

Phenotypic plasticity in tropical butterflies is linked to climatic seasonality on a macroevolutionary scale

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Abstract

Phenotypic plasticity can be adaptive in fluctuating environments by providing rapid environment–phenotype matching and this applies particularly in seasonal environments. African *Bicyclus* butterflies have repeatedly colonized seasonal savannahs from ancestral forests around the late Miocene, and many species now exhibit seasonal polyphenism. On a macroevolutionary scale, it can be expected that savannah species will exhibit higher plasticity because of experiencing stronger environmental seasonality than forest species. We quantified seasonality using environmental niche modeling and surveyed the degree of plasticity in a key wing pattern element (eyespot size) using museum specimens. We showed that species occurring in highly seasonal environments display strong plasticity, while species in less seasonal or aseasonal environments exhibit surprisingly variable degrees of plasticity, including strong to no plasticity. Furthermore, eyespot size plasticity has a moderate phylogenetic signal and the ancestral *Bicyclus* likely exhibited some degree of plasticity. We propose hypotheses to explain the range of plasticity patterns seen in less seasonal environments and generate testable predictions for the evolution of plasticity in *Bicyclus*. Our study provides one of the most compelling cases showing links between seasonality and phenotypic plasticity on a macroevolutionary scale and the potential role of plasticity in facilitating the colonization of novel environments.

Keywords: butterfly eyespot, climatic niche, macroevolution, miocene, seasonal polyphenism, savannahs

Introduction

The role of phenotypic plasticity in adaptive evolution, both at the micro and macroevolutionary scales, is quickly gaining prominence (Bonduriansky, 2012; Chevin & Hoffmann, 2017; Futuyma, 2021; Levis & Pfennig, 2016, 2021; Pfennig et al., 2010; Schneider & Meyer, 2017). For example, phenotypic plasticity can enable rapid environment–phenotype matching by generating novel variants in response to new environments, thus providing ample raw material on which selection can operate (Ghalambor et al., 2007; Levis & Pfennig, 2016; Price et al., 2003). In other words, phenotypic plasticity can allow rapid climbing of new fitness peaks during the early phases of shifting into new environments (Ghalambor et al., 2007; Price et al., 2003). Numerous empirical studies have convincingly demonstrated the adaptive role of phenotypic plasticity in responding to novel selection pressures at a microevolutionary scale (e.g., Corl et al., 2018; Losos et al., 2000; Scoville & Pfrenger, 2010; Wang & Althoff, 2019; Yeh & Price, 2004). However, comparable evidence on a macroevolutionary scale remains scant (but see Kellermann et al., 2018; Relyea et al., 2018, 2021; Susoy et al., 2015) even though this will be crucial in demonstrating how phenotypic plasticity facilitates the conquering of new adaptive zones, for example, in colonization of novel environments.

One dramatic example of a shift into a novel environment by both plants and animals is the Miocene colonization of newly formed seasonal savannah grasslands from ancestral forests. The global expansion of savannahs during this time (Beerling & Osborne, 2006; Edwards et al., 2010; Osborne & Beerling, 2006) represented the origin of a novel niche and its subsequent colonization resulted in rapid diversification across many animal groups (e.g., butterflies, Aduse-Poku et al., 2022; killifishes, Dorn et al., 2014; fan-throated lizards, Deepak & Karanth, 2018; falcons, Fuchs et al., 2015; kangaroos, Couzens & Prideaux, 2018). However, colonizing and persisting in such extremely seasonal habitats is expected to be challenging. In the tropics, seasonal rainfall with alternating wet and dry seasons causes a strong temporal skew in resource availability that typically limits reproduction to the wet season (e.g., Halali et al., 2020; Tauber et al., 1986; Varpe, 2017). Moreover, marked seasonal changes in the general appearance of the environment, especially of savannahs, from green in the wet season to brown in the dry season, impose a strong selection for environment–phenotype matching (e.g., Brakefield & Reitsma, 1991; Crook, 1964; McQueen et al., 2019). In such cyclically fluctuating seasonal environments with reliable environmental cues such as temperature (Brakefield & Reitsma, 1991), phenotypic plasticity

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can provide a tight environment–phenotype matching, especially for insects whose generation times are frequently shorter than the season length (Halali et al., 2021a; Moran, 1992; Nylin & Gotthard, 1998; Reed et al., 2010; Varpe, 2017). From a macroevolutionary perspective, phenotypic plasticity may have played a crucial role by enabling rapid environment–phenotype matching, thus facilitating colonization and subsequently rapid diversification in seasonal environments such as savannahs.

Seasonal polyphenism, an extreme case of phenotypic plasticity where only the alternative phenotypes representing the two extremes of reaction norms are expressed in the wild (Nijhout, 1999, 2003), reflects a remarkable adaptation to seasonal environments, especially in butterflies (Shapiro, 1976). Tropical butterflies of the subtribe Mycalesina (Nymphalidae: Satyrinae) have been particularly well studied providing a textbook example of the phenomenon (Brakefield & Frankino, 2009; Brakefield & Larsen, 1984). Adults of many Mycalesina butterflies show alternative wet- and dry-season forms (WSFs and DSFs, respectively), which differ in a suite of morphological, life-history, and behavioral traits (van Bergen et al., 2017). For example, WSFs show a series of eyespots close to the wing margins which can function in deflecting predator attacks to nonvital body parts (Lyytinen et al., 2004; Prudic et al., 2015). In contrast, DSFs have an inconspicuous brown wing pattern with reduced or completely absent eyespots that enhances crypsis when at rest often on dry leaf litter (Brakefield & Larsen, 1984; Lyytinen et al., 2004; Prudic et al., 2015). Thus, seasonal polyphenism represents a classic case of how phenotypic plasticity enables tight environment–phenotype matching in seasonal environments.

The clear adaptive significance of seasonal polyphenism and the evolutionary history of Mycalesina butterflies allow a direct examination of how phenotypic plasticity is linked with seasonality and its potential role in facilitating colonization of novel seasonal environments. Multiple clades of Mycalesina butterflies have independently colonized seasonal savannahs during the late Miocene and Pliocene (8–3 million years ago) from ancestral forests across three major geographic radiations in Asia, mainland Africa, and Madagascar (Halali et al., 2021b; Aduse-Poku et al., 2022). As seasonal polyphenism represents a crucial adaptation to seasonality, its evolution is expected to be a key factor in facilitating successful colonization and persistence in savannahs.

