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Large-scale spatial variation and phenotypic integration in three *Argynnini* species inform about functions and evolutionary drivers of butterfly wings

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Understanding how large-scale environmental variability may shape the distribution of phenotypic variation remains central to evolutionary biology. Across-species comparisons of trait variation alongside environmental gradients may offer valuable insights into how different species may respond to similar selective pressures. We conducted a comparative morphological study (>32° latitude and >47° longitude) on three closely related *Argynnini* butterfly species, *Speyeria aglaja*, *Fabriciana adippe*, and *F. niobe*. We measured wing size and coloration to assess (1) whether they respond similarly or differently to environmental factors (longitude, latitude, altitude, temperature, precipitation, solar radiation, wind speed); (2) if these factors correspond with those associated with the species' genetic structure based on a previous study; and (3) whether correlations between phenotypic traits within individuals are species-specific. We found common and species-specific associations of climatic (precipitation, wind speed) and geographic (longitude, altitude) factors with the composite phenotypic variation. Wing size was associated with different variables in the studied species, while melanisation mainly increased in cooler regions in all species, suggesting that the need for temperature regulation is a strong selective pressure on melanisation. Wing size was associated with the genetic structure in all species, highlighting the functional importance of this trait. The environmental drivers associated with the phenotypic variation in *S. aglaja* and *F. adippe* were largely the same as those associated with their genetic structure, hinting at a genetic underpinning of the observed morphological variation due to local adaptation. We report some distinct intraspecific trait correlations in *S. aglaja* and *F. adippe*, indicative of independent phenotypic integration. These phenotypes seem to be associated with protection against predators and thermal regulation in the respective habitats of both species, suggesting that similar selective pressures have resulted in the evolution of different trait combinations. Some of the inter-specific differences could be related to diverging niche breadths and dispersal capacities, exemplifying that the evolution of trait integration and spatial phenotypic differentiation may differ between closely related species with overlapping distribution ranges. Our findings highlight the importance of comparative assessments of variation, and demonstrate that the relative effects of drivers of variability may vary between sister species.

KEYWORDS

evolution, geography, melanism, phenotypic integration, thermal environment, wing coloration, wing size

Introduction

There is a seemingly boundless variation in nature among species, populations, and individuals, and to find and explain patterns of biodiversity has been of long-standing interest in evolutionary biology (Darwin, 1859; Wiens, 2015; Rangel et al., 2018). By selecting for characteristics that increase ecological and evolutionary success of individuals, populations, and species, the large-scale variation in environmental conditions can influence the geographic distribution of trait variation across species (Violle et al., 2014). Large-scale comparisons of species' variability in phenotypic characters along environmental gradients thus offer an opportunity to understand the selection pressures involved to explore whether species with different life-histories and legacies show shared or unique responses. If species respond similarly to prevalent environmental conditions, phenotypic patterns might be spatially analogous in sympatric congeneric species as a result of convergent evolution or shared developmental plasticity responses (Losos, 2011; Stern, 2013; Forsman, 2015). Conversely, different spatial patterns and associations of phenotypes with environmental variables in distinct species might either point to species-specific adaptations or plasticity responses owing to differences in ecology and genetic make-up, or reflect important roles of neutral evolution associated with stochastic effects such as genetic drift and founder events (Lande, 1976, 1980; Huynen, 1996; Bahrndorff et al., 2006; Tinnert and Forsman, 2017; Vargas et al., 2017; Qu et al., 2021). Further, distinct phenotypic traits within a species do not develop and evolve independently of each other (Forsman, 2015). This is in part because correlational selection favors individuals with certain combinations of trait values over individuals with other trait value combinations, and this promotes the evolution of genetic correlations and phenotypic integration, aka complex phenotypes, with the optimal solutions depending on environmental conditions and selective regimes (Brodie, 1992; Forsman and Appelqvist, 1998; Forsman et al., 2002; Hagman and Forsman, 2003; McKinnon and Pierotti, 2010; Forsman, 2018; Dingemans et al., 2020). Comparing variation in different phenotypic dimensions across hierarchical levels of organization, and their associations with environmental factors may thus allow for evaluating the interacting roles of external conditions and intrinsic species characteristics for population differentiation and evolutionary modifications of phenotypic integration.

Butterflies are well suited for assessing associations of phenotypes with environmental and geographic factors within and across species. Their wings consist of arrays of colored scales, come in numerous patterns, colors, shapes, and sizes, and have many important functions with ample opportunity for evolutionary modifications. They are important features used for flight, and variation in their shapes and color patterns are involved in inter- and intraspecific visual communication, temperature regulation, protection from UV-radiation, and predator avoidance (Betts and Wootton, 1988; Scoble, 1992; Brunton and Majerus, 1995; Tsai et al., 2020). There is a tremendous variation in wing phenotypes among species, among populations within species, between sexes, among individuals within populations (e.g., polymorphism), and even within individuals (e.g., between fore and hind wings, between dorsal and ventral sides).

Because of the diverse functional roles of butterfly wings, different selective pressures may have contributed to the immense variety in

lepidopteran wing morphology. For example, the variation in the degree of melanism might depend on the ambient temperature and solar radiation, as melanisation is important for thermoregulation and protection against UV-light (Clench, 1966; Matute and Harris, 2013; Heidrich et al., 2018). Melanisation can also increase pathogen resistance in insects, such that darker individuals might be more effective in encapsulating parasitoids, and more resistant against viral and fungal infection (Wilson et al., 2001; True, 2003; Mikkola and Rantala, 2010). Green color scales may reduce predation by providing camouflage while perching on leaves or grass, and aid intraspecific communication, as in *Callophrys rubi* (Michielsen et al., 2010). Structural iridescent scales might act as protection against high UV-radiation, constitute a water repellent, and strengthen the wing structure (Potyrailo et al., 2007; Zheng et al., 2007; Fang et al., 2015), and they are also involved in signaling and communicating during species recognition and mate choice (Brunton and Majerus, 1995; Brunton, 1998; Kemp, 2008). Variation in wing size is also influenced by both biotic and abiotic factors. For example, butterfly populations in areas with longer vegetation periods might have larger wings resulting from a prolonged period of larval feeding and growth (Chown and Gaston, 2010; Zhang et al., 2019). Additional factors believed to influence the size of butterflies are temperature, solar insolation, and the presence of predators (Zeuss et al., 2017; Le Roy et al., 2019). Body size—in butterflies traditionally accounted for by wing size—has previously been associated with variation in flight capacity in insects (Nieminen et al., 1999; Öckinger et al., 2010; Kuussaari et al., 2014), and habitat fragmentation, connectivity, and other environmental stressors acting on mobility might therefore contribute to the diversification of wing length in butterflies. Most previous investigations have attempted to evaluate the contribution and responses to these potential sources of variation using spatial and temporal comparisons of single species. While such approaches can generate important insights, multispecies comparisons provide added value by allowing for the assessment of reproducibility and generality of research findings (Voelkl et al., 2020; Polic et al., 2022).

Fritillary butterflies (i.e., Nymphalidae butterflies with mostly checkered wing patterns) in the tribe *Argynnini* offer suitable model systems for studying and comparing associations of wing morphology with climatic and geographic factors within and among closely related species that are exposed to similar environmental conditions. *Speyeria aglaja*, *Fabriciana adippe*, and *F. niobe* are morphologically similar and have partly overlapping distribution ranges, yet they differ in their degree of ecological generalism, habitat preferences, host plant use, and mobility patterns (Forster and Wohlfahrt, 1955; Higgins and Riley, 1970; Komonen et al., 2004; Eliasson et al., 2005; Fric et al., 2005; Öckinger et al., 2006; Polic et al., 2020). A previously published large-scale comparative study of genetic structure in these three *Argynnini* species (Polic et al., 2022) reports on species-specific associations of geographical and climatic factors with genetic variation. We hypothesize that the same combinations of these environmental variables might also be linked to the large-scale morphological trait distributions. Additionally, individuals from regions that constituted far diverged genetic groups in the phylogeographic comparative study might also be morphologically distinct from other studied regions.

