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Morphological Variability of the Satyrid Butterflies, *Aphantopus hyperantus* and *Erebia ligea* (Lepidoptera, Satyridae) in Allopatric and Allochronous Micropopulations

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Abstract—The variability of morphological traits (the shape and size of the forewing and the location, number, and diameter of eyespots in the submarginal band of the wing pattern) was studied and analyzed in two species of Satyridae, *Aphantopus hyperantus* (Linnaeus, 1758) and *Erebia ligea* (Linnaeus, 1758), occurring sympatrically in Sverdlovsk Province, the Urals. It was originally supposed that micropopulations of the univoltine generalist species *A. hyperantus* would be weakly isolated and phenotypically homogeneous, since their habitats were positioned a small distance apart (about 10 km) within the same forested area and connected by a network of roads and glades serving as potential dispersal corridors; in contrast, micropopulations of the bicyclic specialist species *E. ligea* would be more strongly isolated and, accordingly, would have a higher level of phenotypic differentiation. The variability of morphological traits was analyzed by phenetic methods and also by traditional and geometric morphometrics. In both species, significant differences between micropopulations were found in the wing shape and size, as well as in the location and stability of eyespots in the wing pattern. As expected, temporal and spatial isolation of the micropopulations has led to differentiation in *E. ligea*. At the same time, the initial hypothesis of homogeneity of the *A. hyperantus* population in the studied territory was not confirmed. The latter case demonstrates that spatial isolation may be sufficient for differentiation in a species that is significantly sedentary and does not fully use the existing dispersal corridors.

Keywords: metapopulation, isolation, variation, wing, *Aphantopus hyperantus*, *Erebia ligea*

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It is now evident that destruction of natural environments as the result of economic activity threatens the existence of biodiversity of all the taxa, including insects. The processes related to forest clearing, farmland expansion, and urban development lead to degradation and fragmentation of landscapes (Habel et al., 2019; Cardoso et al., 2020, etc.). Different groups of insects adapt in different ways to fragmentation of their suitable habitats into more or less isolated areas: some continue to exist as local populations (Poniatowski et al., 2018), while others, in particular the mobile and easily dispersing lepidopterans, form metapopulations (Sutcliffe et al., 1997; Hanski, 1999; Powney et al., 2012; Viljur and Teder, 2018, etc.). Of vital importance for insects living in the fragmented environment are dispersal "corridors," i.e., landscape elements that facilitate the movement of individuals between the suitable habitats surrounded by unfavorable biotopes (Gutzwiller, 2002).

Various aspects of ecology and spatial distribution of diurnal butterflies (Rhopalocera) have been studied in sufficient detail at both the population and the community levels, especially in Europe. For instance, dispersal of species along the corridors was found to be considerably affected by the characteristics of the surrounding biotopes, in particular the soil types, plant communities, humidity conditions, etc. (Viljur and Teder, 2018), and also the characteristics of the corridors themselves (Grill et al., 2020; Habel et al., 2020). The generalist and specialist species differ in their use of dispersal corridors:

the former readily enter the corridors and actively move along them, and the latter are less active and more sensitive to various parameters, such as the presence of blossoming plants or the floristic composition of meadow communities (Habel et al., 2020).

This paper is devoted to analysis of variability of morphological traits in two species of the family Satyridae: *Aphantopus hyperantus* (Linnaeus, 1758) and *Erebia ligea* (Linnaeus, 1758), which co-occur in the same localities within the pre-forest-steppe belt of pine-birch forests in the south of Sverdlovsk Province. We studied micropopulations of these species in two open localities positioned a small distance apart within the same forested area and connected by a network of dispersal corridors (roads and glades). The goal of our work was to test the hypothesis that under such conditions, micropopulations of the univoltine generalist species *A. hyperantus* would be weakly isolated and phenotypically homogeneous, while those of the bicyclic specialist species *E. ligea* would be more strongly isolated and, accordingly, more strongly phenotypically differentiated.

Our analysis included such traits as the shape and size of the forewing and the number, location, and diameter of eyespots in the submarginal band of the wing pattern. The selected traits are related to different functions and differ in the level of phenotypic variability.

The wing shape determines the manner of flight and the related behavioral acts, such as seeking mates, avoiding predators, spreading in search of host plants, etc. Geometric morphometrics offers the most convenient and adequate methods of studying the wing shape in an evolutionary ecological context (Breuker et al., 2010; Sanzana et al., 2013; Shkurikhin and Oslina, 2016; Jugovic et al., 2018; Paučulova et al., 2018, etc.).

The wing pattern of Lepidoptera is one of the best studied morphological systems from the viewpoints of genetics, developmental biology, and evolutionary concepts (Beldade and Peralta, 2017; Sekimura and Nijhout, 2017; Marcus, 2019, etc.). It is known that eyespots are involved in communication and also serve to repel predators and divert their attention to the wing margins, where injuries would be less fatal (Kodandaramaiah, 2011). As shown in a number of publications, population variability can be quite adequately assessed by analyzing variation in the size and other characteristics of eyespots, such as their location on the wing (Cassel-Lundhagen et al., 2009, 2020).

MATERIALS AND METHODS

Characteristic of the Study Territory

Our research was carried out in the environs of the biological station of the Ural Federal University, near the interfluve of the Iset and Sysert rivers (Sysertskii District of Sverdlovsk Province) (Fig. 1). At the beginning of the XXI century woodlands in the interfluve area experienced a considerable anthropogenic impact due to recreational activities, berry and mushroom picking, cattle grazing, and selective felling. Analysis based on synanthropization indices showed that most of the forested areas were moderately or strongly disturbed (Mukhin et al., 2003). During the first two decades of the XXI century anthropogenic transformation of forest communities has aggravated as the result of active residential development and increasing recreational load in the form of trampling, berry and mushroom picking, and leisure activities in the environs of the reservoir on the Sysert River. At the same time, such activities as cattle grazing and haymaking have practically ceased.

The butterflies were collected in two localities positioned about 10 km apart (Fig. 1). The first locality, Fomino, comprised a strip of open land stretching for about 1.5–2 km along a power line and also edges of a pine forest and glades extending into the forest for 200–300 m. On its southeastern end the locality included a floodplain meadow on the Sysert River bank. Despite the presence of the river, this locality was drier than the second one, positioned further to the south, in the environs of Lake Karasye. The second locality, Karasye, was also about 1.5 km long and stretched along a forest track, ending in a wet forb-grass meadow on a heavily swamped lake shore. The two localities were not separated by any significant barrier but connected by a well-developed network of potential corridors in the form of forest glades (Fig. 1, *a*) and various kinds of roads (Fig. 1, *b*, *c*).

Characteristic of the Butterfly Species

Aphantopus hyperantus (Linnaeus, 1758) is a widespread trans-Palaearctic meadow-forest species (van Swaay et al., 2006; Settele et al., 2008; Gorbunov and Kosterin, 2007). This is a typical generalist species with a wide range of preferred biotopes, including open areas (dry, forb, grass, wet, and other meadows), glades and edges of deciduous and mixed forests, edges of bogs,

