites of moth origin.

The wings of pinned males were cut from their bodies, cleaned of scales and mounted as dry microscope slides. We measured 12 character distances between the designated landmarks (Fig. 1) in both left and right wings of 50 haphazardly selected individuals (10 per site). The absence of variation in the level of FA among study sites, revealed by the analysis of the obtained data (see Results), prompted us to quantify wing FA in 100 additional individuals (19-22 per site). However, in these individuals, we measured only five of the 12 characters (Data S1 in Supplementary information). All measurements were performed twice.



The first series of measurements was made directly from slides using a stereomicroscope micrometer (accuracy 0.025 mm). The second series of measurements was made from high-resolution digital pictures taken from these slides using the distance measuring tool in Adobe Photoshop (accuracy 0.007 mm). All measurements were performed blindly with respect to the site of moth collection.

When the absolute difference between the first and second measurement exceeded 0.15 mm, a third measurement was performed (with no knowledge of the earlier measurements) to exclude an occasional error. The third measurement was used to replace one of the two earlier measurements that was considered erroneous.

### *Data analysis*

The numbers of moths captured by five pheromone traps during the entire period of their exposure, as well as measurements of forewing length, were averaged for site-specific values. The relationships between these values and the levels of environmental pollution, measured as concentrations of nickel in mountain birch foliage (after Kozlov *et al.*, 1995), were quantified by calculating Pearson product-moment correlation coefficients.

The FA values were calculated as follows:  $FA = 2 \times \text{abs}(WL - WR) / (WL + WR)$ , where WL and WR refer to the distances between the same landmarks measured in the left and right wings of the same moth. This index, labelled FA2 by Palmer & Strobeck (2003), is one of the most frequently used indexes, in spite of its several drawbacks. Its use 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 ( $r = 0.18$ ,  $n = 1083$  measurements,  $P < 0.0001$ ).

We explored the data for presence of directional asymmetry (DA hereafter) and FA

relative to measurement error by mixed model ANOVA. In this analysis, the side (right or left) was considered a fixed factor and the individual moth a random factor. We evaluated the reproducibility of the measurements by calculating the index  $ME5 = [MSi - MSm]/[MSi + MSm]$ , where  $MSi$  and  $MSm$  are the interaction and error mean squares from a side  $\times$  individual ANOVA for two measurements of each trait in each individual (Palmer & Strobeck, 2003). This index expresses FA variation as a proportion of the total variation between the sides, which includes variation due to both FA and measurement error.

Sources of variation in FA were explored with a mixed model ANOVA (SAS GLIMMIX procedure, type III tests: SAS Institute, 2009). We considered the site and character as fixed effects and the moth individual as a random intercept effect. The association between pollution load and moth melanisation level was explored with a multinomial model for ordered data (SAS GLIMMIX procedure, link cumlogit; SAS Institute, 2009), and between pollution load and FA by ANCOVA. To facilitate accurate  $F$  tests of the fixed effects, we adjusted the standard errors and denominator degrees of freedom by the latest version of the method described by Kenward & Roger (2009). The significance of a random factor was evaluated by calculating the likelihood ratio and testing it against the chi-squared distribution (as described in Littell *et al.*, 2006).

## Results

### *Abundance*

The numbers of males collected by sticky pheromone traps decreased five- to ten-fold with a 15-fold increase in environmental pollution, as quantified by the concentrations of nickel in mountain birch foliage. This decline was observed in both 1992 (Fig. 2a) and 1993 (Fig. 2b).

### *Size and melanisation*

The forewing length in moths collected in 1993 correlated negatively with the numbers of moths captured by pheromone traps ( $r = -0.65$ ,  $n = 16$  sites,  $P = 0.0060$ ) and increased (!) significantly when approaching the smelter (Fig. 3). The level of forewing melanisation in moths collected in 1992 did not vary with pollution load (Fig. 4).

### *Fluctuating asymmetry*

The side  $\times$  individual interaction was highly significant in all 12 measured characters (Table 1), thereby confirming the existence of FA in the wing venation of *E. ministrana* and our ability to quantify this FA using repeated measurements of the given accuracy. DA was detected in a single character: the R1 vein was ca. 0.9% longer in the left forewing than in the right forewing (Table 1).