Here, we quantified, characterized, and compared phenotypic (*viz.* morphological) variation in these three *Argynnini* species based on data collected across a large spatial scale that spanned more than

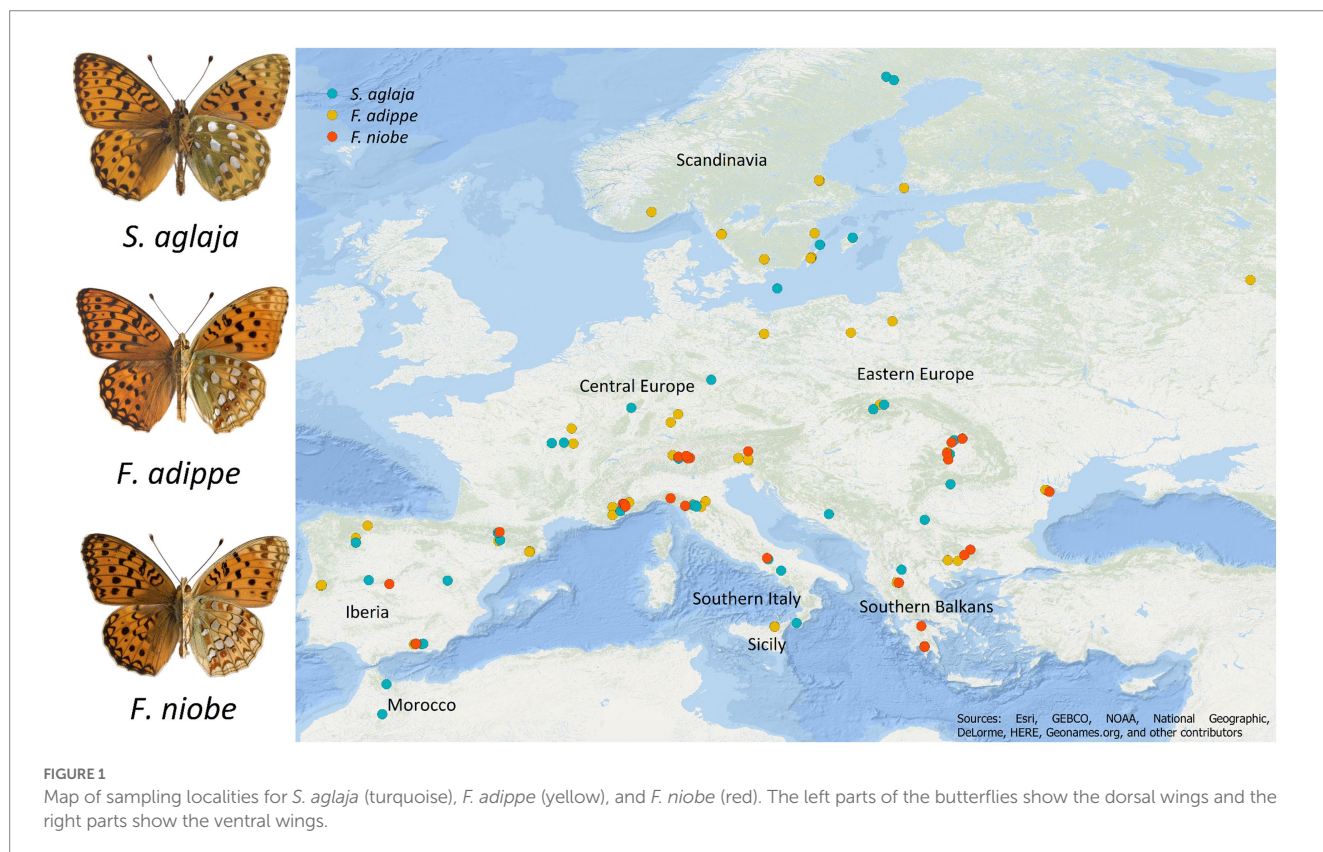
32 degrees latitude and more than 47 degrees longitude (Figure 1), with the overarching aim to identify and compare eco-evolutionary drivers of spatial differentiation and phenotypic integration. To achieve this, we set out to answer the following specific questions. (i) Do different species respond similarly to environmental conditions, such that the spatial variations in wing color patterns and wing size, and their associations with geographic and climatic variables are parallel in the three studied species? Or is the spatial variation in phenotypic traits seen in one species independent of the spatial variation seen in another species? (ii) Is the variation in different phenotypic dimensions (wing size, degree of melanisation, and the amount of white and green color on the wings) independent or correlated within individuals (i.e., between fore and hind wings, or between dorsal and ventral sides of the wings), and are the correlations between different traits species-specific or shared between species? Finally, (iii) Do results from these large-scale morphological analyses parallel findings from a recent comparative phylogeographic study on essentially the same individuals (Polic et al., 2022), such that the same geographic and climatic variables are associated with genetic and phenotypic distribution of variation in the respective species?

Materials and methods

Study species

Speyeria aglaja is a rather widespread, and often abundant species, inhabiting wet and dry grasslands, woodland meadows, coastal areas, and scrublands (Eliasson et al., 2005; Ellis et al., 2010). The species'

distribution area comprises Northern Africa, Europe, Russia, and Southeast Asia, reaching as far north as Southern Lapland (Fox et al., 2011; Tolman and Lewington, 2012). The two *Fabriciana* species do not occur in the Maghreb and are not distributed as far north as *S. aglaja*, possibly due to a higher tolerance of colder temperatures in the latter species (Zimmermann et al., 2009; Ellis et al., 2010). *Fabriciana adippe* reaches as far north as central Sweden (Warren, 1995; Franzén and Johannesson, 2007; Fox et al., 2011; Tolman and Lewington, 2012), while *F. niobe*'s northern range limit is southern Sweden (van Swaay and Warren, 1999; Eliasson et al., 2005; Reinhardt and Bolz, 2011). *Fabriciana adippe* is relatively constrained to open areas within woodland landscapes, and is facing steep declines in some parts of northern Europe, such as the UK (Warren, 1995; Zimmermann et al., 2009; van Swaay et al., 2010; Fox et al., 2011; Ellis et al., 2019), while *F. niobe* prefers nutrient-poor warm micro-habitats within grasslands with a high abundance of *Viola* spp. from sea-level up to sub-alpine altitudes (Salz and Fartmann, 2009; Spitzer et al., 2009; Salz and Fartmann, 2017). During the last decades, *F. niobe* has strongly declined throughout central Europe, and is now considered endangered in some countries, e.g., Germany (van Swaay and Warren, 1999; Reinhardt and Bolz, 2011). Although the overall physical appearance in these *Argynnini* species is similar, there are some differences in wing patterns both between and within species. *Fabriciana adippe*, and *F. niobe*, for example, exhibit an analogous intraspecific color polymorphism, both species having one morph with silvery reflective dots on the ventral side of the hindwings (*F. adippe* f. *adippe*, and *F. niobe* f. *niobe*), and another morph where these dots are yellow (*F. adippe* f. *cleodoxa*, and *F. niobe* f. *eris*) (Eliasson et al., 2005; Tolman and Lewington, 2012). In both species,



the silvery morphs are dominating in the northern parts of the distribution area, whereas the yellow morphs gradually replace the nominate forms in the southern ranges, especially in the Southern Balkans (Coutsis and Ghavalás, 2001; Tolman and Lewington, 2012). Contrastingly, *S. aglaja* is a monomorphic species, morphologically similar to the silvery morphs in the other two species. The dorsal wing sides in all three species are orange with dark (brown/black) spots and they display green scales on the underside of the hindwings (Eliasson et al., 2005; Tolman and Lewington, 2012). A large-scale comparative phylogeography study revealed that the genetic structure in these three species was linked to distinct combinations of climatic and geographic factors, indicative of species-specific responses to shared environments (Polic et al., 2022).

Study area and data collection

We selected 174 *Argynnini* individuals, viz. 53 *S. aglaja*, 85 *F. adippe* (including both morphs *F. adippe f. adippe*, and *F. adippe f. cleodoxa*), and 36 *F. niobe* (including both morphs *F. niobe f. niobe*, and *F. niobe f. eris*) from butterfly collections at the Institut de Biologia Evolutiva (IBE), CSIC-Universitat Pompeu Fabra in Barcelona and the Department of Biology and Environmental Science, Linnaeus University in Kalmar, covering a wide range of locations within their European (and Moroccan for *S. aglaja*, as the other two species do not inhabit the Maghreb) distribution areas (33.542–65.856° latitude, and –8.227–38.875° longitude, Figure 1). Sampling collection took place between 2008 and 2017 and coordinates for each locality were recorded directly in the field. After capture, the wings were either directly cut from the dead specimens and dried, or the whole specimens were dried and wings were cut just before taking pictures for morphological analyses of coloration and size (see below).

Quantification of wing size and color patterns

The butterfly wings were analysed according to coloration and size. For each individual, we placed the ventral side of the left forewing and hindwing and the dorsal side of the right forewing and hindwing (4 wing parts) on a standard white paper sheet, together with a color card and a ruler (Figure 2). Pictures were then taken with a Nikon D3000 camera with an 18–55 mm lens without flash using the following camera settings: focal length 35 mm, ISO 400, shutter speed 1/200 s, aperture *f*/4.5. Each picture was taken in the exact same position with 34 cm distance between the camera lens and the wings. Light was provided by a lamp with a Compact Fluorescent Bulb (FPL 18 W 5400 K) in a fixed position. The same person took all photographs in the IBE laboratory in Barcelona. The pictures were analysed according to wing coloration and wing size using the image analysis software GIMP 2 (The GIMP Development Team, 2019). For each respective wing and side, the pixels containing a specific color were selected using the *select by color* tool with a threshold of 15. Wing colors were assigned into separate categories of interest, “dark brown” (#4a3822), “black” (#0a0907), “white” (#f2f4e7), “yellow” (#c5a035), “light green” (#7b7f20), and “dark green” (#565d17), and the number of pixels covered by each respective color was measured. By measuring the total amount of colored pixels in each wing, the percentage of each color in the wing and respective side was



FIGURE 2

Placement of wings for size and color measurements, here in *F. adippe f. adippe*. Forewing size was measured as the straight-line distance from the base to the apex of the forewing and hindwing size from the hindwing base to the tip of the median vein M3.

calculated. The degree of melanism in each individual was defined as the total percentage of black and dark brown pixels of both dorsal and ventral side of both fore and hind wing. Dorsal and ventral melanism were defined as the total percentages of black and dark brown pixels of dorsal and ventral sides, respectively, of both fore- and hindwing. Similarly, we calculated forewing and hindwing melanism. We added the percentages of light green and dark green in the underside of the hindwing and named the variable green. White hereafter refers to the percentage of white in the underside of the hindwing (silvery white spots only occur in the underside of the hindwings in *S. aglaja*, *F. adippe f. adippe*, and *F. niobe f. niobe*). Forewing size was measured as the linear distance from the base to the apex of the forewing, and hindwing size from the hindwing base to the tip of the median vein M3 (Comstock and Needham, 1898) by using the ruler tool in GIMP 2.