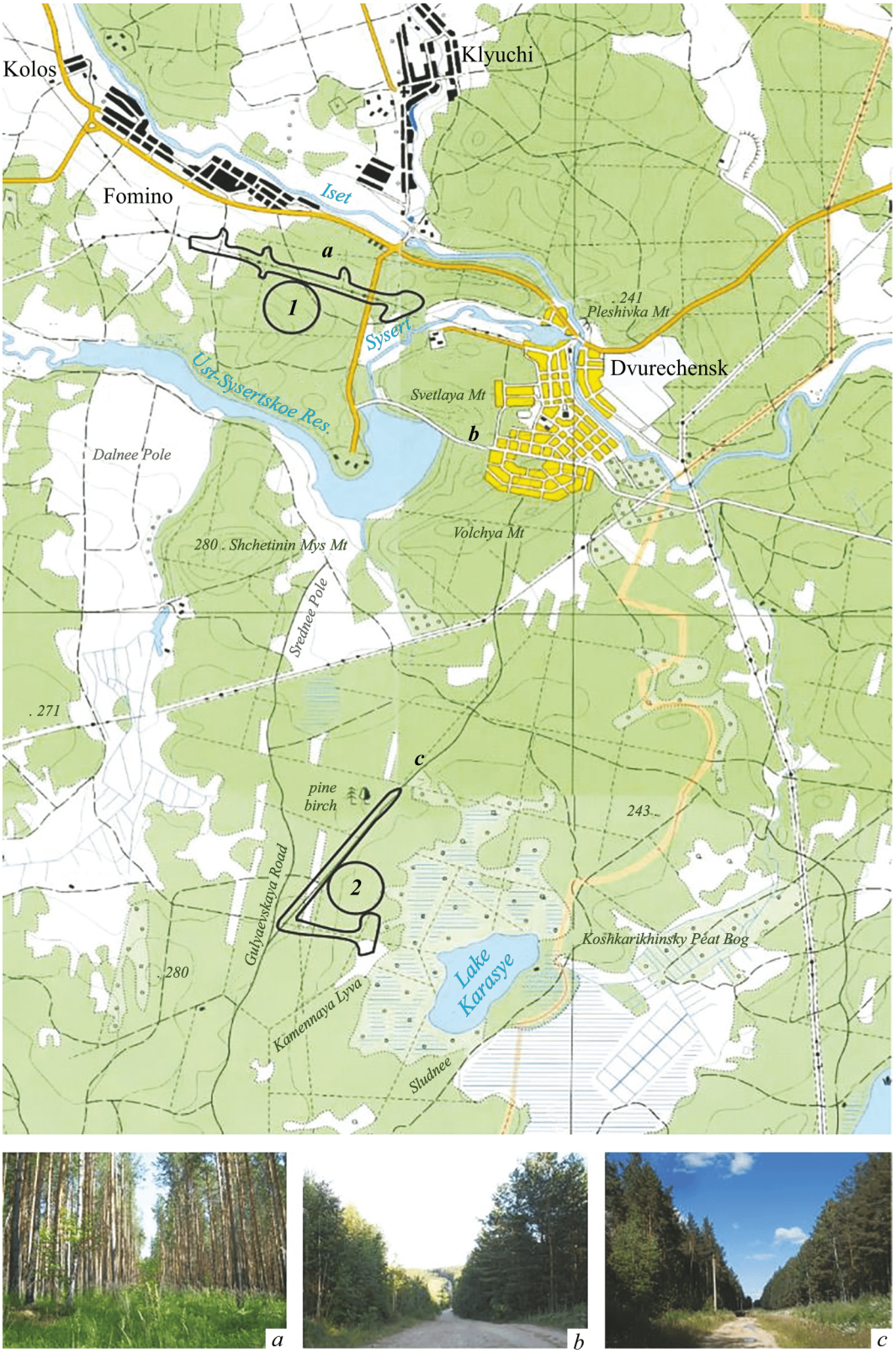

Fig. 1. Map of the study region (Sysertskii District, Sverdlovsk Province). Sampling localities of *Aphantopus hyperantus* and *Erebia ligea*: *1*, Fomino; *2*, Karasye. Examples of dispersal corridors (also marked in the map): *a*, glade; *b*, asphalt road; *c*, dirt road.

etc. It is quite tolerant to anthropogenic impact and can inhabit various agricultural landscapes, shelterbelt forests, planted forest stands, parks, field margins, and roadsides (Saarinen et al., 2005; Valtonen and Saarinen, 2005; van Swaay et al., 2006; Adakhovsky, 2014; Roth et al., 2014, etc.). The species reaches high densities in pastures and hayfields but still prefers natural meadows (Saarinen and Jantunen, 2005).

In the literature *A. hyperantus* is commonly considered a sedendary species, typically forming small and clearly delimited populations (Pollard and Yates, 1993; Sutcliffe et al., 1997; Billeter et al., 2003). In particular, a study of small fragmented territories showed that most individuals (63–79%) of *A. hyperantus* were not prone to dispersal but stayed in the same area. The individual activity radius was about 178.7 ± 136.5 m in males and 175.3 ± 116.2 m in females (Sutcliffe et al., 1997; Valtonen and Saarinen, 2005). Similar results were obtained by other authors (Billeter et al., 2003), who studied metapopulations of *A. hyperantus* in the strongly fragmented landscapes of Switzerland, including quasi-natural elements (banks of drainage canals, hedgerows, etc.) surrounded with agrocenoses. In different areas, sedentary residents comprised from 65 to 85%, migrants, from 7 to 20%; the greatest travel distance was 760 m in males and 970 m in females; the mean individual activity radius was 179 m. Sedentary micropopulations of this species seem to be formed only in fragmented landscapes containing small areas of suitable habitats. By contrast, in a weakly fragmented environment with easily available resources individuals of *A. hyperantus* move freely without staying long in one place (Gorbach, 2012, 2014).

Aphantopus hyperantus is a strictly univoltine species throughout its entire range. Its larvae develop on various grasses (Poaceae) and overwinter at the III or IV instar (Gorbunov and Kosterin, 2007; Ryzhkova and Lopatina, 2016).

The variability of eyespots in the wing pattern of *A. hyperantus* was studied earlier (Novozhenov, 1989, 1997; Zakharova, 1998, 2000; Gorbach, 2012). The number of eyespots on the forewing varies from 0 to 3. The forewing pattern usually includes 3 eyespots (P2, P4, and P5), sometimes 2 (P2 and P4) or 1 eyespot (P2) (Fig. 2, *a–c*). Individuals with no eyespots are rare, comprising no more than 1% in the populations (Novozhenov, 1997; Zakharova, 2000).

Erebia ligea (Linnaeus, 1758) is a widespread trans-Palaearctic specialist species preferring forest habitats, such as edges of dark coniferous and mixed forests, glades, clearings, sides of forest tracts, and also grass-forb meadows and some other types of meadows (Schneider and Fry, 2001; van Swaay et al., 2006; Gorbunov and Kosterin, 2007). The range of inhabited biotopes and the ecological niche of *E. ligea* are 3 times narrower than those of *A. hyperantus* (Komonen et al., 2004). Besides, *E. ligea* is more sensitive to anthropogenic impact (Saarinen and Jantunen, 2005; Adakhovsky, 2014). A recent study of the behavior and movements of several species of the genus *Erebia* Dalman, 1816, including *E. ligea*, carried out in a national park in Austria, showed that roads considerably hindered their dispersal. Although the butterflies were able to cross any wide asphalt road, they did it rarely and reluctantly (Grill et al., 2020).

It is well known that *Erebia* species may be bicyclic or univoltine, but never multivoltine (Warren, 1936). According to the literature data, their life cycle may include two diapauses: the first in the fully developed larva inside the eggshell or in the freshly hatched I instar larva, the second in the IV instar larva.

Earlier we studied the flight timing and the phenotypic wing variability of *E. ligea* in the selected localities in the environs of Fomino Village and Lake Karasye, starting from 2001. It was found out that in the first locality (Fomino) the flight occurred only in odd years (except for sporadic individuals recorded in 2016), and in the second locality (Karasye), only in even years (Zakharova and Shkurikhin, 2017). Thus, the micropopulations inhabiting these two localities were isolated not only in space but also in time, owing to the asynchronous biennial life cycle. Such isolation must have facilitated the establishment of two morphological forms, differing in the position of the eyespot foci in the submarginal band of the forewing pattern (Zakharova and Shkurikhin, 2017).

The variability of the adult size and the eyespots in the wing pattern of *E. ligea* was described by us earlier for the Urals and the adjacent territories (Zakharova, 2008, 2010; Zakharova and Tatarinov, 2016). The forewing pattern may include from 2 to 4, very rarely to 5 eyespots; most commonly there are 3 eyespots: P2, P3, and P5, located in cells M_1–M_2, M_2–M_3, and Cu_1–Cu_2, respectively (Fig. 2, *d–f*).