Here we focus on a crucial wing pattern element acting as an anti-predatory device, namely the size of the most prominent eyespot on the ventral hindwing. This is an important trait in the suite of traits associated with seasonal polyphenism that has been suggested to represent strong fitness consequences (e.g., Brakefield & Frankino, 2009; Lyytinen et al., 2004; Prudic et al., 2015). As such, we expect a strong association between the degree of habitat seasonality and eyespot size plasticity on a macroevolutionary scale. Using the genus *Bicyclus*, the main African radiation of Mycalesina, we quantified the extent of phenotypic plasticity in the eyespot size of 85 species by screening an extensive dataset of museum specimens. To link the observed degree of plasticity to relevant habitat characteristics, we constructed environmental niche models for each species and extracted a range of metrics linked to seasonality. Broadly, we show that species occurring in seasonal environments (such as savannahs) show strong plasticity (including potential polyphenism) in the eyespot size. While species in less seasonal environments

show variable degrees of plasticity ranging from strong plasticity to an apparent absence of plasticity. Unexpectedly, some of the many nonplastic species in less-seasonal forest habitats display a constant DSF, rather than the expected WSF given their habitat. To try and explain the high diversity of plasticity patterns in more stable habitats, we then propose a macroevolutionary framework that may explain the evolution of phenotypic plasticity across habitat types generating testable hypotheses for future studies. Our study provides one of the most compelling examples of how phenotypic plasticity is linked with the degree of climatic seasonality on a macroevolutionary scale and the potential role of plasticity in facilitating the colonization of novel environments.

Methods

All data preparation for the niche modeling, and the phylogenetic comparative analyses, were carried out using R ver. 4.2.3 (R Core Team 2023). Details of the R packages used for specific tasks are given in the description of each step of the analysis. R packages tidyverse ver. 2.0.0 (Wickham et al., 2019), ggplot2 ver. 3.5.0 (Wickham, 2016), ggridges ver. 0.5.6 (Wilke, 2022), gridExtra ver. 2.3 (Auguie 2017), broom ver. 1.0.5 (Robinson et al., 2023), broom.mixed ver. 0.2.9.5 (Bolker & Robinson, 2022), and conflicted ver 1.2.0 (Wickham, 2023) were used for general data processing, plotting, extracting regression estimates, detecting conflicting R functions, etc.

Quantifying species niches

Collection of species distribution data

Presence data for all known *Bicyclus* species were collected based on our records and those of colleagues working in the field, combined with the inspection of thousands of specimens or photographs across multiple museum collections, literature survey, and >1,000 records published on the online citizen science platform (www.inaturalist.org). Many *Bicyclus* species are difficult to identify without expert knowledge, and misidentifications are common in museum material and field records. Therefore, only those records were used for which we either personally knew the skills of the identifier or had detailed photographs or actual specimens so that each record could be identified individually. For the locations gathered via museum labels, a combination of gazetteers and annotated lists provided by the museums themselves, as well as a range of detailed maps and internet searches, were used to verify the coordinates of collection localities. If a locality name could be linked to more than one geographic location, the record was discarded unless some additional information clearly favored one site over another. Obvious errors such as inverted signs for coordinates were first detected by plotting distribution maps and inspecting by eye. Any record well outside the known distribution of a species was removed unless it passed a second assessment of its identity, source, and geo-referencing. We finally removed any duplicate presence records and excluded data for species that were not included in the latest available molecular phylogeny of *Bicyclus* (Aduse-Poku et al., 2022). Following these procedures, a total of 7,957 unique combinations of verified species identities and geo-referenced locations (henceforth called species locations) were gathered (from a total of 94 species) before proceeding with further data preparations.

To minimize bias due to uneven collection efforts, the data were rarefied at the species level using the R package *spThin* ver. 0.2.0 (Aiello-Lammens et al., 2015), with the thinning parameter set to 5, 10, 20, and 50 km with 25 replicates per species/thinning distance, and a method that retained the map with the highest number of remaining records for each selected distance. In general, the 50-km setting was used for species with wide distributions, spanning multiple biogeographic zones; 20 km was used for fairly widespread species linked to a specific region; 10 and 5 km were predominately used for montane species or patchily distributed habitat specialists where clustered location records are likely to reflect actual distributions. The resulting distribution maps were then inspected by eye to

select the dataset that best removed obvious clustering of data, while still retaining as many records as possible (actual thinning parameters applied for the input data of each species are provided in [Supplementary Table S1](#)). Following rarefaction, and the removal of three species that were only represented by a single location point, the final data set contained 4,443 unique species locations. [Figure 1](#) provides an overview of the workflow used for the niche modeling.

Selection of climatic predictors and environmental niche modeling procedures

Climatic data for the environmental niche modeling were extracted from the WorldClim dataset version 2.1 (Fick &