Geographic locations and climatic conditions

For each sampling location, altitude was extracted from raster data attained from U.S. Geological Survey Earth explorer using QGIS 2.18.25 (QGIS.org, 2022). In addition, we obtained climate data (mean annual temperature, mean summer temperature, mean annual solar radiation, mean summer solar radiation, mean annual precipitation, mean summer precipitation, and mean summer wind speed) from WorldClim—Global Climate Data (Fick and Hijmans, 2017) for each sampling point. The R package *raster* 3.5 (Hijmans et al., 2021) was utilized to import these data as raster layers, and the function *extract* to connect the climatic values to each sampling location. We chose these variables as they have previously been reported to influence wing patterns in Lepidoptera (Sullivan and Miller, 2007; Nygren et al., 2008; Heidrich et al., 2018; Zaman et al., 2019). Please see the section “Data analysis” for specifications of which environmental variables were used for each analysis.

Data analysis

To justify the use of linear regression models, we visualized our data by plotting all possible combinations of phenotypic values with geographic and environmental variables. Since the associations shown in the plots followed rather linear relationships, we are confident that all variables should enter the equations linearly (Supplementary Figures S1–S3).

Multivariate analyses of overall phenotypic variation and its association with environmental factors

In addition to the univariate statistical approach outlined below, in which the different phenotypic traits were analysed one by one to test for differences between species and evaluate associations with the geographical and climatic environmental conditions, we also used a multivariate statistical approach. To this end, we used a principal component analysis (PCA, a dimension reducing approach) to describe the total phenotypic variation in all the original phenotypic traits in two dimensions (principal component axes, PC1 and PC2). This was first done for all three species pooled, and the results visualized in a biplot, to evaluate whether composite phenotypic variation overlapped or differed between the three species. To also formally evaluate whether the composite phenotypic variation differed markedly according to species or sex we performed a multivariate analysis of variance (MANOVA) using PC1 and PC2 as dependent variables, and species, sex, and their interaction as explanatory factors. Next, we performed a separate PCA for each species, and used the obtained PC-scores for each species as input to evaluate associations of overall phenotypic variation with environmental factors. To this end, we created biplots according to redundancy analysis (RDA), a constrained version of PCA, of phenotypic data to visualize associations of geographic and climatic variables with the phenotypic structuring in each studied *Argynnini* species.

Analysing spatial variation in wing size

We performed a three-way ANOVA to test for differences in wing size between species, sex, and wing types (forewing, hindwing). We also grouped individuals in each species into geographic regions, representing different European (and Moroccan for *S. aglaja*) climatic, ecological, and biogeographic zones, and, in some cases, glacial refugia during the Last Glacial Maximum (LGM, 26,500–20,000 years ago). We selected these regions to detect potential divergences in wing phenotypes, which are not only associated with environmental or geographic factors following a continuous gradient, but rather caused by large-scale landscape characteristics and/or the biogeographic history of these areas. These regions were also used in the comparative large-scale phylogeography study on essentially the same individuals (Polic et al., 2022). Depending on where the individuals in each species were collected, these regions included Morocco (only for *S. aglaja*, as the other two species do not occur in the Maghreb), Iberia, Central Europe, Eastern Europe, Sicily, Southern Italy, Southern Balkans, and Scandinavia. For each species, we performed 2-way ANOVA to model potential associations of wing type (forewing, hindwing), and geographic region, and used a *post-hoc* Tukey test

to assess which regions differed significantly from each other. Then, we used linear mixed regression models, separately for each species, to explore if wing size was associated with geographic location (longitude, latitude, altitude), mean annual temperature, mean annual solar radiation, mean summer precipitation, mean summer wind speed, sex, or color morph (only for the polymorphic species *F. adippe* and *F. niobe*). We used mean annual temperature and mean annual solar radiation, as these variables are associated with the length of the vegetation period in temperate climates (Piao et al., 2006), which correlates with the duration of the larval food stage, thereby potentially affecting adult size in butterflies. Mean summer precipitation and mean summer wind speed were used, as these variables might affect mobility patterns during the flight period (i.e., summer), which might lead to adaptations in wing architecture and size. Wing type (forewing or hindwing) was included as a dummy variable, and individual identity was entered as a random factor to account for independence of repeated wing measurements of each individual. We also tested for interaction effects of sex, wing type and geographic and environmental variables, but kept only the significant ones ($p < 0.05$) to avoid problems associated with over-parametrization of the statistical models.

Analysing spatial variation in color patterns

We used a four-way ANOVA to test for differences in the degree of melanism between species, sex, wing types, and wing sides. Further, we performed ANOVA separately for each species to test for associations of melanism and geographic regions, equivalently to our models for wing size. Thereafter, we performed mixed linear models, separately for each species, to test for associations of the degree of melanism with geographic location (longitude, latitude, altitude), mean summer temperature, mean summer solar radiation, mean summer precipitation, mean summer wind speed, sex, and color morph (only for *F. adippe* and *F. niobe*). We used mean summer temperature and mean summer solar radiation, since the degree of melanism in the wings might be influenced by these variables during the butterflies' flight period (i.e., summer), when darker wings might be beneficial for temperature regulation and protection from UV radiation. We included wing type (forewing/hindwing) and wing side (dorsal/ventral) as separate dummy variables, and the individual identity was included as a random factor to account for repeated measurements per individual. We also tested for interaction effects of sex, wing side, and wing type with climatic and geographic variables, but we kept and report only those interactions that were significant ($p < 0.05$).

We performed ANOVA and linear regressions to model associations of the amount of green on the ventral side of the hindwings (since green is only present there) with species identity, sex, the above-mentioned geographic regions, and climatic and geographic variables, respectively. Similarly, we analysed associations of the amount of white on the ventral side of the hindwings (white is only present in this part of the wings) with these variables. In the polymorphic species *F. adippe* and *F. niobe*, white spots only occur in the *adippe* and *niobe* morphs, respectively. Because of the small sample size in *F. niobe f. niobe* (8 individuals), this analysis was only performed for *F. adippe f. adippe* and *S. aglaja*.

Analysing phenotypic integration

To study phenotypic integration and evaluate whether evolutionary modifications and the expressions of different phenotypic dimensions are independent or not, we performed pairwise correlation analyses separately for each species. We evaluated all possible pairwise associations (*viz.* total melanism, dorsal melanism, ventral melanism, forewing melanism, hindwing melanism, forewing length, hindwing length, green in the ventral hindwing, and white in the ventral hindwing).

To determine whether phenotypic integration between wing size and the studied species of wing coloration were similar or different in the three studied species, we used the Mantel test (Mantel, 1967) to quantify the correspondence between the pairwise correlation matrices obtained for each species. To assess all possible comparisons, we performed three separate Mantel tests. With this approach, we evaluated whether pairs of phenotypic traits that were highly positively or weakly negatively correlated in one species were also highly positively or weakly negatively correlated in the other species. The statistical significance of the Mantel correlations was assessed using 999 permutations.

Associations of genetic structure with morphological variables

To assess associations of genetic structure with morphological variables (total melanism, forewing length, hindwing length, green, and white), we conducted distance-based redundancy analyses (db-RDA), a constrained version of a PCA (Legendre and Anderson, 1999), for each species separately. The genomic data was derived via double digest restriction-site associated DNA sequencing (ddRADseq). Details about DNA extraction and library preparation can be found in Polic et al. (2022). We generated Manhattan dissimilarity matrices for each species using pairwise distances between all individuals based on the proportion of shared alleles, which was used as input for the db-RDA. To build db-RDA models, we used the *capscale* function in the R package *vegan* 2.5 (Oksanen et al., 2019) to test which morphological variables contribute the most to the PCoA ordination. Using the function *anova.cca* with 999 permutations, we determined the overall statistical significance of the model and the explanatory variables. We used Holm's correction for adjusting the *p*-values for multiple testing (Holm, 1979). We used longitude and latitude as a condition in the model to account for spatial autocorrelation between individuals, through which we partialled out these terms from the test prior to analysing the other predictor variables.