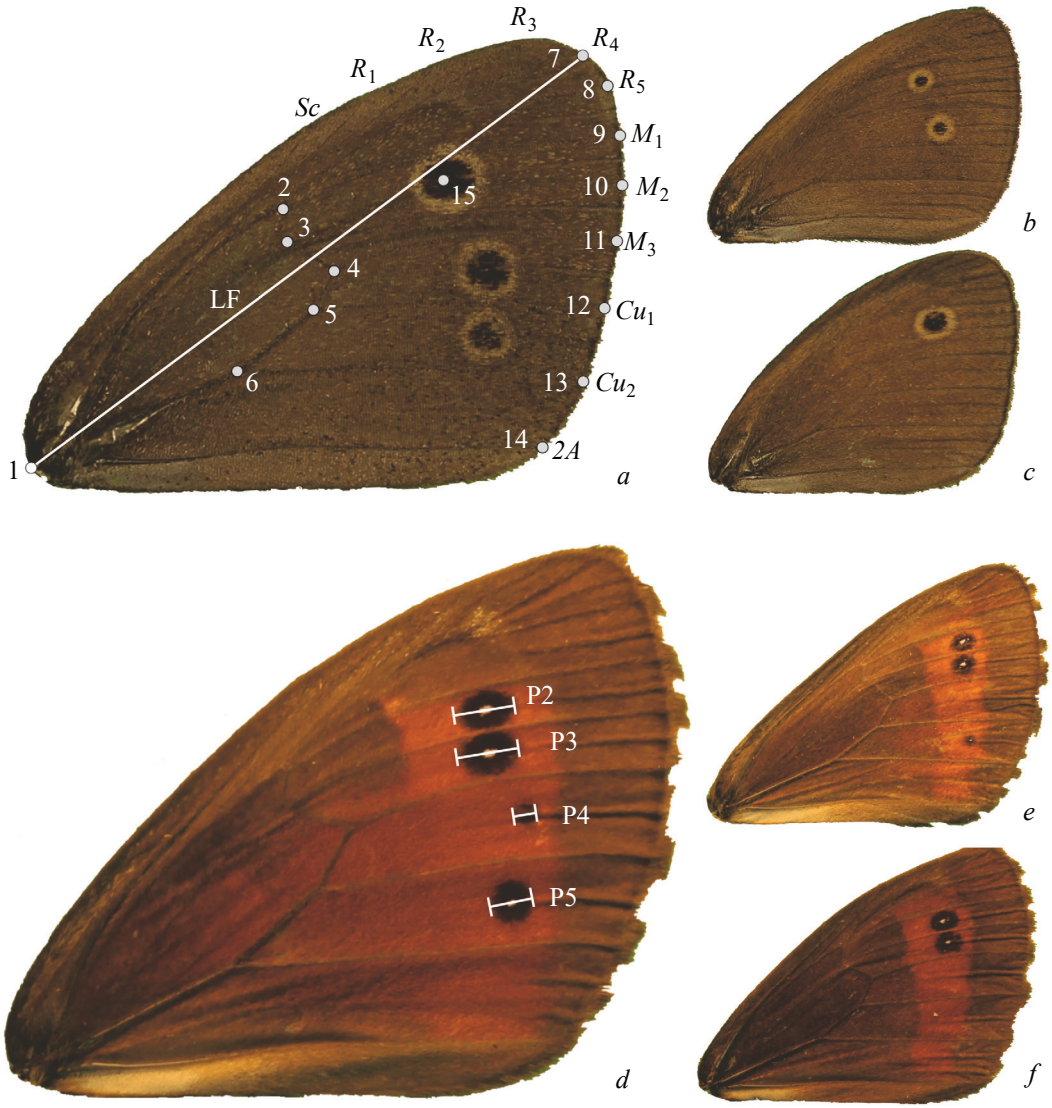

Fig. 2. Forewings of males: (*a–c*) *A. hyperantus*; (*d–f*) *E. ligea*: (*a*) scheme of landmarks and wing measurements (the landmarks are numbered 1–15; LF is the wing length); (*b*) wing pattern with 2 eyespots (P2, P4); (*c*) wing pattern with 1 eyespot (P2); (*d*) scheme of measuring the diameters of eyespots (P2, P3, P4, P5); (*e*) wing pattern with 3 eyespots (P2, P3, P5); (*f*) wing pattern with 2 eyespots (P2, P3).

Laboratory Processing of Material

In this work we used the samples of males of *A. hyperantus* and *E. ligea*. The univoltine species *A. hyperantus* was collected in the same years in both localities, while the bicyclic species *E. ligea* was collected in Karasye in even years and in Fomino in odd years. The amount of material and the years of collection are summarized in Table 1. All the samples are kept in the museum of the Institute of Plant and Animal Ecology, Ural Branch of the Russian Academy of Sciences (Yekaterinburg).

The variability of the forewing shape was studied by the methods of geometric morphometrics, which allow variation in shape to be assessed independently of size (Adams et al., 2004; Zelditch et al., 2004; Mitteroecker and Gunz, 2009). The detached left forewings were photographed from the underside with a Canon Eos 600D digital camera stabilized with a holder. A total of 15 landmarks were placed in the images using the tpsDig 2.29 software (Rolhf, 2017), as shown in Fig. 2, *a*. Landmarks 1–6 were placed along the outline of the medial cell, landmarks 7–14, along the outline of the

wing. Since the set of landmarks had to be identical for all the objects, no landmarks were placed on those eyespots which were absent in some specimens: P3, P4, and P5. Landmark 15 was placed in the focus (or the center, if there was no focus) of eyespot P2 in cell M_1–M_2; this was the only eyespot invariably present in the wing pattern of all the specimens of both species.

The wing length LF was measured between landmarks 1 and 7 (Fig. 2, *a*), using the TMorphGen6 program from the IMP package (Sheets, 2003). The diameters of eyespots P2, P3, P4, P5 in *E. ligea* and P2, P4, P5 in *A. hyperantus* were measured in the ImageJ 1.48v software (Rasband, 2014), as shown in Fig. 2, *d*.

Table 1. Size of samples of *E. ligea* and *A. hyperantus* males from two localities in Sysertskii District, Sverdlovsk Province: number of specimens

Year	*E. ligea*		*A. hyperantus*	
	Karasye	Fomino	Karasye	Fomino
2002	12	–	57	60
2003	–	30	6	55
2008	8	–	21	23
2009	–	30	27	48
2018	19	–	16	55
2019	–	53	14	55

Dash indicates that adult flight did not occur in the given year.

Statistical Methods

The variability of the wing length was studied using multifactorial ANOVA. The model included three factors: *species*, collection *locality*, and collection *year*. The original hypothesis, stating that populations of the bicyclic species *E. ligea* would be more isolated than those of the univoltine species *A. hyperantus*, was tested by estimating the statistical significance of interaction between the factors *species* × *locality*: significant interaction indicated that one species had a greater level of interpopulation variation than the other. The factor *year* was introduced to estimate year-to-year variation. Since the flight of *E. ligea* occurred in odd years in one locality and in even years in the other, interaction between the factor *year* and the two remaining factors could not be analyzed in the given model; instead, *year* was treated as a nested variable (Schielzeth and Nakagawa, 2013). The variability of the wing length LF was analyzed using the Statistica 8.0 software package (StatSoft Inc.), separately for each species.

The wing shape was studied separately for two sets of landmarks, the first set (landmarks 1–14) describing the shape of the wing outline and the venation pattern, and the second set (landmarks 2, 5, 7, 10, 14, and 15) characterizing the position of eyespot P2. In the generalized scheme of the nymphaloid wing pattern (Schwanwitsch, 1924; Nijhout, 1990), the eyespots are arranged in a row proximal to the row of parafocal elements, so that the position of one eyespot (for instance, P2) generally reflects the arrangement of all the eyespots.