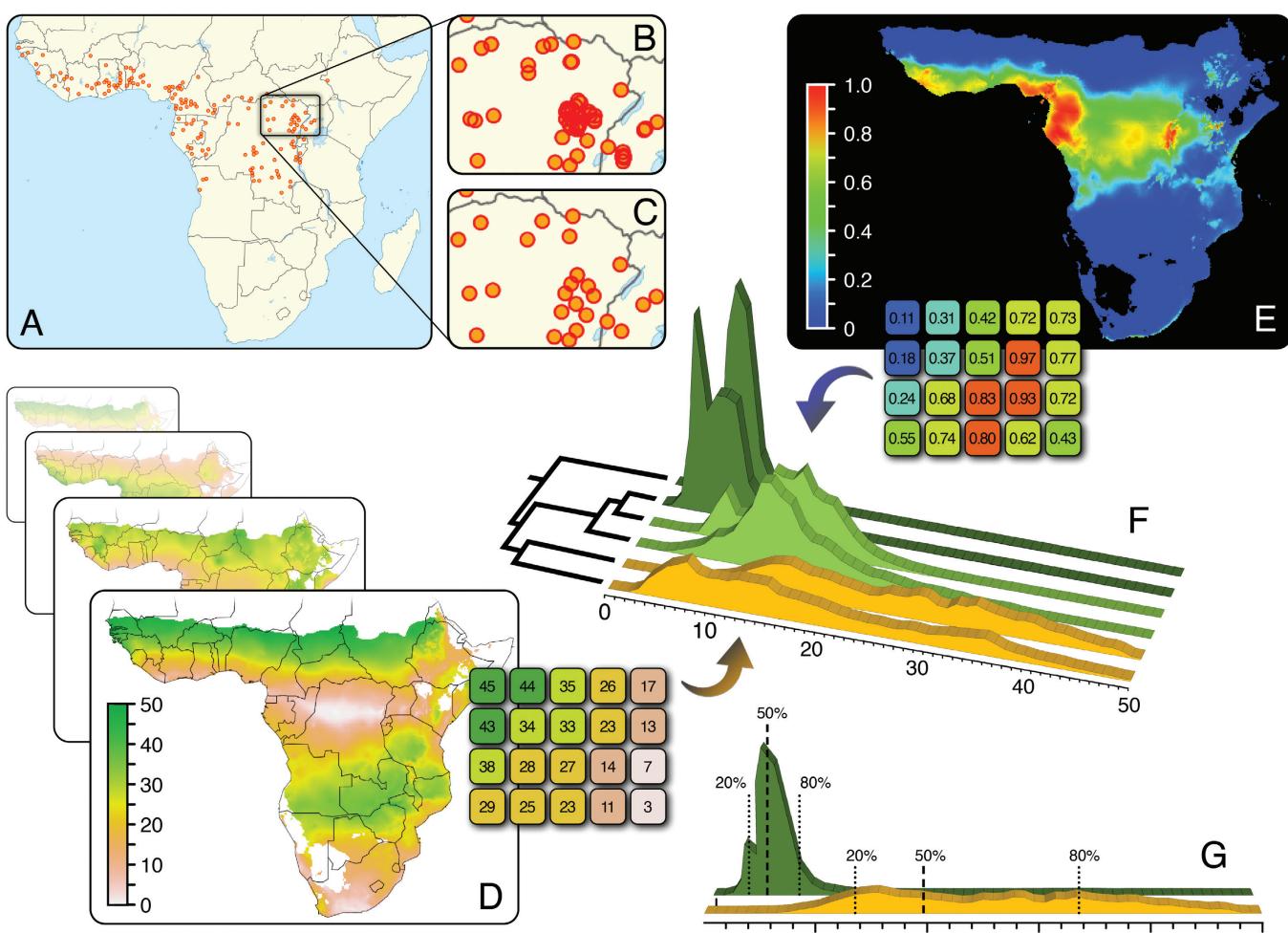


Figure 1. Workflow used for environmental niche modeling and extracting predicted niche occupancy (PNO) profiles from environmental variables linked to seasonality. (A) The input data include a set of verified and geo-referenced occurrence records for each species; (B) due to the diverse nature of the sources for this data (mostly museum specimens), the records are frequently clustered in locations that historically received more attention from collectors; (C) to remove the effect of this sampling bias, the location data are rarefied using the R package *spThin*. The function returns a subset of data for each species that retains the maximum number of occurrence records possible for a given thinning distance (minimum allowed geographical distance between any of the samples); (D) the occurrence data are then analyzed together with a set of climate layers that each contains a data matrix with the same geographic coverage and spatial resolution (numbered cells to the right of the climate map shows the structure of a simplified data matrix); (E) the modeling algorithm Maxent (ver 3.4.1) then compares the climate layers and the occurrence records to produce a map of predicted climate suitability for the full spatial range of the input layers (numbered cells below prediction map show a simplified prediction map). The resolution of the output predictions will match the resolution of the climate data used as input; (F) using PNO profiles enables visual comparison of niche breadth and shape across multiple species for phylogenetic comparisons. A PNO is constructed by summing up the prediction probabilities in each raster cell (upper blue arrow) for a single species with the corresponding environmental data from a selected climate layer (lower brown arrow). The probability surface is then normalized to facilitate comparisons across multiple species; (G) from these PNOs, continuous measurements from the species niche predictions can be extracted. Here, we predominately used the 20% and 80% quantile as an estimate of the climatic tolerances toward the edge of the species niches (see main text for justification). Note that the data shown in the figure are not from this actual study and are only used for demonstration.

(Hijmans, 2017) using the standard 19 WorldClim Bioclimatic variables with a 10-arc minute resolution. These layers were then cropped to match sub-Saharan Africa (20°N – 35°S , 20°W – 60°S). Two additional layers were constructed by first subtracting the mean temperature of the coldest quarter (Bio11) from that of the warmest quarter (Bio10), which indicates the broad mean temperature range over the annual cycle without including data from temporary extreme periods. Furthermore, another layer was created by subtracting the temperature of the driest quarter (Bio09) from that of the wettest quarter (Bio08). This produced a layer that indicates the temperature difference between the wet and dry seasons with positive values suggesting a warmer wet season and negative values suggesting a warmer dry season. These two layers were specifically created because previous studies have shown that temperature can act as an important cue for the expression of phenotypic plasticity in *Bicyclus* (e.g., Brakefield & Reitsma, 1991; van Bergen et al., 2017). Moreover, the seasonal difference in temperature is of special interest since whether the wet or dry season has the highest temperature varies across the African continent, suggesting that species may use different cues across these regions (Roskam & Brakefield, 1999). To enable an informative background point selection in our modeling, all raster cells classified as desert regions (both cold and warm) in the Köppen climate classification were removed as no *Bicyclus* species are known to occur in such arid climates. Finally, islands outside of the known range of *Bicyclus* such as Madagascar and smaller islands located east of Madagascar as well as all Atlantic islands, except those in the Gulf of Guinea, were removed.

To minimize correlation among environmental predictors, pairwise Pearson correlation coefficients for all the bioclimatic raster layers were calculated. Any pairs of climatic layers with a correlation of $\geq|0.75|$ were excluded, while still

trying to retain as many layers as possible that were judged to be biologically meaningful for our system. The selected set included seven climatic layers (average Pearson correlation coefficient = 0.48; range = 0.08–0.75): mean diurnal temperature range (Bio02), mean temperature of coldest quarter (Bio11), the temperature difference between wet and dry seasons (calculated as Bio08 – Bio09), mean temperature difference between warmest and coldest quarter (calculated as Bio10 – Bio11), annual precipitation (Bio12), precipitation seasonality (Bio 15), and precipitation of driest quarter (Bio 17). Note that Bio11 was used twice, once by itself and once to create an annual temperature range layer (Bio10 – Bio11; see Table 1) as both layers capture unique axes of temperature variation. Variable inflation scores were also calculated to ensure that none of the seven layers had a score of >10 (range = 2.13–3.79). All climate data preparation was done using the R packages *raster* ver 3.6-20 (Hijmans, 2023) and *usdm* ver 1.1-18 (Naimi et al., 2014).