Model selection

For all regression and RDA models, we applied a backward elimination process, *viz.* we started with the most complex model and removed one variable at a time, starting with the least significant one. Non-significant interactions were removed before non-significant main effects. If non-significant main effects were included in a significant interaction, the main effect was kept in the model. The final most informative model included the highest number of significant predictor variables. The Akaike Information Criterion (AIC) was used for model comparisons for univariate analyses, and adjusted R^2 was used for RDA model selection. To check for multi-collinearity, we calculated the variance inflation factor (VIF) for our explanatory variables, which were, depending on the analysis, mean annual temperature, mean summer

temperature, mean annual solar radiation, mean summer solar radiation, mean annual precipitation, mean summer precipitation, mean summer wind speed, altitude, latitude, longitude, color morph (only for *F. adippe* and *F. niobe*), total melanism, forewing length, hindwing length, green, and white. The presence of multi-collinearity indicates that two or more predictor variables are highly correlated, and thus, the impact of one predictor on the response variable might be inaccurately estimated and not informative (James et al., 2013; Bruce and Bruce, 2017). Variables with a VIF > 10 were therefore excluded from the final model as this implies a potentially problematic level of collinearity (James et al., 2013). When we analysed all three species together, and when *F. adippe* and *F. niobe* were analysed separately, the variable green (underside of hindwing) was square root transformed to achieve normal distribution. The same was done for the variable altitude for *S. aglaja* and *F. adippe*, and for the variable melanism in all regression analyses. All statistical analyses were performed in R 3.6.1 (R Core Team, 2020).

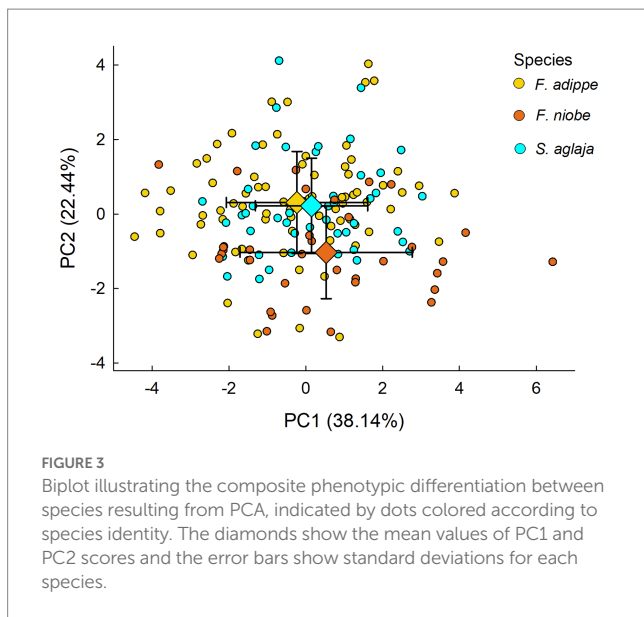
Results

Associations of overall multivariate phenotypic variation with geography and climatic conditions

Principal component analysis on all three species pooled illustrated that the overall phenotypic variation based on the studied morphological variables was similar in the studied species (Figure 3). The results from multivariate analysis of variance suggest that composite phenotypic variation (as estimated using PC1 and PC2 in combination) did not vary to an important degree according to species or sex (MANOVA: effect of species, Pillai's Trace = 0.04, $F_{4,306} = 1.78$; $p = 0.13$; effect of sex: Pillai's Trace = 0.01, $F_{2,152} = 0.92$, $p = 0.40$; effect of species*sex: Pillai's Trace = 0.07, $F_{4,306} = 2.79$, $p = 0.03$). Performing RDA based on PCA scores for each species separately to link the overall phenotypic variation to geographic and climatic variables revealed some similarities and some differences between the species (Figure 4). For *S. aglaja*, the final selected model included longitude and mean summer wind speed (adjusted $R^2 = 0.08$), of which longitude was significantly associated with the overall phenotypic variation ($F_{1,44} = 4.8$, $p < 0.01$). For *F. adippe*, the final selected model included longitude, altitude, mean annual temperature, and mean summer precipitation (adjusted $R^2 = 0.22$), of which longitude ($F_{1,72} = 8.2$, $p < 0.001$), altitude ($F_{1,72} = 9.6$, $p < 0.001$), and mean summer precipitation ($F_{1,72} = 4.9$, $p < 0.01$) were significantly associated with the general phenotypic variation. For *F. niobe*, the final selected model included mean annual temperature, mean summer precipitation, and mean summer wind speed (adjusted $R^2 = 0.19$), of which mean summer precipitation ($F_{1,30} = 4.8$, $p = 0.01$), and mean summer wind speed ($F_{1,30} = 3.5$, $p = 0.04$) were significantly associated with the composite phenotypic structure.

Wing size in relation to species, sex, geography, and climatic conditions

Wing size differed significantly between the studied species, with *F. adippe* being largest, *S. aglaja* intermediate and *F. niobe* smallest, but



the relative size difference between fore- and hindwings did not vary between species. Females were significantly larger than males, which was most pronounced in *F. niobe*, and in the hindwings (ANOVA, main effect of species identity, $F_{2,329} = 21.08$, $p < 0.001$; main effect of sex, $F_{1,329} = 71.18$, $p < 0.001$; main effect of wing type, $F_{1,329} = 621.71$, $p < 0.001$; interaction effect of species identity and sex, $F_{2,329} = 4.46$, $p = 0.01$; interaction effect of sex and wing type, $F_{1,329} = 4.11$, $p = 0.04$; interaction effect of species identity and wing type, $F_{2,329} = 1.1$, $p = 0.33$, Table 1; Figure 5). According to results from ANOVA and linear mixed models for each species, the large-scale spatial variation in wing size and its association with environmental factors was largely species-specific (details in the Supplementary material, and the statistical results and their visualization in Tables 2, 3; Supplementary Table S1, Supplementary Figure S4).

Dark coloration (melanism) in relation to species, sex, geography, and climatic conditions

The degree of wing melanisation varied in a complex interactive manner among species, and according to wing type (between the fore and hind wings) and wing side (between the dorsal and ventral sides), as evidenced by a significant three-way interaction between species, wing type and wing side. Sex was excluded from the analysis, as it did not have a significant effect (main or interaction) on the degree of melanism. In all three species, the dorsal wing side was darker than the ventral side. Dorsal hindwings were darker than dorsal forewings in all species, while ventral forewings were darker than ventral hindwings in *S. aglaja* and *F. adippe*, but not in *F. niobe* (3-way ANOVA, main effect of species identity, $F_{2,655} = 2.78$, $p = 0.06$; main effect of wing type, $F_{1,655} = 6.18$, $p = 0.01$; main effect of wing side, $F_{1,655} = 1369.44$, $p < 0.001$; interaction effect of species identity and wing type, $F_{2,655} = 5.5$, $p < 0.01$; interaction effect of species identity and wing side, $F_{2,655} = 14.26$, $p < 0.001$; interaction effect of wing type and wing side, $F_{1,655} = 122.34$, $p < 0.001$; interaction effect of species identity, wing type, and wing side, $F_{2,655} = 7.95$, $p < 0.001$; Table 1;

Figure 5). According to results from ANOVA and linear mixed models for each species, the large-scale spatial variation in dark coloration was mainly associated with aspects of the thermal environment (details in the Supplementary material, and the statistical results and their visualization in Tables 3, 4; Supplementary Table S2, Supplementary Figure S5).