The measured characters differed six-fold in the level of their FA (Table 2), but this variation was not associated with any variation in the repeatability of our measurements ( $r = 0.14$ ,  $n = 12$  characters,  $P = 0.66$ ). The FA of any measured character did not vary among the study sites (Table 2). The level of FA (averaged across all measured characters) did not depend on pollution (Fig. 4b) and did not differ among moth individuals (Table 2).

## **Discussion**

The monitoring of *E. ministrana* during the summers of 1991–1993 (for data from 1991 consult Kozlov, 1997) consistently demonstrated a substantial decline in the abundance of

this species with increasing pollution. This finding contrasts with multiple reports of increases in the densities of leafrolling/leaf-tying moths near industrial polluters (Kozlov & Haukioja, 1993; Jones *et al.*, 2008; Selikhovkin, 2013; Kozlov *et al.*, 2017) and demonstrates that responses to pollution may greatly differ among ecologically and taxonomically similar insect species.

Another unusual pattern discovered in the course of our study was a significant positive correlation between moth size and environmental pollution. This pattern contrasts with the observations made on several other moth species with leafrolling/leaf-tying larvae, where the moth size was reported to decrease in surroundings of several industrial polluters (Kozlov & Haukioja, 1993; Selikhovkin, 2013). It also contrasts with the previous reports of a general decrease in body size of insects near industrial polluters relative to those in unpolluted areas (Zvereva & Kozlov, 2010). If we were to base our conclusions on the first morphological indicator, namely moth size, we would have to predict that density of *E. ministrana* will increase near the smelter due to an increase in moth fecundity.

The temporal and spatial changes in the occurrence of melanic forms were reported in populations of several moth and beetle species, although the immediate drivers of these changes remain obscure (Brakefield, 1987; Talloen *et al.*, 2004; Mikkola & Rantala, 2010; Riley, 2013). Surprisingly, this phenomenon (i.e. the increase in frequencies of dark individuals in large industrial cities), which is called industrial melanism, has been never reported from the surroundings of polluters emitting sulphur dioxide, fluorine or heavy metals. The late Kauri Mikkola arranged bait-trapping of Geometridae and Noctuidae near Monchegorsk from 1991–1994, but none of the collected specimens appeared to be melanic (Kozlov, 1997). In the present study, we collected sufficient numbers of dark (melanic) specimens of *E. ministrana*, but their proportions in the catches did not depend on pollution level at the study sites. A similar pattern (i.e. the absence of a correlation between pollution

and melanisation) was reported earlier in a polymorphic leaf beetle *Chrysomela lapponica* L. in the same environmental gradient. (Zvereva *et al.*, 2019). Therefore, we conclude that neither pollution nor the associated environmental disturbance in the surroundings of the Monchegorsk smelter affect the proportion of melanic individuals in insect populations. Thus, our second morphological indicator, wing melanisation, did not predict any effect of industrial pollution on populations of *E. ministrana*.

In our moth species, the FA of individual traits varied inconsistently; consequently, individual moths did not differ in the levels of FA averaged across all traits. Consistently, the third morphological indicator, the FA of wing venation, did not change along the pollution gradient. Thus, our study represents one more example of a lack of the expected increase in FA in organisms collected from polluted environments or subjected to pollutants in controlled conditions (Hardersen & Frampton, 1999; Szentgyorgyi *et al.*, 2017; Sandner, 2020).

None of the studied morphological characters suggest that populations of *E. ministrana* should decline in polluted habitats. Of course, pollution reduced the foliar biomass of deciduous trees and shrubs to 55% of values measured from unpolluted forests, whereas foliar biomass of dwarf shrubs was reduced to 20% (Manninen *et al.*, 2015). Keeping in mind the ratio between foliar biomass of trees and dwarf shrubs in our study region, we estimate that the potential food resources for *E. ministrana* larvae in our gradient decreased three-fold. Thus, although the decrease in foliar biomass may have contributed to the decline of *E. ministrana*, it cannot explain the magnitude of this effect.