Procrustes superimposition was carried out for each set of landmarks in order to eliminate the differences among the objects not related to shape (Zelditch et al., 2004; Vasil'ev et al., 2018). Then partial deformations were calculated based on the Procrustes coordinates in the Past 4.02 software (Hammer et al., 2001) and used for further comparative analysis. The hypothesis about the stronger isolation of populations in *E. ligea* as compared to *A. hyperantus* was tested by multifactorial MANOVA. The model design was the same as that described above for the wing length, with the difference that analysis included the whole set of partial deformation scores, i.e., the wing shape was treated as a multidimensional trait. Accordingly, we used MANOVA rather than ANOVA. In this model we also analyzed the influence of the factors *species*, *locality*, *year*, and interaction between the factors *species* × *locality*. Since the wing shape is a multidimensional trait, statistical significance of interaction between the factors *species* × *locality* was not sufficient to confirm the initial hypothesis. The measure of difference between two shapes in geometric morphometrics is the Procrustes distance (for detail, see Zelditch et al., 2004; Vasil'ev et al., 2018). However, two shapes showing the same total level of difference (expressed as the Procrustes distance) may differ in a variety of ways, for example, due to different landmarks making the greatest contribution to the total difference. Accordingly, in the cases when interaction between the factors *species* × *locality* was found to be significant, we additionally assessed the level of difference between the localities for both species. For this purpose, Procrustes distances between the samples from different localities for each species were calculated in MorphoJ 1.06d (Klingenberg, 2011); then

Table 2. Results of three-way MANOVA of variability of the wing shape in *A. hyperantus* and *E. ligea*

Factor	Wilks' λ	df1	df2	*p*
Species	0.29	24	549	< 0.01
Locality	0.92	24	549	< 0.01
Species × Locality	0.91	24	549	< 0.01
Year	0.29	336	6706	< 0.01

the sets of Procrustes distances for *E. ligea* and *A. hyperantus* were compared by the Mann–Whitney test. To visualize the differences in wing shape between the localities for each species, discriminant analysis was carried out in MorphoJ 1.06d.

The variability of eyespots in the wing pattern was studied as follows. First, the eyespots were treated as discrete nonmetric traits (phenes), and their frequencies in the samples were compared by the Pearson's χ^2 test, using the Past 4.02 software. Second, variation of the metric trait *sum of diameters* was analyzed by multifactorial ANOVA in the same way as when studying the wing length variation. The sum of diameters was calculated for eyespots P2 + P4 + P5 in *A. hyperantus* and for P2 + P3 + P4 + P5 in *E. ligea*. Third, the indices describing the eyespot size relative to the wing length were calculated: P2/LF, P4/LF, and P5/LF for *A. hyperantus* and P2/LF, P3/LF, P4/LF, and P5/LF for *E. ligea*. Statistical significance of differences between the samples in the set of indices was estimated separately for each species by canonical discriminant analysis, using the Statistica 8.0 package (StatSoft Inc.). The squared Mahalanobis distance D^2 was used as a measure of difference.

RESULTS

Variability of the Wing Length

Unlike the body mass, the wing length evidently remains constant during the adult life (except for cases of mechanical damage); at the same time, it depends on the conditions of preimaginal development. We can suppose that for oligophagous species developing on grasses, the availability of food does not vary from year to year and does not constitute a limiting factor. Interannual variation in size is most probably determined by the weather conditions during development of late-instar larvae and their growth before pupation. It was experimentally shown (Ryzhkova and Lopatina, 2016) that higher air temperatures led to a decrease in size of *A. hyperantus*; this result is consistent with the "temperature-size rule" (Atkinson, 1994). Under natural conditions the effects of individual ecological factors on the final size of the adults are difficult to assess, since all these factors act concurrently and interact with one another. Our regression analysis did not reveal any significant dependence of the wing length on such climate parameters as the mean temperatures and precipitation levels of May and June for the years of our research (based on archived data of the weather site http://www.pogodaiklimat.ru/ for Weather Station 28440 in Yekaterinburg). However, it is possible that analysis of more extensive data would demonstrate correlations between the adult body size and the weather parameters.

The wing length variation in males of both species is shown in Fig. 3. According to the ANOVA results, the effects of all the three tested factors proved to be significant: *species* ($F = 3822.5$, df = 1, $p < 0.01$), *locality* ($F = 25.7$, df = 1, $p < 0.01$), and *year* ($F = 6.7$, df = 14, $p < 0.01$), while interaction between the factors *species × locality* was non-significant ($F = 1.3$, df = 1, $p = 0.25$). Although it had been earlier shown that interannual variation in size could exceed the geographic differences (Zakharova and Tatarinov, 2016), in this study adults of both species from the environs of Fomino were found to be on average larger than those from the environs of Lake Karasye. The mean forewing length in males of *A. hyperantus* was 19.5 ± 0.1 mm in Fomino and 19.2 ± 0.1 mm in Karasye; that of *E. ligea* was 24.9 ± 0.1 mm in Fomino and 24.5 ± 0.1 mm in Karasye. Since interaction between the factors *species × locality* was non-significant, we may conclude that the populations from two closely positioned localities demonstrated a similar level of differences in wing length in both species.

Variability of the Wing Shape and the Eyespot Position

The results of MANOVA of the partial deformations describing the shape variability of the wing outline and the medial cell showed that the effects of all the factors were statistically significant (Table 2). Since interaction between the factors *species × locality* was also significant, we may conclude that differences in the wing

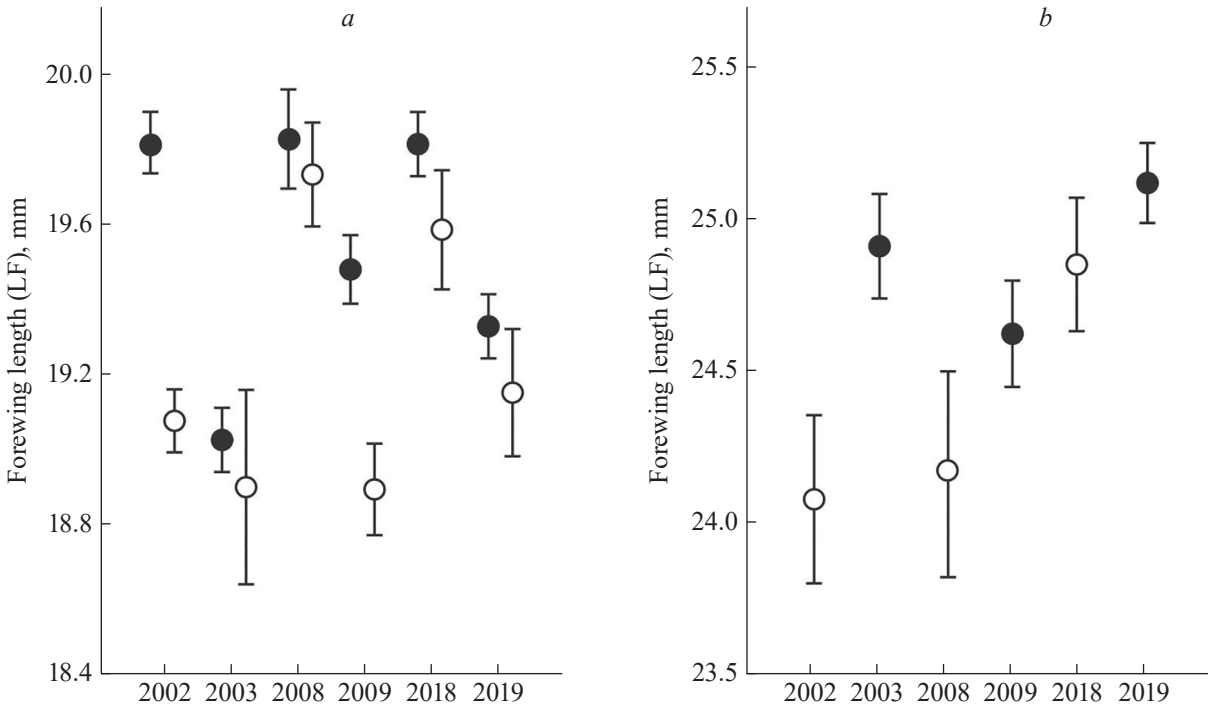

Fig. 3. Variability of the forewing length (LF) in samples of males from the environs of Fomino (black circles) and Karasye (white circles): (*a*) *A. hyperantus*; (*b*) *E. ligea*. The means and standard errors are shown.

shape between the two localities were not the same in *A. hyperantus* and *E. ligea*. The mean Procrustes distance describing the difference in wing shape between the samples from Fomino and Karasye was 0.014 in *A. hyperantus* and 0.018 in *E. ligea*. The results of the Mann–Whitney test (U = 77, $p < 0.05$) confirmed that adults from two sampling localities were significantly more different in *E. ligea* than in *A. hyperantus*.