Amount of white on the ventral hindwings

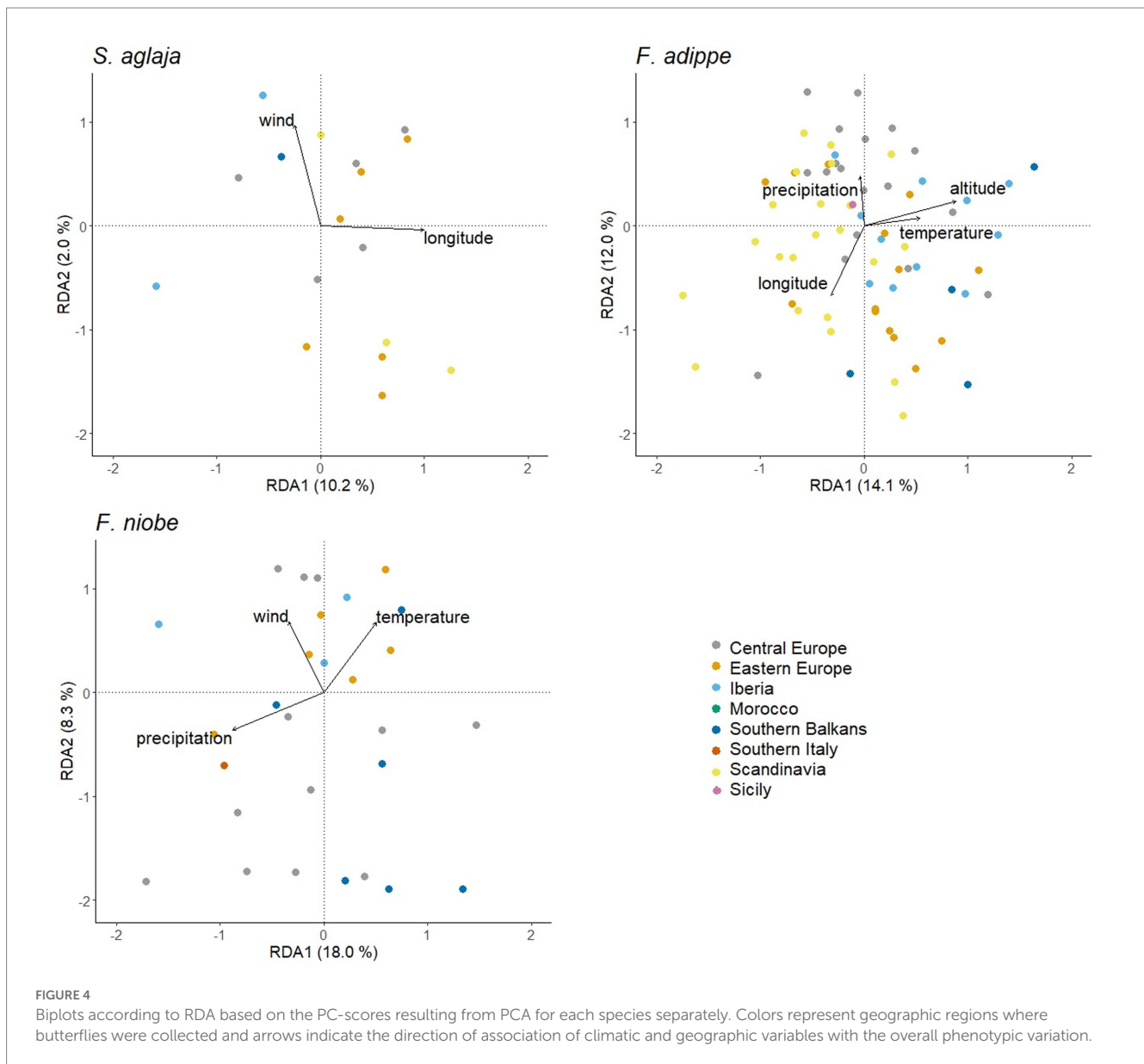
The amount of white in ventral hindwings (only present there) differed significantly between *F. adippe f. adippe*, *F. niobe f. niobe*, and *S. aglaja*, while sex did not affect this trait (ANOVA, effect of species identity, $F_{2,111} = 7.16$, $p < 0.01$; effect of sex, $F_{1,111} = 2.46$, $p = 0.12$), i.e., in both *F. adippe f. adippe* ($p < 0.01$) and *S. aglaja* ($p = 0.04$) the amount of white was higher than in *F. niobe f. niobe*. Results from ANOVA and linear regression analyses for each species revealed that the associations of white wing coloration with geography and climatic conditions were species-specific (details in the Supplementary material, and the statistical results in Table 3 and Supplementary Table S3).

Amount of green on the ventral hindwings

The amount of green in the ventral hindwings (only present there) differed significantly between the three species, while sex did not have an effect on this trait (ANOVA, effect of species identity, $F_{2,160} = 27.11$, $p < 0.001$; effect of sex, $F_{1,160} = 1.69$, $p = 0.2$). *Speyeria aglaja* showed a significantly higher amount of green than *F. adippe* ($p < 0.001$) and *F. niobe* ($p < 0.001$), but there was no difference between *F. adippe* and *F. niobe*. ANOVA and regression analyses for each species suggested that the associations of green wing coloration with geography and climatic conditions were species-specific (details in the Supplementary material, and the statistical results in Table 3 and Supplementary Table S4).

Pairwise correlations between traits—phenotypic integration

The analyses of pairwise correlations between traits indicate that phenotypic integration was both general and species-specific. In all three species, dorsal, ventral, forewing, and hindwing melanism were positively correlated, although some of these associations were not significant in *S. aglaja* and *F. adippe* (Figure 6, for correlation coefficients please see Supplementary Table S5). Forewing and hindwing length correlated positively and significantly with each other in all species. In *S. aglaja*, the amount of green in the ventral hindwing was significantly and positively associated with both forewing and hindwing length, while it was significantly and negatively associated with ventral melanism and the amount of white in the ventral hindwing (Figure 6). Dorsal melanism was significantly and positively correlated with hindwing length, while there was a significant and positive correlation of forewing melanism with forewing and hindwing length in *S. aglaja*. In *F. adippe*, the amount of green in the ventral hindwing was significantly and negatively associated with ventral melanism, forewing melanism and total melanism (Figure 6).



Forewing length was significantly and positively associated with the amount of white in the ventral hindwing in *F. adippe*. While there were some apparent differences in phenotypic integration between species as described above, results from Mantel correlation analyses based on comparisons of the matrices of estimated pairwise trait correlations for each species suggested significant and positive correlations between the three matrices (Mantel test, *S. aglaja* – *F. adippe*, $r=0.58$, $p<0.01$, *S. aglaja* – *F. niobe*, $r=0.47$, $p=0.01$, *F. adippe* – *F. niobe*, $r=0.45$, $p=0.03$).

Associations of genetic structure with morphological variables

In *S. aglaja* and *F. adippe* (but essentially not in *F. niobe*), the phenotypic trait variation was largely associated with similar environmental variables that were previously found to be associated

with their genetic structure (Polic et al., 2022). Wing size and melanism were associated with the genetic structure in all three species or in both *Fabriciana* species, respectively, hinting at a strong genetic signal and emphasizing the functional importance of these traits, e.g., for flight performance and temperature regulation. For *S. aglaja*, the final selected model included total melanism, forewing length, hindwing length, white, and green (adjusted $R^2=0.02$). The genetic structure was significantly associated with forewing length ($F_{1,30}=1.42$, $p=0.01$). For *F. adippe*, the final selected model included color morph, total melanism, forewing length, hindwing length, and green (adjusted $R^2=0.02$). The genetic structure was significantly associated with color morph ($F_{1,60}=1.27$, $p=0.01$), total melanism ($F_{1,60}=1.19$, $p=0.05$), forewing length ($F_{1,60}=1.31$, $p=0.01$), hindwing length ($F_{1,60}=1.38$, $p<0.01$), and green ($F_{1,60}=1.17$, $p=0.05$). For *F. niobe*, the final selected model included color morph, total melanism, forewing length, and hindwing length (adjusted $R^2=0.03$). The genetic structure was

TABLE 1 Mean values of phenotypic variables included in the analyses with standard deviations in brackets for *S. aglaja*, *F. adippe*, and *F. niobe*.

Species	<i>S. aglaja</i>	<i>F. adippe</i>	<i>F. niobe</i>
forewing length [mm]	27.4 (± 2.2)	28.2 (± 2.2)	26.1 (± 2.0)
hindwing length [mm]	22.4 (± 2.0)	22.8 (± 2.0)	21.3 (± 2.1)
total melanism [%]	22.2 (± 4.7)	20.7 (± 5.4)	23.0 (± 7.0)
dorsal melanism [%]	35.2 (± 8.2)	30.3 (± 8.7)	35.9 (± 10.2)
ventral melanism [%]	9.3 (± 5.0)	11.0 (± 4.3)	11.1 (± 4.8)
forewing melanism [%]	21.3 (± 5.8)	19.4 (± 5.5)	20.4 (± 7.9)
hindwing melanism [%]	23.9 (± 4.7)	22.5 (± 7.0)	26.4 (± 7.8)
white ventral hindwing [%]	22.1 (± 4.8)	25.4 (± 9.4)	14.4 (± 12.1)
green ventral hindwing [%]	35.3 (± 11.5)	17.3 (± 12.5)	20.7 (± 14.0)

White in the ventral hindwings is only shown for *S. aglaja*, *F. adippe* f. *adippe*, and *F. niobe* f. *niobe*.

significantly associated with total melanism ($F_{1,22} = 1.25$, $p = 0.04$), and forewing length ($F_{1,22} = 1.34$, $p = 0.02$).

Discussion

In this study, we analysed how phenotypic variation (wing size, wing melanisation, and the proportion of green and white coloration in the ventral hindwings) varied between species and how it was associated with climatic and geographic factors to identify and compare eco-evolutionary drivers of large-scale spatial differentiation and phenotypic integration in three *Argynniini* species, viz. *S. aglaja*, *F. adippe* and *F. niobe*. Notably, the results of our multivariate analyses show that the three species are phenotypically largely overlapping and nearly indistinguishable based on the morphological variables studied here. This overlap is in sharp contrast to the results of a recent comparison of genetic structure (based on analyses of ddRADseq data for the same set of individuals as used in the present study) which showed that these three species are genetically clearly distinct (cf Figure 3 in this paper with Figure 3F in Polic et al., 2022). While our analyses of drivers of phenotypic variation revealed some parallel trends between species (e.g., the composite phenotypic variation was associated with longitude in *S. aglaja* and *F. adippe*, and with precipitation in *F. adippe* and *F. niobe*), most associations of wing size and different aspects of wing coloration with climatic and geographic factors were species-specific, showcasing that even closely related species with similar distribution ranges may respond differently to common environments. We hypothesized that the combinations of environmental variables that shape the large-scale morphological trait distributions in these three species might be similar to those previously reported to be associated with the genetic structure (Polic et al., 2022). For *S. aglaja* and *F. adippe*, this hypothesis was largely qualitatively supported, while the phenotypic trait variation in *F. niobe* was mainly associated with different factors than the genetic structure. Wing size and melanism stood out as morphological variables that were associated with the genetic structure in all three or the two *Fabriciana* species, respectively. Further, we report some distinct intraspecific trait correlations for *S. aglaja* and *F. adippe*, suggesting that phenotypic integration is in part species-specific.