The only plausible explanation for the detected decline in this moth species near the Monchegorsk smelter is the severity of winter conditions in the industrial barrens for this particular moth species. An absence of field layer vegetation and a low amount of plant litter in most polluted sites, combined with a thin snow layer, result in deep soil freezing. Consequently, the minimum soil temperatures in the industrial barrens reach -20°C, whereas

the soil temperatures in the surrounding forests never drop below  $-1^{\circ}\text{C}$  during the wintertime (Kozlov & Haukioja, 1997). The most common leafrolling/leaf-tying moths, such as *Apotomis sororculana* (Zett.), *Hedya atropunctana* (Zett.), *Carpatolechia proximella* (Hbn.) and *Swammerdamia caesiella* (Hbn.), whose densities increase near Monchegorsk (Zvereva *et al.*, 2014; Kozlov *et al.*, 2017), overwinter as pupae, whereas *E. ministrana* overwinters as a larva. We suggest that, as with some other insects (Hain & Ben Alya, 1985), moth larvae are more vulnerable to low winter temperatures than are moth pupae, and that this difference in life histories may be responsible for the exceptional behaviour of *E. ministrana* populations in the severe pollution gradient.

In conclusion, insect populations can decline without any visible signs of stress: none of the three morphological indices examined in this study signalled a vulnerability of *E. ministrana* to industrial pollution and/or pollution-induced environmental disturbance. Therefore, the use of morphological proxies of insect fitness to predict the consequences of human impact on insect populations is risky until the causal relationships between these proxies and insect abundance are deciphered.

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## **Disclosure**

The authors declare no conflicts of interest.

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### Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Characteristics of study sites and numbers of *Eulia ministrana* males collected by individual sticky traps with synthetic sex attractant.

**Data S1.** Values of wing venation characters measured from males of *Eulia ministrana* collected by non-sticky Multi-Pher I traps in 1992.

**Table 1.** Basic statistics on measurements of differences between the left and right wings of leafroller *Eulia ministrana* (samples of 1992; 50 males in total).

Character <sup>†</sup>	DA <sup>‡</sup> , mm	Source of variation:		FA <sup>§</sup>	Source of variation:		Reproducibility (ME5)
		Side			Side × Individual		
		$F_{1,49}$	$P$		$F_{49,98}$	$P$	
F02-F03	0.0389	5.29	0.03	0.0142	6.56	<0.0001	0.736
F04-F05	0.0014	0.03	0.87	0.0198	7.23	<0.0001	0.759
F06-F07	-0.0165	1.92	0.17	0.0112	3.93	<0.0001	0.595
F09-F10	0.0124	2.00	0.16	0.0108	43.12	<0.0001	0.515
F14-F15	0.0015	0.18	0.68	0.0088	4.53	<0.0001	0.637
F04-F06	0.0085	0.51	0.48	0.0537	7.07	<0.0001	0.753
F12-F14	0.0061	0.42	0.52	0.0266	3.65	<0.0001	0.570
F11-F13	0.0065	0.09	0.77	0.0238	3.86	<0.0001	0.589
H01-H02	0.0106	0.67	0.42	0.0221	19.4	<0.0001	0.902
H04-H05	0.0012	0.01	0.91	0.0175	13.6	<0.0001	0.863
H06-H07	-0.0078	0.46	0.50	0.0161	13.0	<0.0001	0.858
H03-H05	-0.0056	0.39	0.53	0.0292	4.55	<0.0001	0.639

<sup>†</sup> Each character is identified by two landmarks. For positions of landmarks, consult Fig. 1.

<sup>‡</sup> DA, directional asymmetry.

<sup>§</sup> FA, fluctuating asymmetry.

**Table 2.** Sources of variation in fluctuating asymmetry in wing venation of leafroller *Eulia ministrana* (samples of 1992).

Source of variation	Group 1 <sup>†</sup>		Group 2 <sup>‡</sup>		Groups 1+2	
	Test statistics	<i>P</i>	Test statistics	<i>P</i>	Test statistics	<i>P</i>
Site	$F_{4,45.3} = 1.26$	0.30	$F_{4,89.0} = 0.26$	0.91	$F_{4,110.3} = 1.70$	0.15
Character	$F_{11,485.8} = 11.8$	<.0001	$F_{4,360.0} = 5.47$	0.0003	$F_{11,923.5} = 12.8$	<.0001
Site × Character	$F_{44,485.6} = 0.80$	0.81	$F_{16,360.3} = 0.83$	0.65	$F_{44,925.7} = 0.81$	0.81
Individual	$\chi^2_1 = 0.51$	0.24	$\chi^2_1 = 0.58$	0.22	$\chi^2_1 = 1.27$	0.13

<sup>†</sup> Group 1: 50 males (10 per site; 12 characters measured).