The results of MANOVA of the partial deformations describing the variability of eyespot P2 position showed that the effects of all the factors were statistically significant (Table 3). Interaction between the factors *species* × *locality* was also significant; therefore, differences between the adults from two sampling localities in the position of eyespot P2 were not the same in *A. hyperantus* and *E. ligea*. As can be seen in Fig. 4, the vectors of landmark shifts were codirectional in the two species. In adults of both species from the environs of Lake Karasye, eyespot P2 and, correspondingly, the whole row of eyespots were positioned closer to the outer wing margin, as compared with the adults collected in the environs of Fomino. The mean difference between the localities in the position of eyespot P2, expressed as the Procrustes distance, was 0.021 in *A. hyperantus* and 0.032 in *E. ligea*. The results of the Mann–Whitney test (U = 63.5, $p < 0.05$) showed that the samples of *E. ligea* from two localities were more different than the corresponding samples of *A. hyperantus*.

Variability of the Eyespots

The wing pattern of all the studied *A. hyperantus* males included eyespot P2, and that of *E. ligea* males included eyespots P2 and P3. The remaining eyespots, i.e., P4 and P5, were found with different frequencies (Table 4). These eyespots were recorded significantly

Table 3. Results of three-way MANOVA of variability of eyespot P2 position in *A. hyperantus* and *E. ligea*

Factor	Wilks' λ	df1	df2	*p*
Species	0.40	8	565	< 0.01
Locality	0.88	8	565	< 0.01
Species × *Locality*	0.97	8	565	0.02
Year	0.63	112	3974	< 0.01

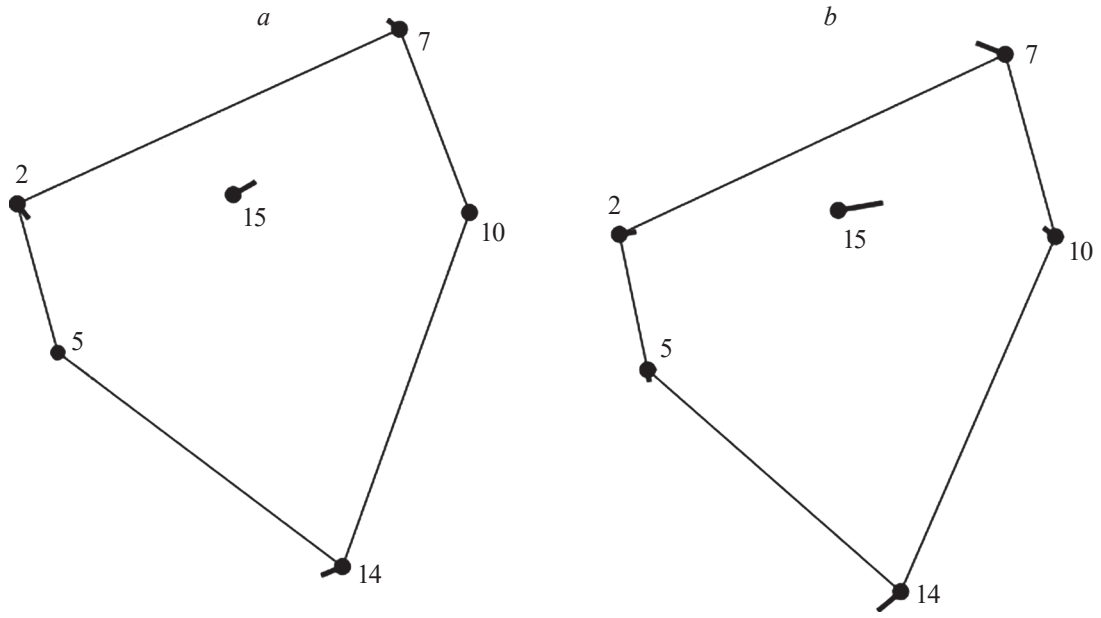

Fig. 4. Results of discriminant analysis of eyespot P2 position within the marginal wing area: (*a*) *A. hyperantus*; (*b*) *E. ligea*. The dots show the mean configuration for each species; the landmark shift vectors illustrate the differences between the mean configuration and the configuration describing the samples from Karasye.

more frequently in the samples of *A. hyperantus* from Fomino than in those from Karasye: $\chi^2 = 20.1$, df = 5, $p = 0.001$ for P4; $\chi^2 = 92.7$, df = 5, $p < 0.0001$ for P5. In *E. ligea*, differences between the samples from two localities were found only in the frequency or eyespot P4: $\chi^2 = 26.1$, df = 2, $p < 0.0001$.

The diameters of all the eyespots in the forewing pattern of both species are given in Table 4. The trend of the spot size coincided with that of the wing length, so that adults of both species from Fomino had larger eyespots than those from Karasye. The differences between the samples of *A. hyperantus* males in the trait *sum of diameters* were estimated by two-way ANOVA including the factors *locality* ($F = 19.3$, df = 1, $p < 0.0001$) and *year* ($F = 2.5$, df = 5, $p = 0.03$); interaction of these factors was non-significant. Analysis of *E. ligea* samples for the trait *sum of diameters* revealed a significant effect of the factor *year*: $F = 2.9$, df = 5, $p = 0.02$.

The variability of the relative size of eyespots in the male forewing patterns of both species was studied by canonical discriminant analysis, based on the calculated eyespot indices (eyespot diameter to wing length ratios). The results are shown in Fig. 5, in which the most different samples correspond to the centroids separated by the greatest distance in the space of canonical axes. For instance, *A. hyperantus* samples of 2018 from Fomino and Karasye were significantly different: $D^2 = 0.66$, $F = 2.12$, $p < 0.05$. However, on some occasions chronographic differences were greater than geographic ones: D^2 (Fomino 2002 vs Fomino 2009) = 0.92, $F = 8.1$, $p < 0.0001$; D^2 (Fomino 2008 vs Karasye 2008) = 0.38, $F = 1.38$, $p = 0.2$. The observed year-to-year variation may be explained by a number of reasons, including the different weather conditions, a small sample size, and also the fact that the butterflies were captured during different phases of the flight period. It is known that the phase of flight may contribute to the total variation in the metric traits of the wing pattern (Zakharova and Tatarinov, 2016). Comparison of the combined *A. hyperantus* samples for all the years revealed the following difference between the two localities: Wilks' $\lambda = 0.95$, $F = 7.00$, df1 = 3; df2 = 433; $p < 0.0001$.

The results of discriminant analysis of the eyespot indices in *E. ligea* are shown in Fig. 5, *b*. Certain geographic differences were detected along the second canonical axis, explaining 16.1% of the variance, but these differences were found to be statistically non-

Table 4. Diameter (mean ± standard error, mm) and frequency of occurrence of discrete eyespots in the forewing pattern of males of *E. ligea* and *A. hyperantus*