Large-scale patterns of spatial variation in wing size were mostly incongruent among species

Assessing and comparing large-scale associations of phenotypic characters with climatic and geographic variables revealed mostly divergent patterns between the three studied fritillary species. For example, only altitude was associated with wing size in more than one species, while the associations of wing size with the other factors were species-specific. Moroccan *S. aglaja* and Iberian *F. adippe* individuals were on average larger than from other regions, which coincides with the findings of a large-scale comparative phylogeographic study on the essentially same set of individuals, in which Moroccan *S. aglaja* and Iberian *F. adippe* samples appeared to be genetically distinct from other European sampling locations (Polic et al., 2022). That these regions constituted important glacial refugia during the LGM, when populations retracted to warmer areas during the ice ages, is still apparent in the phylogeography of many species (e.g., Cheddadi et al., 2006; Ferrero et al., 2011). Today, southern regions within our study area have longer vegetation periods and therefore offer more time for larval development, which may translate into larger adult butterflies. Further, the Strait of Gibraltar and the Pyrenees, respectively, might act as barriers to dispersal for both species, which might increase differences in wing size and genetic structure between individuals from these regions and the rest of the study area.

At higher altitudes, *F. adippe* individuals had larger wings, while this association was stronger in the forewings of *S. aglaja* whose hindwing length did not increase substantially with altitude. In high altitude habitats with low temperatures, larger wings with higher surface areas might be beneficial for thermoregulation, as the cooling rates are reduced in larger individuals (Shelly and Ludwig, 1985; Heinrich, 1993). Hindwings are not necessary for flight in lepidopterans, although maneuverability is substantially decreased when hindwings are missing or significantly reduced (Jantzen and Eisner, 2008). The varying associations of wing length with altitude in *S. aglaja* and *F. adippe* might reflect differences in ecological specialization and mobility patterns. For the highly specialized *F. adippe*, a high flight capacity may be required to locate potentially suitable new habitat patches, while this might not be as crucial for the relative generalist *S. aglaja* that can occupy a broad range of habitats. In line with this argument, a small-scaled comparative study on these two fritillary species found higher dispersal distances and lower population densities in *F. adippe* than in *S. aglaja* (Polic et al., 2020). The spatial variation in wing size was positively correlated with temperature in *F. adippe*, but not in the other two species. These differences might again reflect distinct ecological niche breadths and dispersal capacities, such that the specialist *F. adippe* might rather invest in large wings in order to locate potentially suitable new habitat patches as opposed to the relative generalist *S. aglaja*. The sampling strategy for *F. niobe* (less samples than in the other two species and a smaller latitudinal range) might have masked associations of wing size and most environmental factors. Our results suggest that the evolution of wing size in these *Argynniini* butterflies is influenced in part by selection for temperature regulation in combination with environmental constraints on the time (mediated by the duration of the vegetation period) available for development and growth, and that these different drivers are modulated by geographic and climatic conditions. That this has contributed to species-specific evolutionary

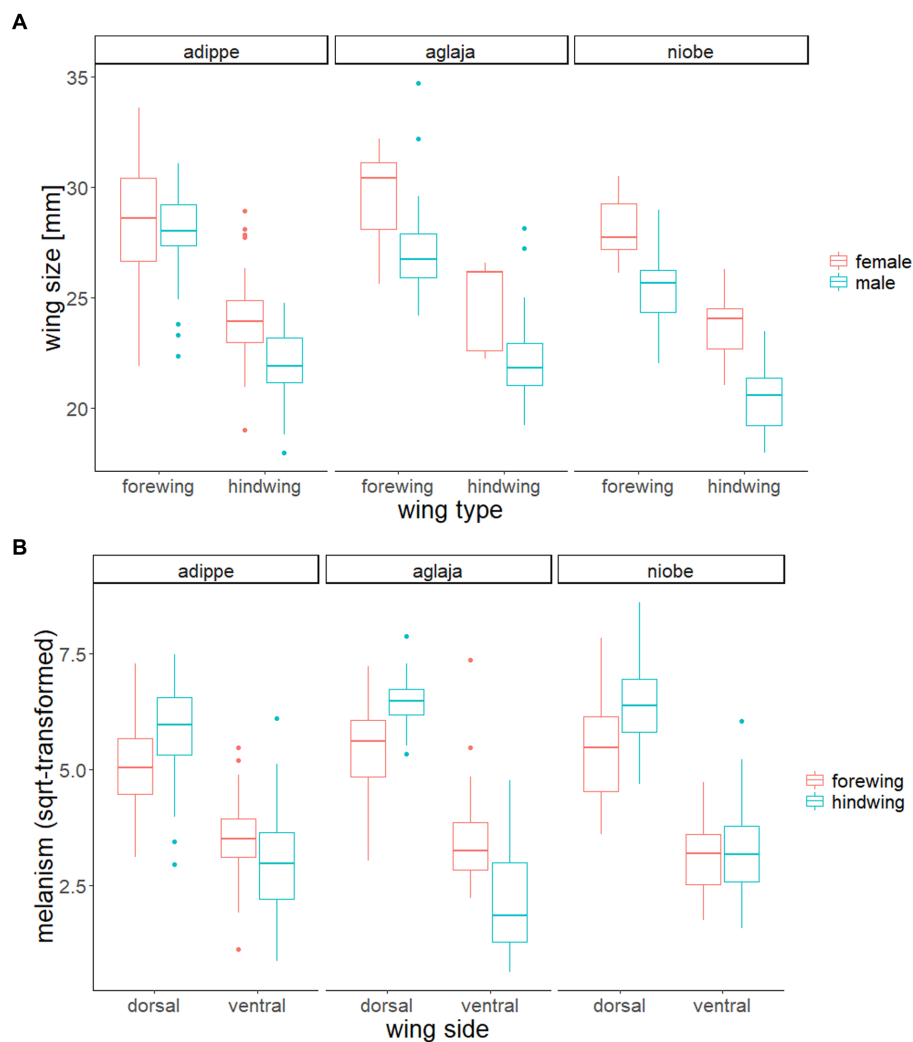


FIGURE 5

(A) Boxplot showing the significant differences in wing size between species, sexes and wing types. There was a significant interaction effect of sex with species identity and wing type, but the interaction of species identity and wing type was not significant. (B) Boxplot showing the significant 3-way interaction of species identity, wing type, and wing side on melanism (square root-transformed). Sex did not have an effect on the degree of melanism.

modifications of wing size in closely related sympatric species is possibly owing to differences in ecological niche breadth and dispersal strategies as mentioned above.

Variation in dark coloration (melanisation) was mainly linked to aspects of the thermal environment across species

The degree of melanism was associated with similar environmental factors in all three fritillary species, showing that this trait is likely influenced by similar selective pressures across species. Dorsal wings were darker than ventral wings in all species, probably reflecting that the dorsal side is exposed during sun basking and melanised scales increase the solar heat absorption (Watt, 1968; Kingsolver, 1985, 1987). Melanisation mainly increased in cooler regions (e.g., with lower solar radiation in *S. aglaja* and lower temperature in *F. niobe*), which is in line with several studies on different ectothermic taxa (ants, butterflies, moths, dragonflies, beetles, and reptiles) that found

darker individuals in colder environments, presumably owing to more efficient solar absorption with increasing melanism (Clusella Trullas et al., 2007; Bishop et al., 2016; Schweiger and Beierkuhnlein, 2016; Pinkert et al., 2017; Heidrich et al., 2018; Rosa and Saastamoinen, 2020; Kozlov et al., 2022). In *F. adippe* and *F. niobe*, melanisation increased with precipitation. We propose that because precipitation is often negatively correlated with temperature in European summers (Lhotka and Kysely, 2022), exhibiting darker wings might be beneficial in areas with high precipitation. Together, these results strengthen the conclusion that the thermal milieu is a crucial selective driver for the degree of melanism in insects, and ectotherms generally. In contrast to these previous studies, however, we also found some opposing trends, i.e., melanisation increased at lower latitudes (*S. aglaja*) and altitudes (*F. adippe*), which might highlight the important anti-pathogenic properties of melanised scales. Melanin protects against pathogens and UV radiation, which would select for darker phenotypes at warmer and low latitude localities, where these pressures intensify (Mackintosh, 2001; Burt and Ichida, 2004; Caro, 2005). We argue that the selective pressures on wing melanisation

TABLE 2 Associations of wing size with geographic regions and wing type for *S. aglaja*, *F. adippe*, and *F. niobe* based on results from two-way ANOVA.