<sup>‡</sup> Group 2: 100 males (19-22 per site; 5 characters measured).

## Figure captions

**Fig. 1.** Wing venation of *Eulia ministrana* and landmarks used in measurements. Characters measured in forewing: wing length (F01 to F08), length of R1 (F02 to F03), length of R2 (F04 to F05), length of R3 (F06 to F07), length of R5 (F09 to F10), length of CuA2 (F14 to F15), distance between bases of R2 and R3 (F04 to F06), distance between bases of CuA1 and CuA2 (F12 to F14), distance between apices of M1 and M3 (F11 to F13). Characters measured in hindwing: length of R (H01 to H02), length of M3 (H04 to H05), length of CuA2 (H06 to H07), distance between apices of M1 and M3 (H03 to H05).

**Fig. 2.** Numbers of *Eulia ministrana* males captured by sticky traps with synthetic sex attractant (mean  $\pm$  SE, n = 5 traps per site) in 1992 (a) and 1993 (b) plotted against concentrations of the principal metal pollutant (nickel) in birch leaves.

**Fig. 3.** Forewing length of *Eulia ministrana* males (mean  $\pm$  SE, n = 9–25 moths per site) captured by sticky traps with synthetic sex attractant in 1993 plotted against concentrations of the principal metal pollutant (nickel) in birch leaves.

**Fig. 4.** The level of forewing melanisation (a; mean  $\pm$  mean absolute deviation) and relative fluctuating asymmetry of wing venation (b; the estimated marginal means from the SAS GLIMMIX procedure and CI<sub>95</sub>). Sample sizes are shown in parentheses.