Species	Locality	Year	P2	P3	P4		P5		Sum of eyespot diameters
			mm	mm	mm	%	mm	%	
E. ligea	Karasye	2002	1.7 ± 0.1	1.8 ± 0.1	–	0.0	1.0 ± 0.1	91.7	4.4 ± 0.3
		2008	1.8 ± 0.1	1.9 ± 0.1	–	0.0	1.1 ± 0.2	87.5	4.6 ± 0.4
		2018	1.7 ± 0.1	1.8 ± 0.1	0.6 ± 0.4	5.3	1.1 ± 0.1	89.5	4.5 ± 0.3
	Fomino	2003	1.9 ± 0.1	1.9 ± 0.1	1.4 ± 0.2	9.7	1.4 ± 0.1	93.5	5.2 ± 0.2
		2009	1.8 ± 0.1	1.8 ± 0.1	0.5 ± 0.2	10.0	1.1 ± 0.1	80.0	4.5 ± 0.2
		2019	2.0 ± 0.1	1.9 ± 0.0	0.7 ± 0.1	17.0	1.3 ± 0.1	98.1	5.2 ± 0.2
A. hyperantus	Karasye	2002	1.8 ± 0.1	–	1.6 ± 0.1	78.9	1.1 ± 0.1	29.8	3.2 ± 0.2
		2003	1.6 ± 0.1	–	1.7 ± 0.3	66.7	1.2 ± 0.3	33.3	3.0 ± 0.5
		2008	1.9 ± 0.1	–	1.6 ± 0.1	90.5	0.8 ± 0.2	38.1	3.5 ± 0.3
		2009	1.7 ± 0.1	–	1.6 ± 0.1	88.9	1.1 ± 0.1	55.6	3.6 ± 0.2
		2018	1.7 ± 0.1	–	1.7 ± 0.2	81.3	0.9 ± 0.2	31.3	3.2 ± 0.3
		2019	1.8 ± 0.1	–	1.5 ± 0.2	92.9	1.2 ± 0.2	35.7	3.6 ± 0.3
	Fomino	2002	1.9 ± 0.1	–	1.5 ± 0.1	88.3	0.9 ± 0.1	45.0	3.6 ± 0.2
		2003	1.9 ± 0.1	–	1.6 ± 0.1	98.2	1.1 ± 0.1	63.6	4.0 ± 0.2
		2008	1.8 ± 0.1	–	1.7 ± 0.1	91.3	1.0 ± 0.1	52.2	3.8 ± 0.2
		2009	1.9 ± 0.1	–	1.8 ± 0.1	95.8	1.1 ± 0.1	70.8	4.3 ± 0.2
		2018	2.0 ± 0.1	–	1.7 ± 0.1	98.2	1.0 ± 0.1	56.4	4.1 ± 0.2
		2019	1.9 ± 0.1	–	1.8 ± 0.1	94.5	1.1 ± 0.1	67.3	4.2 ± 0.2

significant. Only two pairs of samples were significantly different: D^2 (Fomino 2003 vs Fomino 2009) = 0.76, $F = 2.8$, $p = 0.02$; D^2 (Fomino 2009 vs Fomino 2019) = 0.66, $F = 3.11$, $p = 0.02$.

DISCUSSION

When starting this research, we expected that under the conditions of a fragmented landscape the population of *A. hyperantus* would be phenotypically homogeneous, as opposed to that of *E. ligea*. This assumption was based on the specific features of ecology and life cycles of these species considered above.

However, the initial hypothesis about phenotypic homogeneity of *A. hyperantus* population was not confirmed. Analysis of phenotypic variability of the forewing in these two species by the methods of traditional and geometric morphometrics revealed significant differences between the micropopulations of both species in all the studied traits: the size and shape of the forewing and the parameters of eyespots in the wing pattern. The differences in a number of traits were found to be codirectional in the two species. At the same time, the level of phenotypic differentiation was higher in the specialist species *E. ligea* than in the generalist species *A. hyperantus*.

Adults of both species collected in Fomino were larger than those from the environs of Lake Karasye. In an earlier study, adults of *Coenonympha oedippus* (Fabricius, 1787) were found to be smaller in arid territories than in more humid ones (Jugovic et al., 2018). By contrast, according to our results, males of *A. hyperantus* and *E. ligea* were larger in the drier locality (Fomino) than in the environs of Lake Karasye. In our opin-

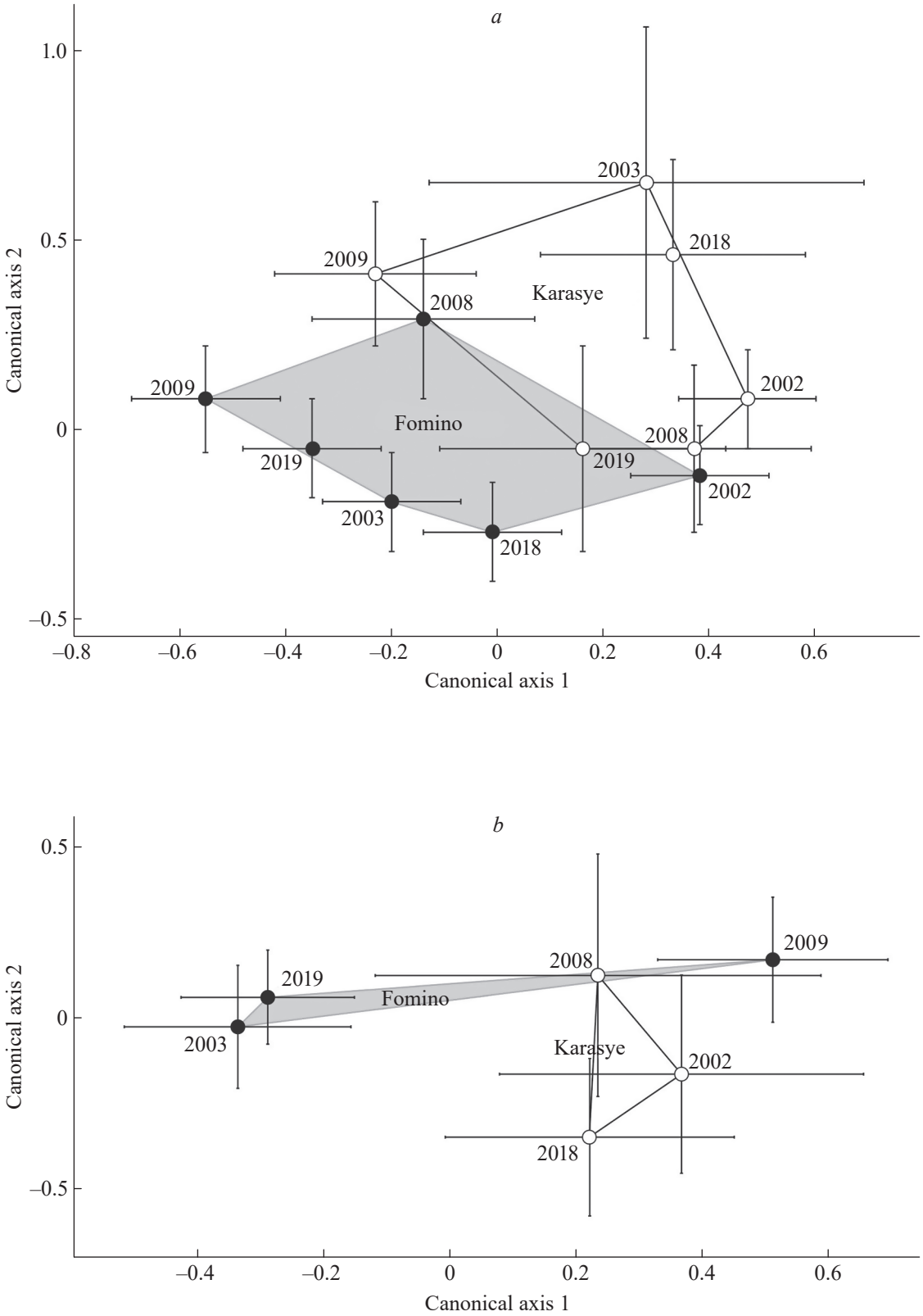

Fig. 5. Results of canonical discriminant analysis of the relative size of eyespots in the wing pattern of butterflies from two studied localities, Fomino (black circles) and Karasye (white circles): (*a*) *A. hyperantus*; (*b*) *E. ligea*. The sample centroids with standard errors are shown.

ion, the different humidity levels of the two localities most probably affect the body size of adult butterflies indirectly, via the trophic resources of their larvae, i.e., the species composition and relative abundance of grasses, the phytomass density, etc. However, testing this hypothesis would require a complex ecological study aimed at the most complete assessment of the microlandscape, climatic, and geobotanical parameters of the two sampling localities.

Adults of both species from Fomino had larger eyespots shifted toward the median cell, whereas adults from the environs of Lake Karasye had smaller eyespots positioned closer to the wing margin. In addition, individuals from the former locality on average had a greater total number of forewing eyespots due to more frequent occurrence of the discrete eyespots (phenes) P4 and P5. It is known that eyespots can perform various functions and, accordingly, can be selected for a smaller or greater size (Kodandaramaiah, 2011; Dapporto et al., 2018), and that the discrete eyespots in the wing pattern are epigenetically controlled threshold traits (Brakefield and van Noordwijk, 1985; Zakharova, 2010).