Predictors	df	F-value	p
<i>S. aglaja</i>			
geographic region	6, 87	8.31	<0.001
wing type	1, 87	187.57	<0.001
interaction geographic region × wing type	6, 87	0.52	0.8
<i>F. adippe</i>			
geographic region	5, 157	12.64	<0.001
wing type	1, 157	360.91	<0.001
interaction geographic region × wing type	5, 157	0.79	0.56
<i>F. niobe</i>			
geographic region	4, 61	1.32	0.27
wing type	1, 61	94.51	<0.001
interaction geographic region × wing type	4, 61	0.07	0.99

related to temperature regulation and protection from pathogens and solar radiation interact in a complex manner, and that the relative importance of these two mechanisms strongly depend on environmental factors. Dark coloration and immune defense are developmentally and genetically associated, partly because they derive from differentiation of the same cells at early embryonic stages, and darker individuals are therefore better protected against some pathogens (True, 2003; McKinnon and Pierotti, 2010). The need for temperature regulation might be a stronger driving force for melanisation than its anti-pathogenic function, as has been suggested for moths and grasshoppers (Heidrich et al., 2018; Srygley and Jaronski, 2022). This might become detrimental to insects in a changing climate: if ectotherms suppress melanisation in warmer climates to absorb less sunlight, their innate immune response can be lowered and they might be more vulnerable to, e.g., fungal attacks (Srygley and Jaronski, 2022).

Latitudinal trend in white coloration

The proportion of white scales increased in northern latitudes in *S. aglaja*, a pattern that parallels the geographic color morph frequency distribution in *F. adippe*. *Fabriciana adippe f. adippe* has silvery-white spots on the ventral hindwings (similar to *S. aglaja*) and occurs more frequently in northern Europe, while the yellow form *F. adippe f. cleodoxa* becomes progressively more frequent toward the south, and is dominating in the Balkans and Greece (Tolman and Lewington, 2012). The silvery-reflective patterns might represent an adaptation to forest habitats, where they may complicate the chase and capture of butterflies by visually oriented predators through the reflection of light in the prevalent complex light conditions (Olofsson et al., 2010; Kjærsmo et al., 2020). This is in line with results from a study on neotropical nymphalid butterflies, which found that species with iridescent wing patterns were more likely to occur in forest habitats

(Douglas et al., 2007). The evolution of polarized light signaling in forest dwelling species may be favored due to the complex light environment and the lack of polarization in most forest backgrounds (Endler, 1993; Sweeney et al., 2003). In Europe, the forest cover increases toward higher latitudes (Schuck et al., 2002), which might render butterflies with more iridescent white scales better suited for northern European landscapes, as showcased in both *S. aglaja* and *F. adippe*.

Phenotypic integration in *S. aglaja* and *F. adippe*

The results for our analyses of trait correlations showed that fore- and hindwing length, as well as different aspects of melanisation were correlated in all studied species. This might suggest that the different dimensions of size and melanism, respectively, are inherited together in these species, e.g., via pleiotropic effects or linked genes, or influenced by similar environmental cues as a result of plasticity (Andersson, 2001; Schlichting and Wund, 2014; Fruciano et al., 2016). We detected some signs of species-specific phenotypic integration in *S. aglaja* and *F. adippe*, but essentially not in *F. niobe* (Figure 6). The question arises which ecological and selective pressures are involved in the evolution of these trait correlations in *S. aglaja* and *F. adippe*. Phenotypic trait integration may evolve when certain combinations of characteristics are advantageous in a specific environment, or it may result from developmental constraints or trade-offs (Forsman and Appelqvist, 1998; McKinnon and Pierotti, 2010; Dingemans et al., 2020). In *S. aglaja*, but not in *F. adippe* or *F. niobe*, wing length and melanism were positively correlated, which possibly makes *S. aglaja* better adapted to colder habitats compared to the other two fritillary species, as both wing size and darkness can be beneficial for thermoregulation (Heinrich, 1993; Pinkert et al., 2017; Heidrich et al., 2018). *Speyeria aglaja* can breed at lower temperatures than the other two studied species, and its distribution range reaches further north (Warren, 1995; Ellis et al., 2010; Fox et al., 2011; Tolman and Lewington, 2012). The observed trait correlation might contribute to *S. aglaja*'s ecological generalism (it occupies a broad range of habitats), while the studied *Fabriciana* species are restricted to warm microclimates (Salz and Fartmann, 2009; Ellis et al., 2019).

That the proportion of green scales increased with wing size in *S. aglaja* might represent an adaptation to open grassland habitats where larger individuals might be more easily detected by predators, while the green scales might provide camouflage during perching in grassland, as in the butterfly *C. rubi* (Michielsen et al., 2010). An adaptation to grassland habitats might also have driven a trait correlation in *F. adippe*, namely that paler individuals also had more green pigments on the wings. Individuals in shady woodland habitats might have a higher need to efficiently absorb solar light, which would make darker individuals better suited for such habitats. Lighter and greener individuals might thus be better adapted in open grassy areas, which also corresponds with the findings from a study on two *F. adippe* color morphs showing a parallel trait value combination of the yellow spotted *cleodoxa* morph, which is likely better adapted to grassland habitats as opposed to the silvery spotted *adippe* morph (Polic, 2023). That wing size increased with the proportion of white-reflective scales in *F. adippe* might constitute an

TABLE 3 Overview of geographic and climatic factors associated with phenotypic variables and interaction effects in the three studied fritillary species according to linear (mixed) regression models.

<i>S. aglaja</i>	Wing size	Melanism	White	Green
Longitude	* (-)	---	---	---
Latitude	---	* (-)	* (+)	---
Altitude	---	---	---	---
Solar radiation	---	---	---	---
Temperature	---	---	---	---
Precipitation	---	---	---	---
Wind speed	---	---	---	---
Hindwing	*** (-)	*** (+)	N.A.	N.A.
Ventral wing	N.A.	---	N.A.	N.A.
Male	*** (-)	---	---	---
Hindwing × altitude	* (-)	---	---	---
Hindwing × ventral	---	*** (-)	---	---
Ventral × solar radiation	---	* (-)	---	---
Ventral × precipitation	---	* (-)	---	---
<i>F. adippe</i>				
Longitude	---	*** (-)	---	---
Latitude	---	---	---	---
Altitude	*** (+)	** (-)	---	---
Solar radiation	---	---	---	* (+)
Temperature	*** (+)	---	---	---
Precipitation	---	*** (+)	---	---
Wind speed	---	---	---	---
Hindwing	*** (-)	*** (+)	N.A.	N.A.
Ventral wing	N.A.	*** (-)	N.A.	N.A.
Male	*** (-)	---	---	---
Color morph (<i>cleodoxa</i>)	* (-)	---	N.A.	---
Hindwing × male	*** (-)	---	---	---
Hindwing × temperature	* (-)	---	---	---
Hindwing × ventral	---	*** (-)	---	---
Ventral × precipitation	---	** (-)	---	---
<i>F. niobe</i>				
Longitude	---	---	N.A.	** (-)
Latitude	---	---	N.A.	---
Altitude	---	---	N.A.	---
Solar radiation	---	---	N.A.	---
Temperature	---	* (-)	N.A.	---
Precipitation	* (-)	** (+)	N.A.	---
Wind speed	---	---	N.A.	---
Hindwing	*** (-)	*** (+)	N.A.	N.A.
Ventral wing	N.A.	*** (-)	N.A.	N.A.
Male	*** (-)	---	N.A.	---
Color morph	---	---	N.A.	---
Hindwing × ventral	---	** (-)	N.A.	---
Ventral × precipitation	---	** (-)	N.A.	---

Significance codes are as follows: ****p* < 0.001, ***p* < 0.01, **p* < 0.05, ---*p* > 0.05. Minus or plus signs in brackets show the direction of association of variables. Models on the amount of white in the ventral hindwings were only performed for *S. aglaja*, and *F. adippe* *F. adippe*.

TABLE 4 Associations of the degree of melanism with geographic regions, wing type and wing side for *S. aglaja*, *F. adippe*, and *F. niobe* based on results from 3-way ANOVA.