Environmental niche modeling was carried out using the Maxent program (Ver. 3.4.1) (Phillips et al., 2006) choosing the settings according to previous studies (e.g., Estrada-Peña et al., 2013; Kolanowska et al., 2017; Merow et al., 2013; Pérez et al., 2014) and based on pilot runs. Ten thousand background points were extracted using a maximum of 100,000 iterations, and convergence was set to the threshold of 0.00001. We validated the models by using a bootstrap approach with 100 replicates per species (setting 20% of the data aside for testing in each replicate). To avoid overfitting, only linear, quadratic, and product features were allowed when creating the response curves, and the curves were allowed to be extrapolated outside of the training data range. Following completion of the modeling procedure and examination of the resulting distribution maps, all species with fewer than five occurrence points were excluded from

Table 1. List of climatic variables used for environmental niche modeling and predicted niche occupancy quantiles (PNO, lower = 20%, median = 50%, upper = 80%) used for extracting climatic value of variables.

Climatic variable	Bioclim code/description	PNO quantile used	Rational for choosing a specific quantile
Mean diurnal range	Mean of monthly (max temperature–min temperature) (Bio2)	80	Higher value indicates higher range, hence stronger seasonality
Mean temperature of coldest quarter	Bio11	20	Lower value indicates colder temperatures
Annual precipitation	Bio12	20	Lower values indicate lower precipitation
Precipitation seasonality	Coefficient of variation (Bio15)	80	Higher values indicate stronger variation, hence seasonality
Precipitation of driest quarter	Bio17	20	Lower values indicate drier conditions
Temperature range	Mean temperature warmest quarter (Bio10)–mean temperature coldest quarter (Bio11)	80	Higher values indicate higher range, hence stronger seasonality
Seasonal temperature difference	Mean temperature wettest quarter (Bio8)–mean temperature driest quarter (Bio9)	50	Values can be both positive (wet season is warmer) and negative (dry season is warmer), hence we choose median of PNO values
Absolute seasonal temperature difference (absolute)	Absolute value of (Bio8–Bio9)	80	Same as the above variable but absolute value. Here, higher values indicate higher range hence stronger seasonality

Note. Specific quantiles for each PNO were chosen to capture stronger seasonality or harsher climate (see Figure 1 for more details). The final variable was not used in the niche modeling.

further analyses, resulting in a final data set of 87 species. The test area under the curve score, one of the metrics used for examining model performance, for all the retained models, was consistently high (mean = 0.933; range = 0.768–0.999).

Extracting of predicted niche occupancy values

Continuous values from the environmental niche models that could be used to examine the association with the eyespot size plasticity (see below) were extracted. This was done by using the predicted niche occupancy (PNO) profiles for each species, following the method developed by Evans et al. (2009) as implemented in the R package *phyloclim* Ver 0.9.5 (Heibl & Calenge, 2018) (see Figure 1). The process involves integrating Maxent probability distributions with respect to each original climate variable and normalizing the data to enable straightforward comparisons between species of different predicted suitable range sizes. The PNO profiles provide both graphical and numerical representations of niche breadth and shape (see Figure 1). PNOs were generated using the average Maxent model prediction for each species and a bin width of 100 was used. Three different quantile boundaries, lower (20%), median (50%), and upper (80%), were then extracted for each species and climatic variable (see Figure 1) depending on the variable that best describes the more seasonal or extreme conditions. For example, the lower end of the distribution (lower 20% quantile) for annual precipitation includes drier habitats within a species range (see Figure 1 and Table 1 for the rationale behind choosing a specific quantile for each environmental variable). This procedure further allowed us to gain an estimate of the climatic tolerances toward the edge of the species niches but with the exclusion of long distribution tails with low probabilities of occurrence on either side of the PNOs (see Figure 1). In addition to the seven climatic variables used in constructing the niche models, we created an additional variable generating a PNO based on the absolute values in the seasonal temperature difference layer (Table 1).

Quantifying plasticity in the eyespot size

Bicyclus butterflies, like most other Mycalesina, typically show some degree of plasticity in their wing patterns; WSFs always have comparatively large and distinct eyespots compared to the small and inconspicuous eyespots of the DSFs that can sometimes be missing entirely (Brakefield & Larsen, 1984; Halali et al., 2021a; van Bergen et al., 2017). Moreover, morphological changes such as the tone of colors and the width of contrasting bands also vary between forms, but eyespot size has been consistently found to be a good predictor of overall changes in the wing pattern, behavior, and life-history traits (e.g., van Bergen & Beldade, 2019; van Bergen et al., 2017). To quantify the plasticity of eyespots across *Bicyclus*, specimens were photographed from eight museum collections (see Acknowledgments) and the personal collections of one of the authors were used (see Supplementary Material for details on photographing the specimens).

We acknowledge the limitation of quantifying plasticity using museum specimens as the overall variation may not be entirely captured, especially when species are rare in museum collections or sampled only during a specific period of the year. However, to minimize such bias one of the authors screened >30,000 museum specimens aiming to choose, and photograph sets of individuals displaying the extreme ends of the phenotypic spectrum (i.e., seasonal forms) normally found in each species to capture as much of the variation as

possible, but excluding any obvious aberrations. Furthermore, to ensure that photographed specimens were representative of their species and captured the phenotypic spectrum, standard taxonomic literature (e.g., Condamin, 1973; Larsen, 2005; Vandeweghe, 2010) was used and we additionally relied on our own extensive field experience. Analysis was restricted only to males because of their higher abundance in collections, and the fact that females of some species are difficult to identify to species level solely based on wing pattern.

Based on our experience from the field, many species show true polyphenism in nature with intermediate forms being infrequent or very rare (Brakefield & Reitsma, 1991; Halali et al., 2021a; Windig et al., 1994), even when laboratory experiments have shown that species can potentially have continuous reaction norms (e.g., van Bergen et al., 2017). Some species show a rather high degree of plasticity but without exhibiting distinct WSF and DSF phenotype, while some species display a more or less constant WSF- or DSF-like phenotype (e.g., Brakefield & Frankino, 2009).

To more effectively capture the full variation in wing patterns, both continuous measurement and discrete classifications of plasticity were derived. First, the diameter of the hindwing CuA₁ eyespot along a line parallel to the hindwing CuA₁ and CuA₂ veins was measured using the outer edge of the yellow eyespot ring used as end points (Supplementary Figure S1). Additionally, the straight-line distance between the proximal and distal ends of the forewing CuA₂ vein was also measured as a proxy for wing size (e.g., Brattström et al., 2020, Supplementary Figure S1). The relative eyespot size was then calculated as the ratio of eyespot diameter and the proxy of wing size. The hindwing CuA₁ eyespot was chosen because it is present in all Mycalesina taxa and has also been extensively used as a proxy for plasticity in reaction norm experiments (e.g., Brakefield et al., 1996; Wijngaarden & Brakefield, 2001) and evo-devo studies (e.g., Bhardwaj et al., 2020; Brattström et al., 2020). The photos were measured using a custom-written macro implemented in the software ImageJ (Schneider et al., 2012). In total, wing traits were measured for 1,418 males from 87 *Bicyclus* species. However, two species (*Bicyclus dekeyseri* and *Bicyclus heathi*) for which <5 individuals were measured were excluded from the dataset resulting in a total of 85 species, which were used for phylogenetic analyses (see below; number of specimens measured per species is provided in the Supplementary Table S2).