At the present stage of research it would be difficult to determine the exact factors and mechanisms underlying the observed morphological differences. In our opinion, the differences between the micropopulations, preserved over a relatively long period of time (about 20 years), indicate synchronous responses of the two species to the environmental and selective factors. Since morphological differences occurred on the local scale, between the localities positioned about 10 km apart, we may conclude that the fragmented environment constituted quite an effective barrier for these species and that the existing network of corridors did not allow complete mixing of individuals from the neighboring micropopulations. However, it remains an open question whether the observed differences between the micropopulations are stable and genetically or epigenetically fixed (i.e., the micropopulations have indeed become differentiated), or these differences are reproduced every year as similar phenotypic reactions to similar environmental conditions within a single population with limited movement of individuals.

Our results agree with the original assumption that the populations of the specialist species *E. ligea* are more strongly isolated that those of the generalist species *A. hyperantus*. However, we have studied only two species, and the observed differences between them may be coincidental. To confirm this conclusion in a more reliable way, a greater number of generalist–specialist pairs of species should be studied in different taxa of diurnal butterflies.

CONCLUSIONS

Thus, micropopulations sufficiently differentiated in the shape and size of the wing and in the position and stability of the eyespots were distinguished in both the species studied. Temporal and spatial isolation of the micropopulations creates preconditions for establishment of distinct forms, as it was demonstrated in the bicyclic specialist species *E. ligea*. The initial hypothesis about population homogeneity of the univoltine generalist species *A. hyperantus* in the studied territory was not confirmed. It was found out that spatial isolation might be sufficient for differentiation in a significantly sedentary species.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. All the applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All the procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

REFERENCES

Adakhovsky, D.A., An ecological characteristic of diurnal butterflies (Lepidoptera, Rhopalocera) of Udmurtia: the topical aspect, *Vestn. Udmurt. Univ*., 2014, no. 4, p. 44.

Adams, D.C., Rohlf, F.J., and Slice, D.E., Geometric morphometrics: ten years of progress following the "revolution," *Ital. J. Zool.*, 2004, vol. 71, p. 5.

Atkinson, D., Temperature and organism size – a biological law for ectotherms? *Adv. Ecol. Res.*, 1994, vol. 25, p. 1.

Beldade, P. and Peralta, C.M., Developmental and evolutionary mechanisms shaping butterfly eyespots, *Curr. Opin. Insect Sci.*, 2017, vol. 19, p. 22.

Billeter, R., Sedivy, I., and Diekötter, T., Distribution and dispersal patterns of the ringlet butterfly (*Aphantopus hyperantus*) in an agricultural landscape, *Bull. Geobot. Inst. ETH*, 2003, vol. 69, p. 45.

Brakefield, P.M. and van Noordwijk, A.J., The genetics of spot pattern characters in the meadow brown butterfly *Maniola jurtina* (Lepidoptera: Satyrinae), *Heredity*, 1985, vol. 54, p. 275.

Breuker, C.J., Gibbs, M., van Dongen, S., Merckx, T., and van Dyck, H., The use of geometric morphometrics in studying butterfly wings in an evolutionary ecological context, in *Morphometrics for Nonmorphometricians*, Berlin-Heidelberg: Springer, 2010, p. 271.

Cardoso, P., Barton, P.S., Birkhofer, K., Chichorro, F., Deacon, C., et al., Scientists' warning to humanity on insect extinctions, *Biol. Conserv.*, 2020, vol. 242, art. 108426. https://doi.org/10.1016/j.biocon.2020.108426

Cassel-Lundhagen, A., Tammaru, T., Windig, J.J., Ryrholm, N., and Nylin, S., Are peripheral populations special? Congruent patterns in two butterfly species, *Ecography*, 2009, vol. 32, p. 591.

Cassel-Lundhagen, A., Schmitt, T., Wahlberg, N., Sarvašová, L., Konvička, M., Ryrholm, N., and Kaňuch, P., Wing morphology of the butterfly *Coenonympha arcania* in Europe: Traces of both historical isolation in glacial refugia and current adaptation, *J. Zool. Syst. Evol. Res.*, 2020, vol. 58, p. 929. https://doi.org/10.1111/jzs.12360

Dapporto, L., Hardy, P.B., and Dennis, R.L.H., Evidence for adaptive constraints on size of marginal wing spots in the grayling butterfly, *Hipparchia semele*, *Biol. J. Linn. Soc.*, 2018, vol. 20, p. 1.

Gorbach, V.V., Intrapopulation variability of the wing pattern elements in the ringlet, *Aphantopus hyperantus* (Lepidoptera, Satyridae), *Uch. Zap. Petrozavodsk. Gos. Univ.*, 2012, no. 6, p. 27.

Gorbach, V.V., Fauna and ecology of butterflies (Lepidoptera: Hesperioidea et Papilionoidea) of Eastern Fennoscandia, *Extended Abstract of Doctoral Dissertation in Biology*, Petrozavodsk, 2014.

Gorbunov, P. and Kosterin, O., *The Butterflies (Hesperioidea and Papilionoidea) of North Asia (Asian Part of Russia) in Nature, Vol. 2*, Moscow: Rodina & Fodio, 2007.

Grill, A., Polic, D., Guariento, E., and Fiedler, K., Permeability of habitat edges for Ringlet butterflies (Lepidoptera, Nymphalidae, *Erebia* Dalman 1816) in an alpine landscape, *Nota Lepidopterol.*, 2020, vol. 43, p. 29.

Gutzwiller, K.J., Ed., *Applying Landscape Ecology in Biological Conservation*, N.Y.: Springer, 2002.

Habel, J.C., Trusch, R., Schmitt, T., Ochse, M., and Ulrich, W., Long-term large-scale decline in relative abundances of butterfly and burnet moth species across south-western Germany, *Sci. Rep.*, 2019, vol. 9, art. 14921. https://doi.org/10.1038/s41598-019-51424-1

Habel, J.C., Ulrich, W., and Schmitt, T., Butterflies in corridors: quality matters for specialists, *Insect Conserv. Diversity*, 2020, vol. 13, p. 91.

Hammer, Ø., Harper, D.A.T., and Ryan, P.D., PAST version 2.17. Paleontological Statistics software package for education and data analysis, *Palaeontol. Electron.*, 2001, vol. 4, no. 1, p. 1.

Hanski, I., *Metapopulation Ecology*, N.Y.: Oxford University Press, 1999.

Jugovic, J., Zupan, S., Bužan, E., and Čelik, T., Variation in the morphology of the wings of the endangered grass-feeding butterfly *Coenonympha oedippus* (Lepidoptera: Nymphalidae) in response to contrasting habitats, *Eur. J. Entomol.*, 2018, vol. 115, p. 339.

Klingenberg, C.P., MorphoJ: an integrated software package for geometric morphometrics, *Mol. Ecol. Resour.*, 2011, vol. 11, p. 353.

Kodandaramaiah, U., The evolutionary significance of butterfly eyespots, *Behav. Ecol.*, 2011, vol. 22, p. 1264.

Komonen, A., Grapputo, A., Kaitala, V., Kotiaho, J., and Paivinen, J., The role of niche breadth, resource availability and range position on the life history of butterflies, *Oikos*, 2004, vol. 105, p. 41.

Marcus, J.M., Evo-Devo of butterfly wing patterns, in *Evolutionary Developmental Biology*, Nuno de la Rosa, L. and Müller, G., Eds., Cham: Springer, 2019, p. 1.

Mitteroecker, P. and Gunz, P., Advances in geometric morphometrics, *Evol. Biol.*, 2009, vol. 36, p. 235.

Mukhin, V.A., Tretyakova, A.S., Teptina, A.Yu., Kutlunina, N.A., Zimnitskaya, S.A., Goncharova, Yu.V., Yudin, M.M., and Berezina, A.Ya., *Flora i rastitel'nost' biologicheskoi stantsii Ural'skogo gosudarstvennogo universiteta: uchebnoe posobie po letnei polevoi praktike dlya studentov biologicheskogo fakul'teta* (Flora and Vegetation of the Biological Station of the Ural State University: A Study Guide for the Summer Field Practical Course at the Faculty of Biology), Yekaterinburg: Ural. Univ., 2003.