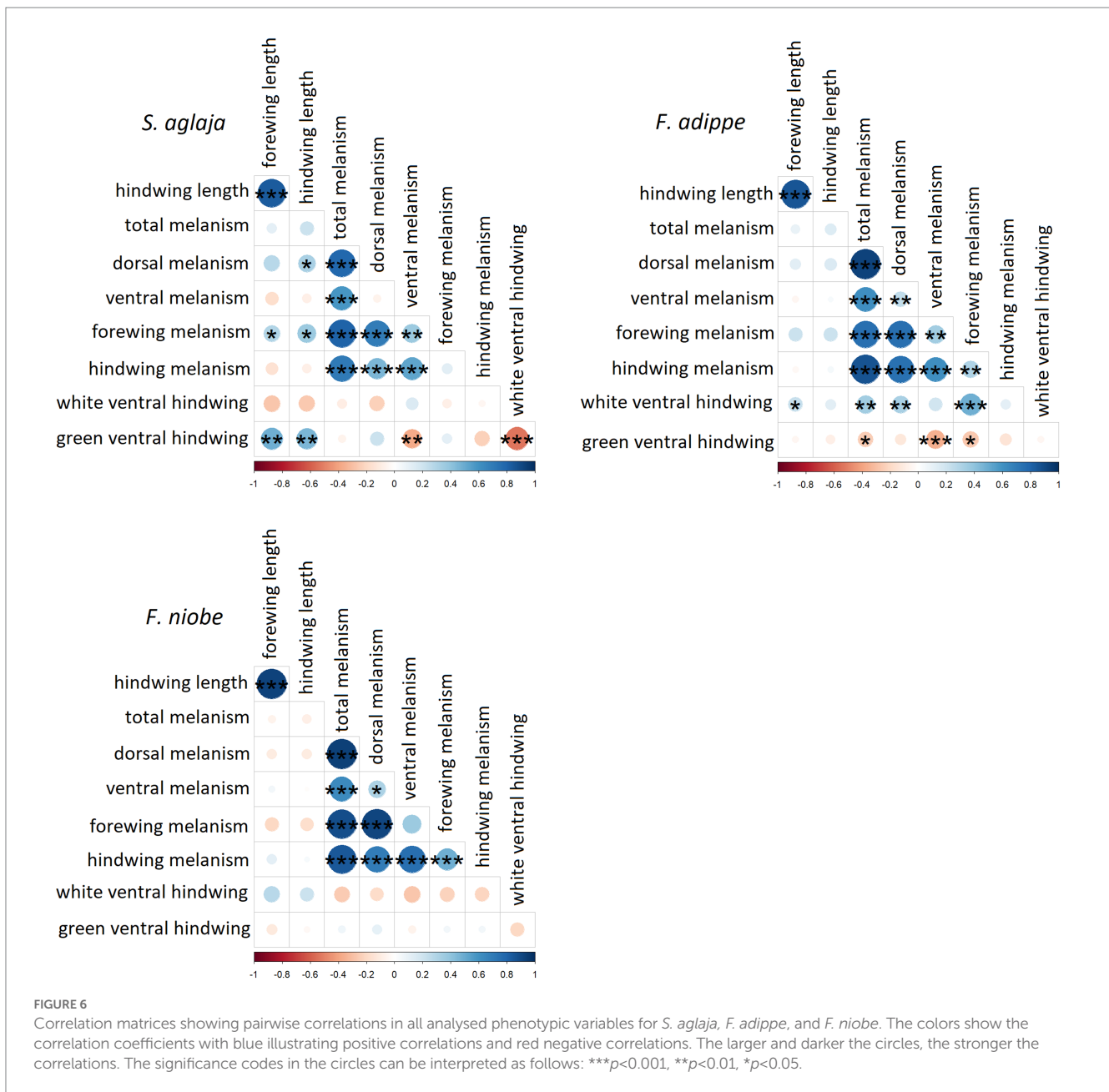
Predictors	df	F-value	p
<i>S. aglaja</i>			
Geographic region	6, 174	1.45	0.2
Wing type	1, 174	0.53	0.47
Wing side	1, 174	606.56	<0.001
Interaction geographic region × wing type	6, 174	1.1	0.37
Interaction geographic region × wing side	6, 174	0.79	0.58
Interaction wing type × wing side	1, 174	84.39	<0.001
Interaction geographic region × wing type × wing side	6, 174	0.99	0.43
<i>F. adippe</i>			
Geographic region	5, 301	3.89	<0.01
Wing type	1, 301	3.41	0.7
Wing side	1, 301	542.62	<0.001
Interaction geographic region × wing type	5, 301	0.94	0.46
Interaction geographic region × wing side	5, 301	2.68	0.02
Interaction wing type × wing side	1, 301	52.95	<0.001
Interaction geographic region × wing type × wing side	5, 301	0.8	0.55
<i>F. niobe</i>			
Geographic region	4, 120	1.84	0.13
Wing type	1, 120	12.46	<0.001
Wing side	1, 120	288.72	<0.001
Interaction geographic region × wing type	4, 120	1.25	0.29
Interaction geographic region × wing side	4, 120	0.57	0.69
Interaction wing type × wing side	1, 120	6.13	0.02
Interaction geographic region × wing type × wing side	4, 120	0.38	0.82

adaptation to forest habitats. Individuals with larger wings can accumulate solar heat faster, as shown for example for the forest dwelling butterfly *Pararge aegeria* (Berwaerts et al., 2001). The silvery scales in *F. adippe* are likely beneficial for camouflage in complex light conditions as in forest dominated areas (Olofsson et al., 2010; Kjærsmo et al., 2020), and have been suggested to function as intra-specific signals during mate choice (Wilts et al., 2013). This is in line with findings from the above-mentioned study (Polic, 2023), which revealed that the *adippe* morph was also larger than the *cleodoxa* morph, possibly resulting from adaptation to distinct microhabitats in this species.

Associations of phenotypic variation with genetic structure, and some common environmental factors linked with both phenotypic and genetic variation

In all three species, forewing length was significantly associated with the genetic structure. That this association was observed across species and that it was the only one in *S. aglaja*, suggests a strong genetic signal (which might have masked other associations in *S. aglaja*) and

highlights the importance of this trait, e.g., for flight capacity and temperature regulation. Similarly, the degree of melanism was associated with the genetic structure in both *Fabriciana* species, which might emphasize this trait's relevance in this genus, such as for temperature regulation, UV protection, and pathogen resistance. In *S. aglaja* and *F. adippe*, there was a broad overlap of environmental variables associated with the large-scale variation in phenotypic traits and the genetic structure as assessed on the same set of individuals in a phylogeographic study (Polic et al., 2022). In *S. aglaja*, some variables were associated with the genetic structure, the degree of melanism (latitude, solar radiation, precipitation), and the amount of white (latitude). This might hint at adaptive modifications with respect to melanisation and white coloration along these geographic and climatic factors, although a direct association of these phenotypic traits with the genetic structure could not be verified. Alternatively, plastic responses in accordance to these environmental drivers might be involved in the trait variation distribution. In *F. adippe*, almost all variables that were associated with the genetic structure were also correlated with different aspects of the phenotypic appearance, viz. wing size (altitude, temperature), the degree of melanism (altitude, precipitation), and green coloration (solar radiation), suggesting that these environmental factors pose selective pressures on these phenotypic traits. These



phenotypic dimensions were also associated with the genetic structure in *F. adippe*, hinting at adaptive genetic modifications of these traits in congruence with spatial environmental variation. Further, color morph was significantly associated with the genetic structure, corroborating results from a small-scaled study on color polymorphism in *F. adippe* (Polic, 2023).

Conclusions and future directions

This study revealed some parallel (e.g., similar responses to selective pressures related to the thermal environment regarding melanisation) and diverging (e.g., species-specific responses to environmental factors regarding size) large-scale patterns of variation in the three studied fritillary species. Some of the observed differences could be related to diverging ecological niche breadths and dispersal capacities, thereby highlighting the importance of comparative

assessments of variation, as the relative effects of drivers of variability may vary between species.

The species-specific combinations of climatic and geographic drivers associated with the phenotypic variation in *S. aglaja* and *F. adippe* were widely the same variables that shape their large-scale genetic structure (Polic et al., 2022), which might suggest a genetic underpinning of the observed trait distribution in these species as a result of adaption to prevalent environmental conditions. This could be further tested by using a combination of rearing experiments, and phenotypic and genomic analyses. Such studies could help understand whether phenotypic trait variation stems from evolutionary genetic modifications or from plasticity responses.

Associations of wing size and environmental conditions were species-specific, posing the questions if different selective drivers shape this trait depending on the species' biology and if responses to environmental conditions are modulated by species-specific developmental constraints. The above-mentioned approaches could further the identification of the

specific mechanisms involved, and help clarify if there are trade-offs between size and other traits depending on environmental conditions. That melanism was mainly correlated with factors shaping the thermal milieu in all three species is in line with the thermal melanism hypothesis (Clusella Trullas et al., 2007; Pinkert et al., 2017). Since the need for temperature regulation seems to impose a substantial selective pressure on melanisation, the trade-off between thermoregulatory and anti-pathogenic properties of melanised scales might become disadvantageous on a warming planet. Host-pathogen interactions have changed due to global warming, in part because of shifts in species range distributions (Harvell et al., 2002; Traill et al., 2010), and in part because the replication and the transmission of viruses, bacteria, and fungi are temperature dependent (Altizer et al., 2013). The increased pathogenic pressure and the suppression of melanisation in warmer climates might thus pose a threat to ectotherms (Caro, 2005; Srygley and Jaronksi, 2022). Monitoring changes in ectotherm infections over time and along spatial temperature gradients could offer important insights for making informed decisions for management and conservation plans.

Finally, our results suggest that although phenotypic integration was overall similar in the different species, *S. aglaja* and *F. adippe* exhibit some distinct trait correlations with respect to color and size of the wings, possibly driven by a combination of correlational selective pressures related to susceptibility to visually oriented predators and thermoregulatory requirements in their respective habitats. Again, rearing experiments combined with genomic assessments could help identify the underlying mechanisms involved in shaping these complex phenotypes, including an assessment of the relative roles of genetic correlations and plasticity integration.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DP, AF, MF, RV, and YY conceived the study. PR photographed the butterflies. PR and DP collected data on wing size and wing coloration. DP analysed the data and wrote the first draft of the manuscript, under supervision by AF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2023.1087859/full#supplementary-material>

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