We acknowledge that using ratios can be problematic when the allometric scaling deviates from isometry (Nakagawa et al., 2017). To ensure that the variation in the relative eyespot size is not arising purely due to changes in the wing length, several exploratory analyses were carried out. First, performing linear regression (\log_{10} eyespot size ~ \log_{10} wing length) including all species suggested that there was no allometric relationship between the eyespot size and wing length (Supplementary Figure S2). Furthermore, fitting linear regressions separately for each species, being aware of the fact that sample sizes varied widely across species (Supplementary Table S2), also suggested that the majority of fits were non-significant (Supplementary Figure S3). This lack of allometry is not surprising, especially for highly plastic or polyphenic species, as the eyespot size can show a strong bimodal distribution for a similar wing length (Supplementary Figure S4; Halali et al., 2021a). Second, density distribution for the raw eyespot size and relative eyespot size was visualized for a subset of species for which >20 specimens were measured and for

three wild species with an adequate sample size (data from Halali et al., 2021a). These distributions were all extremely similar comparing both measurements across all species (Supplementary Figures S4 and S5). Overall, we acknowledge that isometric scaling between the eyespot and the wing length would have been ideal for using ratios. However, our exploration suggests that the variation in the relative eyespot size in this study is to a major extent driven by the variation in the absolute eyespot size, and is not simply driven by the variation in the wing length.

For categorical classification of plasticity patterns, we first explored the overall span of the relative eyespot size (i.e., the difference between the largest and smallest eyespots in each species) (Figure 2B). Small and large eyespot size spans indicate narrow and wide variations in the eyespot size, respectively. It should be noted that these discrete classifications are only used for visualization and do not affect any of the analyses, but remain crucial in demonstrating the spectrum of plasticity species can exhibit. For discrete classification, two arbitrary thresholds (lower = 0.1, upper = 0.2) were set for classifying species into five categories based on the extent of eyespot span (Figure 2B–D): (a) constant or invariant (DSF) that only ever show small eyespots resembling dry season phenotypes and the eyespot span does not cross the lower threshold; (b) plasticity small (PLS)—small eyespots that cross the lower threshold but do not reach the upper one; (c) potentially polyphenic (POLY) (henceforth polyphenic)—species that cross both thresholds showing clear dry-season and wet-season phenotypes, and generally having the greatest eyespot size span; (d) plasticity large (PLL)—species with large wet-season eyespots, but that cross the upper threshold toward smaller spots; and finally (e) constant WSF—species that only show large eyespots such that even their smallest eyespots never cross the upper threshold. One species (*Bicyclus rhacotis*) did not fit any of these criteria; hence, this species was left unclassified. This species was still used in all analyses using the eyespot range data but not represented as a group in categorical comparisons.

Phylogenetic comparative analyses

A recently published phylogeny of *Bicyclus* butterflies from Aduse-Poku et al. (2022) was used for carrying out phylogenetic analysis. The phylogenetic signal for the eyespot range and all environmental variables was estimated using Pagel's lambda (function: *phyl.sig*) with 999 randomization tests to assess whether lambda was significantly different from zero. The value of Pagel's lambda ranges from 0 to 1, where 0 indicates that there is no phylogenetic information in the data and 1 indicates that the trait follows the Brownian motion model. Furthermore, five different homogenous-rate evolutionary models (function: *fitContinuous*) were fitted for the eyespot range: the Brownian motion, Brownian motion with trend, Ornstein–Uhlenbeck, early burst, and white noise, and the best model was chosen based on Akaike information criteria (AIC) score. Ancestral state reconstruction for eyespot range (function: *contMap*) was carried out using the Brownian motion model. The R package *phytools* ver 1.9.9 (Revell, 2012, 2024) was used to compute phylogenetic signal and for ancestral reconstruction and *geiger* ver. 2.0.11 (Harmon et al., 2008; Pennel et al., 2014) for fitting evolutionary models for continuous traits.

Phylogenetic generalized least squares regression (PGLS) (Grafen, 1989) was used to test the correlation between

the degree of eyespot size plasticity (or eyespot range) as a response variable and the extracted seasonality measurements from the eight PNOs as predictors (see Table 1). All predictors were standardized to have a mean of zero and an SD of 1 before fitting the model. Each PGLS model was fitted with the Brownian motion, Ornstein–Uhlenbeck, and Pagel's lambda correlation structure. An additional model was fitted where Pagel's lambda was fixed to zero which is equivalent to the ordinary least squares regression. The best model was chosen based on the AIC score, and when the score was similar, models were compared using the likelihood ratio test. PGLS models were fitted using *nlme* ver. 3.1.162 (Pinheiro et al., 2023).

Sensitivity analyses

Sensitivity analyses were carried out to test how alternative ways of measuring eyespot size plasticity were correlated with the original measure of plasticity (quantified as the range of the relative eyespot size, see above). First, coefficient of variation (CV) in relative eyespot size was calculated for each species with the expectation that highly plastic/polyphenic species will have higher CV than nonplastic species. Second, instead of using the highest and lowest relative eyespot size for calculating the eyespot range, 20% highest and lowest eyespot size values were chosen, averaged, and then the range was calculated from the averaged values.

Next, sensitivity analyses were carried out to test whether the PGLS estimates and significance were robust to different PNO quantiles and sample size. In addition to 20% or 80% quantiles (as in the original analyses, see above), 15% and 25% quantiles (when 20% quantile was used for a variable), and 75% and 85% quantiles (when 80% quantile was used for a variable) were extracted. PGLS were then performed on these additional quantiles and their estimate values, and significance was compared to the original quantiles. Furthermore, PGLS were performed on a separate dataset including only those species for which 15 or more individuals were measured. For all PGLS, Brownian motion, Ornstein–Uhlenbeck, and Pagel's lambda correlated were fitted with an additional OLS model where Pagel's lambda was fixed to zero. Best-fitted model was chosen based on the AIC score (see Supplementary Tables S7 and S8).