Nijhout, H.F., A comprehensive model for colour pattern formation in butterflies, *Proc. R. Soc. London B*, 1990, vol. 239, p. 81.

Novozhenov, Yu.I., Chronographic variability in a population, *Zh. Obshch. Biol.*, 1989, vol. 50, no. 2, p. 171.

Novozhenov, Yu.I., Sexual dimorphism in the color polymorphism of some insect species, *Zh. Obshch. Biol*., 1997, vol. 58, no. 1, p. 26.

Paučulova, L., Dzurinka, M., Semeláková, M., Csanády, A., and Panigaj, L., Phylogeography, genetic structure and wing pattern variation of *Erebia pronoe* (Esper, 1780) (Lepidoptera: Nymphalidae) in Europe, *Zootaxa*, 2018, vol. 4441, no. 2, p. 279.

Pogoda i klimat (Weather and Climate), 2020 (accessed 1.06.2020).
http://www.pogodaiklimat.ru/weather.php?id=28440

Pollard, E. and Yates, T.J., *Monitoring Butterflies for Ecology and Conservation*, London: Chapman and Hall, 1993.

Poniatowski, D., Stuhldreher, G., Löffler, F., Fartmann, T., Patch occupancy of grassland specialists: habitat quality matters more than habitat connectivity, *Biol. Conserv.*, 2018, vol. 225, p. 237.

Powney, G.D., Broaders, L.K., and Oliver, T.H., Towards a measure of functional connectivity: local synchrony matches small scale movements in a woodland edge butterfly, *Landscape Ecol.*, 2012, vol. 27, p. 1109.

Rasband, W.S., ImageJ: Image Processing and Analysis in Java, 2014 (accessed 2.08.2020).
https://imagej.nih.gov/ij/

Rohlf, F.J., TpsDig version 2.29, 2017 (accessed 2.08.2020).
https://life.bio.sunysb.edu/morph/

Roth, T., Strebel, N., and Amrhein, V., Estimating unbiased phenological trends by adapting site-occupancy models, *Ecology*, 2014, vol. 95, no. 8, p. 2144.

Ryzhkova, M.V. and Lopatina, E.B., The seasonal development cycle of *Aphantopus hyperantus* (L.) (Lepidoptera, Nymphalidae: Satyrinae) in Leningrad Province, *Entomol. Rev*., 2016, vol. 96, no. 7, p. 831.

Saarinen, K. and Jantunen, J., Grassland butterfly fauna under traditional animal husbandry: contrasts in diversity in mown meadows and grazed pastures, *Biodiversity Conserv.*, 2005, vol. 14, p. 3201.

Saarinen, K., Valtonen, A., Jantunen, J., and Saarnio, S., Butterflies and diurnal moths along road verges: does road type affect diversity and abundance? *Biol. Conserv.*, 2005, vol. 123, p. 403.

Sanzana, M.-J., Parra, L.E., Sepúlveda-Zúňiga, E., and Benítez, H.A., Latitudinal gradient effect on the wing geometry of *Auca coctei* (Guérin) (Lepidoptera, Nymphalidae), *Rev. Bras. Entomol.*, 2013, vol. 57, no. 4, p. 411.

Schielzeth, H. and Nakagawa, S., Nested by design: model fitting and interpretation in a mixed model era, *Methods Ecol. Evol.*, 2013, vol. 4, p. 14.

Schneider, C. and Fry, G., The influence of landscape grain size on butterfly diversity in grasslands, *J. Insect Conserv.*, 2001, vol. 5, p. 163.

Schwanwitsch, B.N., On the groundplan of the wing pattern in nymphalids and certain other families of rhopalocerous Lepidoptera, *Proc. Zool. Soc. London Ser. B*, 1924, vol. 34, p. 509.

Sekimura, T. and Nijhout, H.F., Eds., *Diversity and Evolution of Butterfly Wing Patterns. An Integrative Approach*, Singapore: Springer, 2017.

Settele, J., Kudrna, O., Harpke, A., Kühn, I., van Swaay, C., et al., *Climatic Risk Atlas of European Butterflies*, Sofia–Moscow: Pensoft, 2008.

Sheets, H., IMP: Integrated Morphometrics Package, 2003 (accessed 17.06.2021).
https://www.animal-behaviour.de/imp/

Shkurikhin, A.O. and Oslina, T.S., Seasonal variation in the forewing of polyvoltine whites *Pieris rapae* L. and *P. napi* L. (Lepidoptera: Pieridae) in the forest-steppe zone of the Southern Urals, *Russ. J. Ecol.*, 2016, no. 3, p. 296.

Sutcliffe, O.L., Thomas, C.D., and Peggie, D., Area-dependant migration by ringlet butterflies generates a mixture of patchy population and metapopulation attributes, *Oecologia*, 1997, vol. 109, p. 229.

Valtonen, A. and Saarinen, K., A highway intersection as an alternative habitat for a meadow butterfly: effect of mowing, habitat geometry and roads on the ringlet (*Aphantopus hyperantus*), *Ann. Zool. Fenn.*, 2005, vol. 42, p. 545.

Van Swaay, C.A.M., Warren, M.S., and Lois, G., Biotope use and trends of European butterflies, *J. Insect Conserv.*, 2006, vol. 10, p. 189.

Vasil'ev, A.G., Vasil'eva, I.A., and Shkurikhin, A.O., *Geometricheskaya morfometriya: ot teorii k praktike* (Geometric Morphometrics: from Theory to Practice), Moscow: KMK Scientific Press, 2018.

Viljur, M-L. and Teder, T., Disperse or die: Colonisation of transient open habitats in production forests is only weakly dispersal-limited in butterflies, *Biol. Conserv.*, 2018, vol. 218, p. 32.

Warren, B.C.S., *Monograph of the Genus Erebia*, London: British Museum of Natural History, 1936.

Zakharova, E.Yu., The use of variation spectra in analysis of heterogeneity in natural populations, by the example of *Aphantopus hyperantus* L. (Lepidoptera, Satyridae), in *Bespozvonochnye zhivotnye Yuzhnogo Zaural'ya i sopredel'nykh territorii. Materialy Vseros. konf., 14–16 aprelya 1998 g.* (Invertebrates of the Southern Trans-Ural Region and Adjacent Territories: Proc. of All-Russian Conf., April 14–16, 1998), Kurgan: Kurgan. Univ., 1998, p. 146.

Zakharova, E.Yu., Phenotypic variability of eyespots as homologous wing pattern elements in satyrs (Lepidoptera: Nymphalidae: Satyrinae), in *Biodiversity and Dynamics of Ecosystems in North Eurasia. Section "Diversity of the fauna of North Eurasia" (August 21–26, 2000), Vol. 3, Part 1*, Novosibirsk: IC&G, 2000, p. 113.

Zakharova, E.Yu., Seasonal variability of the wing pattern in *Erebia ligea* L. (Lepidoptera: Satyridae) in the Middle Urals, in *Sovremennye problemy evolyutsii (sbornik dokladov): XXII Lyubishchevskie chteniya* (Current Problems of Evolution: Lyubishchev Memorial Lectures, Issue 22), *Vol. 2*, Ulyanovsk: Ulyanovsk. Gos. Ped. Univ., 2008, p. 30.

Zakharova, E.Yu., Seasonal variability of wing length and eyespots in populations of *Erebia ligea* (L.) (Lepidoptera, Satyridae) in the Middle Urals, *Entomol. Rev*., 2010, vol. 90, no. 6, p. 669.

Zakharova, E.Yu. and Shkurikhin, A.O., Wing morphological variation in *Erebia ligea* (Linnaeus, 1758) (Lepidoptera: Satyridae) bicyclic and univoltine populations in the Urals, Russia, *Euroasian Entomol. J.*, 2017, vol. 16, no. 4, p. 344.

Zakharova, E.Yu. and Tatarinov, A.G., A chronogeographic approach to analysis of variability in a bicyclic species *Erebia ligea* (L.) (Lepidoptera: Satyridae) in the Urals, *Sib. Ekol. Zh*., 2016, no. 3, p. 322.

Zelditch, M.L., Swiderski, D.L., Sheets, H.D., and Fink, W.L., *Geometric Morphometrics for Biologists: a Primer*, New York: Elsevier, 2004.