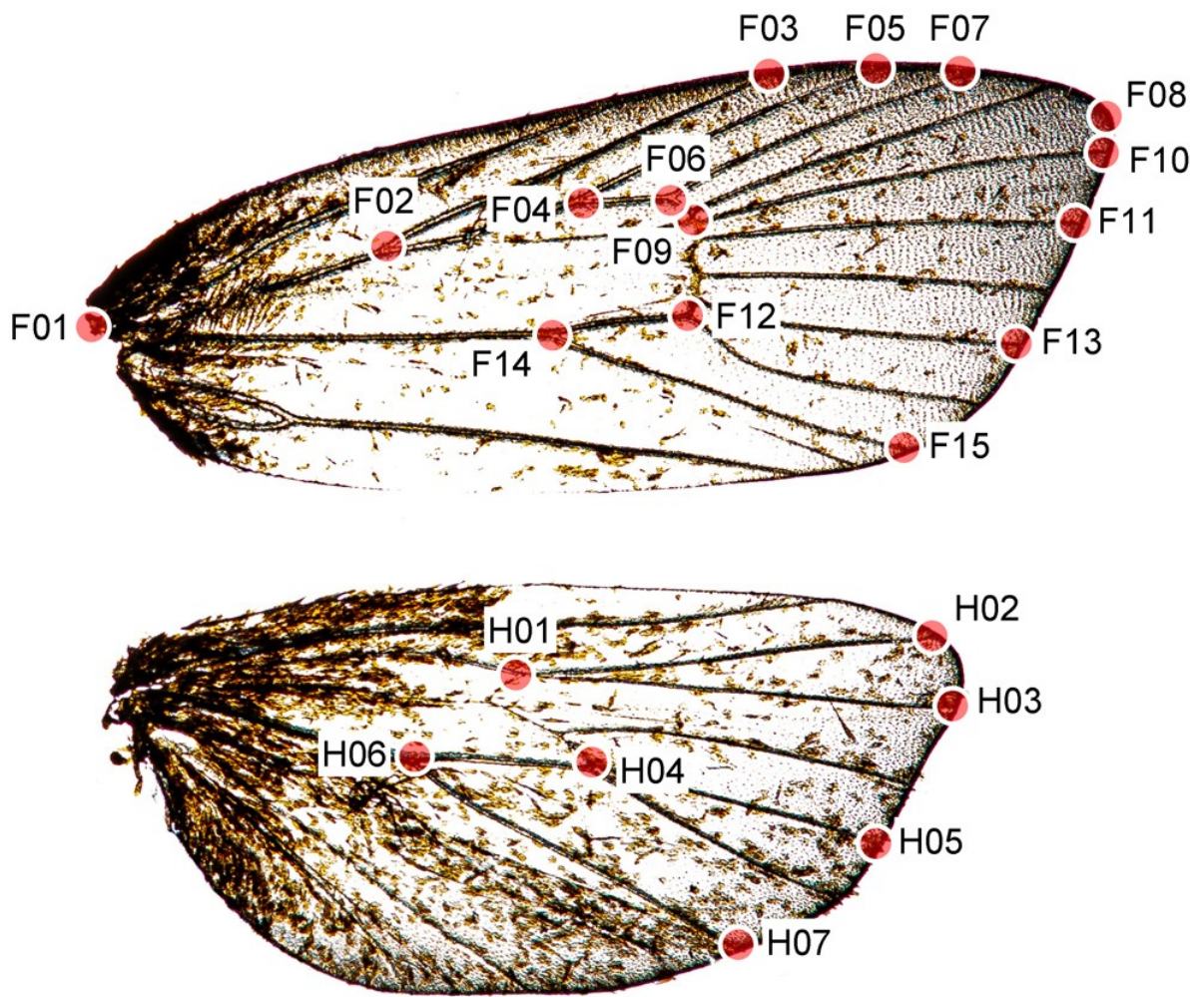


Fig. 1