Finally, sensitivity analyses were carried out to detect influential species, the effect of sample size, and phylogenetic uncertainty on the phylogenetic signal and estimate values and significance of regressions (at $\alpha = 0.05$), using the R package *sensiPhy* ver 0.8.5 (Paterno et al., 2018). Pagel's lambda correlation structure (which was the best-fitting correlation structure for all phylogenetic regressions) was used for all regressions in the sensitivity analyses. Influential species were detected by sequentially removing species from the dataset and then estimating the magnitude of change in the parameter estimates (Pagel's lambda, estimate and p values of regressions). For testing the effect of the sample size on phylogenetic signal and significance of regressions, 10%, 20%, 30%, or 40% of species were removed in turn from the dataset and each removal was simulated 100 times. Finally, the effect of phylogenetic uncertainty was quantified by randomly choosing 500 posterior trees from Aduse-Poku et al. (2022). This procedure was simulated 100 times such that a random set of 500 trees was chosen each time. Note that sensitivity analyses were only carried out on significant regressions (7 of 8 regressions, see *Results*) when the entire dataset was used.

A) Examples of *Bicyclus* species from each plasticity category

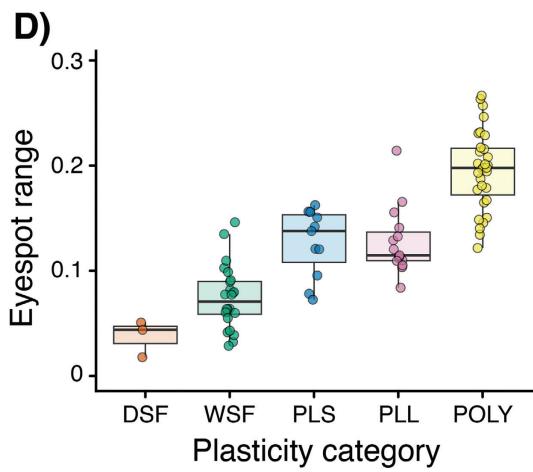
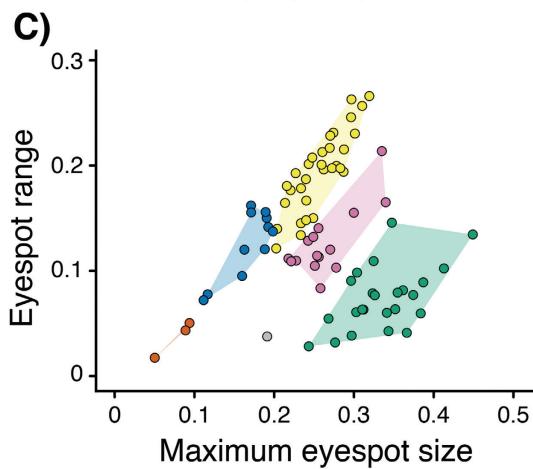
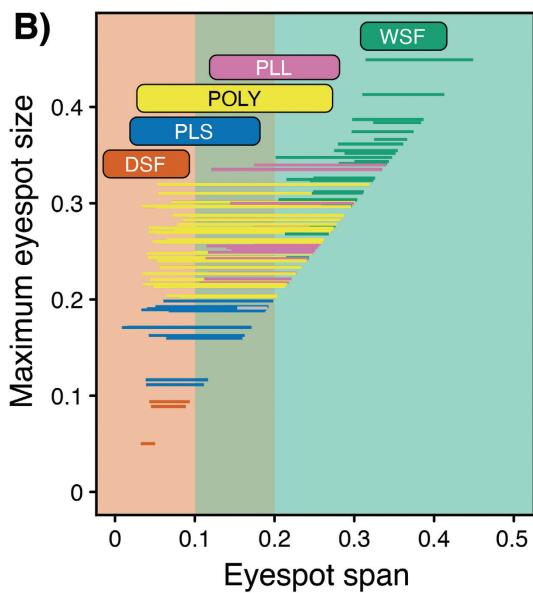
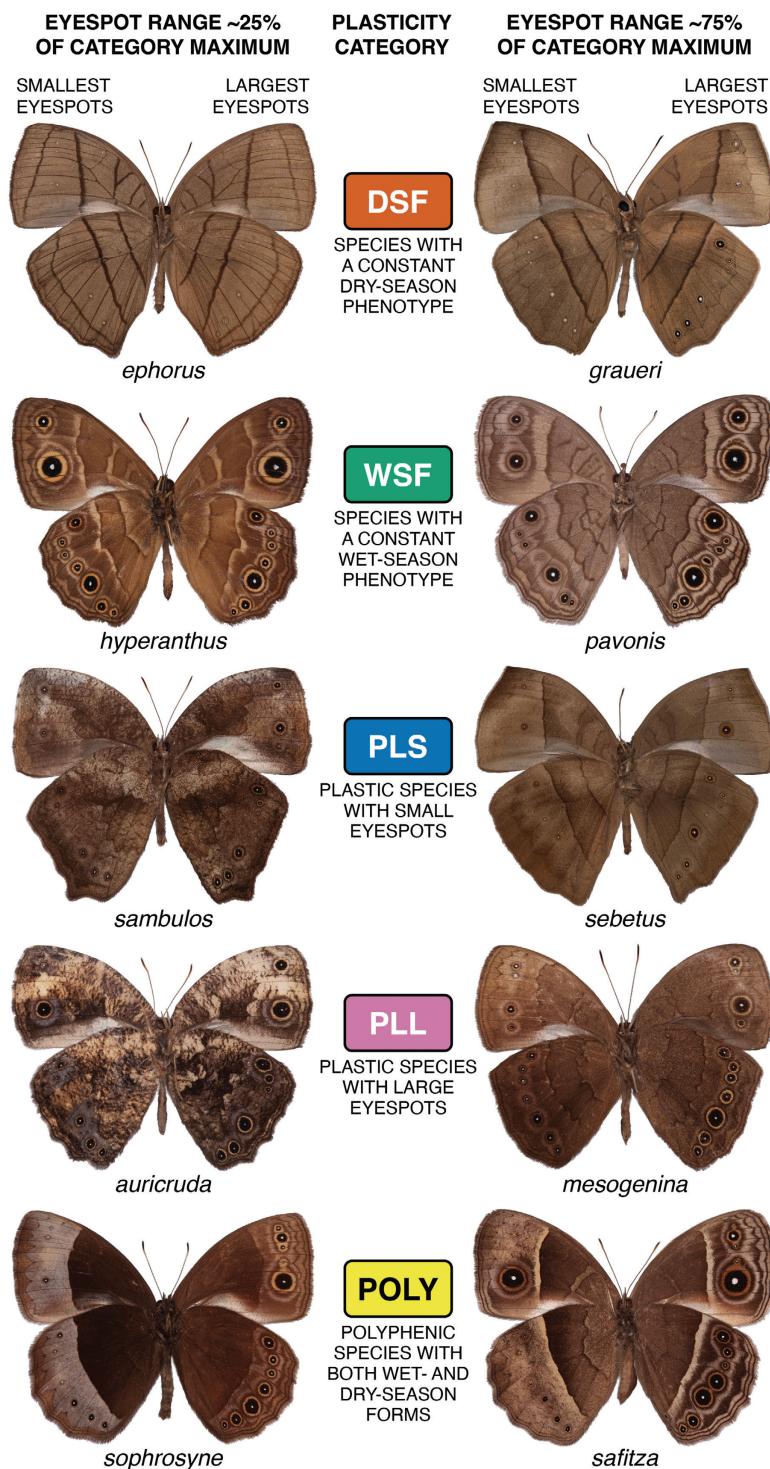