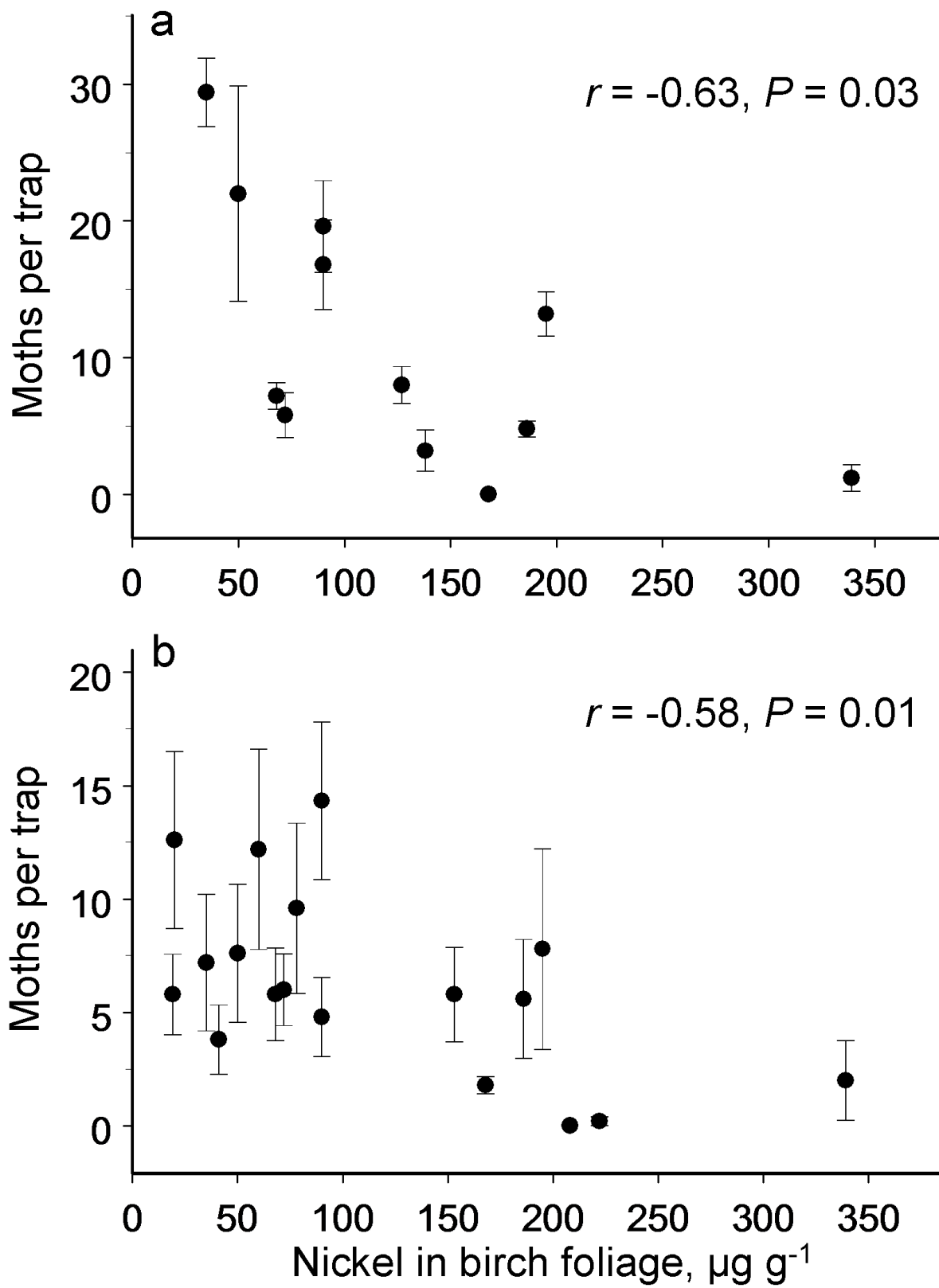


Fig. 2

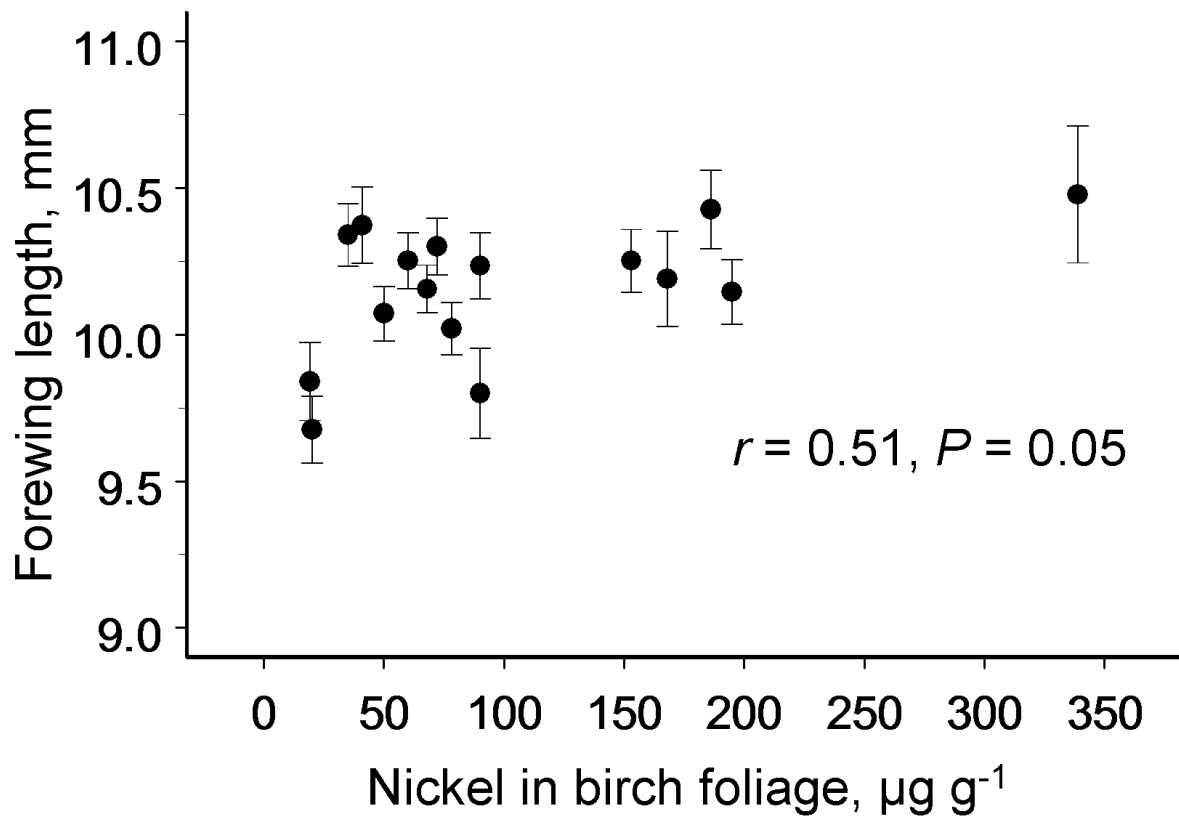


Fig. 3

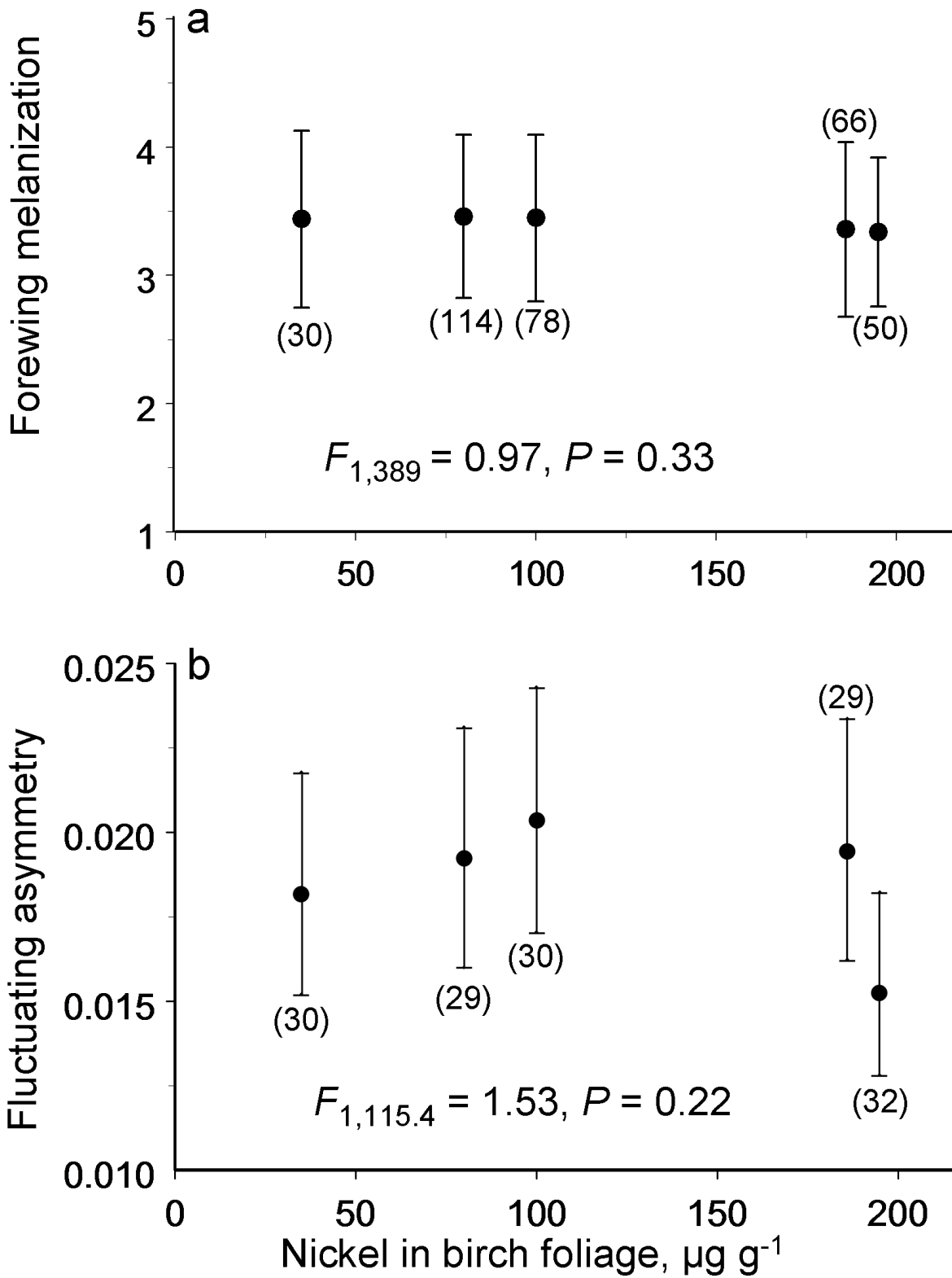


Fig. 4