Figure 2. (A) Examples of representative *Bicyclus* species from the five categories for plasticity pattern as used in this study. Each butterfly in the image is a photomontage of two different specimens, with left and right wings, and then depicting the smallest and largest eyespots in our dataset found within each of these species. Within each category, the pairs of species depicted as examples have eyespot ranges corresponding to ~25% (left column) and ~75% (right column) of the maximum range found within their respective plasticity categories; (B) span of the eyespot size (colored lines) and thresholds used for classifying the five categories (overlay color) are indicated together with examples of species from the categories and how they relate to the upper and lower thresholds (wider colored boxes); (C) polygons showing the distinct clusters of plasticity categories with respect to the relative size of the largest spots found in a species and the range of observed plasticity; (D) box plot depicting how the measurement of plasticity as a continuous variable (as eyespot range) align with the discrete plasticity categories. For all plots, the color of each point or line matches its plasticity category.

Results

The span of the eyespot range across 85 *Bicyclus* species shows that they exhibit the entire spectrum of plasticity from strongly plastic (including potential polyphenism) to nonplastic or an invariant phenotype either showing WSF- and DSF-like wing pattern (Figure 2A). The eyespot range, a proxy for the eyespot size plasticity, exhibited a moderate but significant phylogenetic signal (Pagel's lambda = 0.46; likelihood ratio test, $p < 0.001$, likelihood profile is shown in Supplementary Figure S6). The phylogenetic signal for all environmental variables was rather high with Pagel's lambda ranging between 0.68 and 0.92, except for a single variable (precipitation of driest quarter) with a value of 0.28 (Supplementary Table S3).

Fitting several homogenous-rate trait evolution models for the eyespot range provided the best support for the Ornstein–Uhlenbeck model (AIC weight = 0.91) (Supplementary Table S4). However, we interpret this result with caution as there were issues with convergence in the maximum likelihood estimate for Ornstein–Uhlenbeck, early burst, and Brownian motion with trend models (Supplementary Table S4).

Ancestral state reconstruction for the eyespot range further suggested that ancestral *Bicyclus* species likely exhibited moderate eyespot size plasticity and that strong plasticity (and likely polyphenism) evolved multiple times with timings approximately corresponding to the late Miocene–Pliocene period (8–3 MYA), and even earlier in some species (Figure 3).

In all regressions save one, Pagel's lambda correlation structure had the best fit (Supplementary Table S5) and there was a significant association between the eyespot range and the degree of seasonality in the environmental variables (Figure 4, Table S6). That is, all species that occurred in highly seasonal environments or savannahs were in the polyphenic category (POLY) with the single exception of the nonpolyphenic (category WSF) species, *Bicyclus pavonis* (Figure 4). Polyphenic species also had the widest distribution occurring in both seasonal and less seasonal environments (Figure 4). In contrast, species from all five categories including strongly plastic to nonplastic (WSF, DSF, PLL, PLS, POLY) occur in less seasonal habitats (Figure 4). Furthermore, plotting species occurrence points according to plasticity pattern categories complemented the patterns observed in the regressions (Figure 5). For example, species in the nonplastic DSF and WSF categories occurred in more evergreen habitats, except *B. pavonis* (WSF category), which extended into much seasonal environments, while species from the POLY category had the widest distribution.

Sensitivity analyses suggested that two alternative measures of plasticity (CV and eyespot range calculated by averaging 20% highest and lowest values) correlated strongly with the original measure of eyespot plasticity (Supplementary Figure S7). Next, sensitivity analyses for testing the effect of using additional PNO quantiles (15/25% or 75/85% quantiles) suggested that the PGLS estimates and significance were similar to the original estimates (Supplementary Figure S8). Sensitivity analyses for testing the effect of sample size (i.e., choosing species for which ≥ 15 individuals were measured) resulted in slightly different estimates but significant regressions (Supplementary Figure S9). This was because of using the OLS regressions (i.e., Pagel's lambda = 0), which had the best fit compared to the Brownian or Ornstein–Uhlenbeck correlation structure (Supplementary Table S8). Pagel's

lambda correlation structure could not be used in the model comparison due to convergence issues.

Sensitivity analyses of Pagel's lambda value for the eyespot range identified four influential species, and the removal of these species one by one either increased the lambda value to a maximum of 0.59 or decreased it to a minimum of 0.37 and was significant in all cases (Supplementary Table S9, Supplementary Figure S10). Moreover, and as expected, variance in lambda value increased with the percentage of species removed from the data but remained significant in 81% of simulations even after removing 40% of species from the dataset (Supplementary Figure S12). Finally, lambda values remained similar and significant across 500 posterior trees (Supplementary Figure S14). Furthermore, sequentially removing each species and then fitting the OU model to the eyespot range had a strong effect on the AICc score with most values close to the white noise model (Supplementary Figure S11).

In most instances, sensitivity analyses conducted to identify influential species and quantify their effect on the estimate and significance of phylogenetic regressions had only a minor to moderate effect on estimate values (Supplementary Table S10). In some cases however, for example, when the mean diurnal range and mean temperature of the coldest quarter were used as predictors, the removal of a single species (*Bicyclus sylvicolus*) resulted in nonsignificant regressions (Supplementary Table S10). Furthermore and unsurprisingly, the percentage of significant regressions decreased with the percentage of species removed from the dataset (Supplementary Figure S13). However, even when 40% of species were removed from the dataset, >80% of regressions remained significant when four environmental variables were used as predictors, and overall, >60% of regressions remained significant (Supplementary Figure S13). Finally, incorporating phylogenetic uncertainty had only a minor effect on the regression estimates, and in all cases, regressions remained significant (Supplementary Figure S15).

Discussion

Seasonal polyphenism, a special case of strong phenotypic plasticity and evolutionary innovation, is a remarkable example of natural selection that reflects a crucial adaptation to alternating seasonal environments in butterflies (Brakefield & Larsen, 1984; Kingsolver, 1995; Shapiro, 1976). Despite being one of the clearest examples of adaptive phenotypic plasticity, investigations of the evolution of seasonal polyphenism in a macroevolutionary framework are largely lacking (but see Fric et al., 2004; Bhardwaj et al., 2020; for other examples of polyphenism, see Ledón-Rettig et al., 2008 and Susoy et al., 2014). Here, for the first time, we show that all *Bicyclus* species from markedly seasonal habitats (with one notable exception) exhibit strong plasticity with regard to eyespot size, most of them likely being truly polyphenic. However, we also find that species from less seasonal habitats exhibit a wide range of plasticity patterns from nonplastic (with both static dry- and wet-season-like patterns) to strongly plastic, including the potential to express true polyphenism (Figure 4). Furthermore, our ancestral state reconstruction suggests that early *Bicyclus* species likely exhibited moderate plasticity in eyespot size, an expected finding as plasticity in eyespot size is deep rooted in butterflies (Bhardwaj et al., 2020). The reconstruction also shows that strong plasticity,

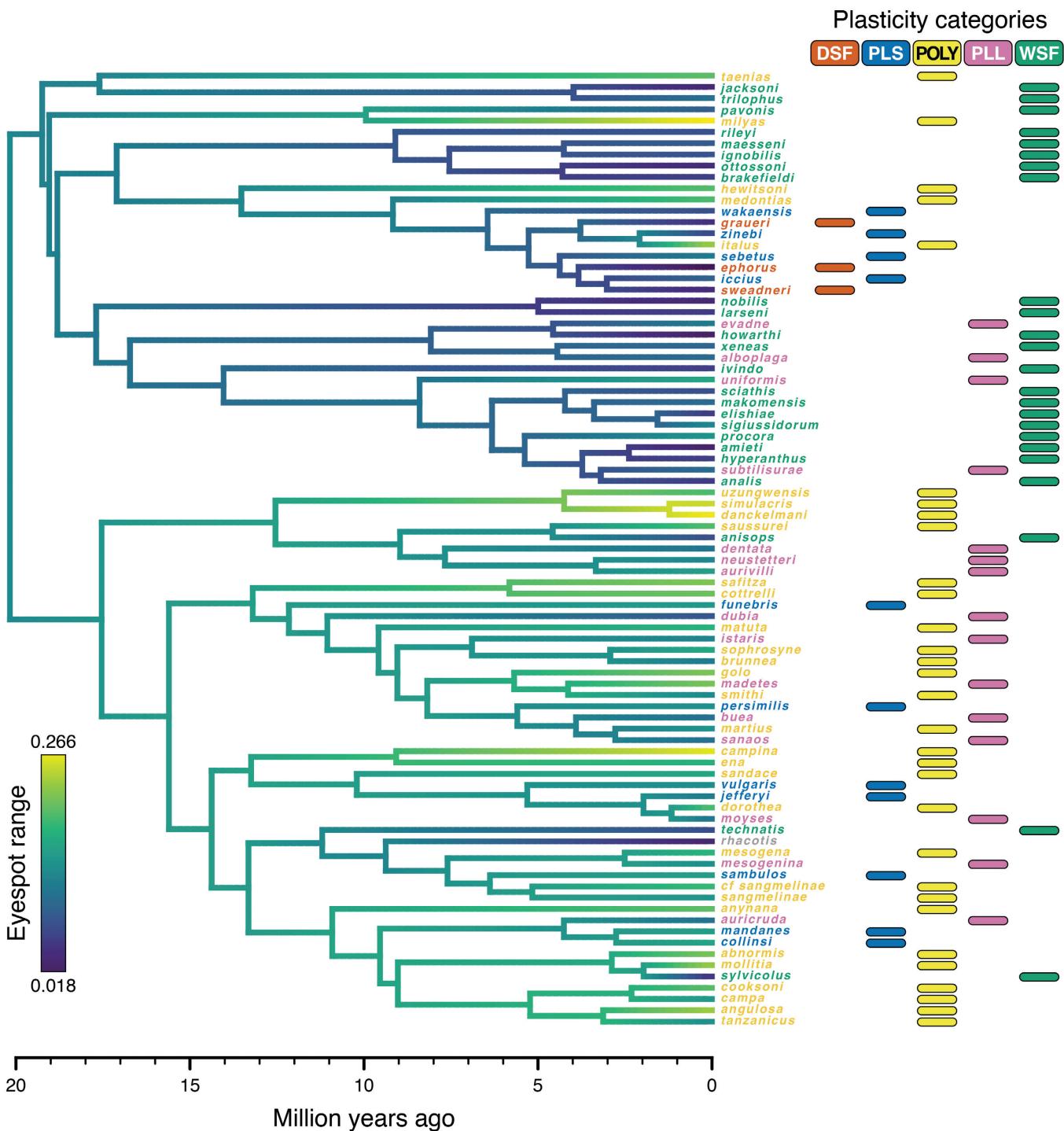


Figure 3. Ancestral state reconstruction for eyespot range for 85 species of African *Bicyclus* butterflies. The colored symbols to the right and the color of the species names indicate the plasticity pattern category of each species. A single species (*Bicyclus rhacotis*) did not fit into any of the five categories, showing an intermediate form in all investigated specimens.

potentially enabling the evolution of true polyphenism, began to evolve more frequently from moderately plastic ancestors during the late Miocene and Pliocene, coinciding with the time when *Bicyclus* began to invade savannahs (Halali et al., 2021b; Aduse-Poku et al., 2022). This pattern needs to be interpreted with some caution as ancestral states were reconstructed assuming the Brownian motion model, which was not the best-fitting model for our measure of plasticity. Moreover, in the absence of a fossil record, any estimate of

ancestral state is merely a phylogenetic-weighted average and would never estimate value outside the extant diversity.

We acknowledge that our study is based on the survey of wild-collected specimens, and we cannot state with certainty that those species not exhibiting plasticity in the wild are truly nonplastic without performing common garden experiments. But nevertheless, species habitat characteristic (such as degree of seasonality), at least to some extent if not entirely, can offer some explanation on why such diverse patterns

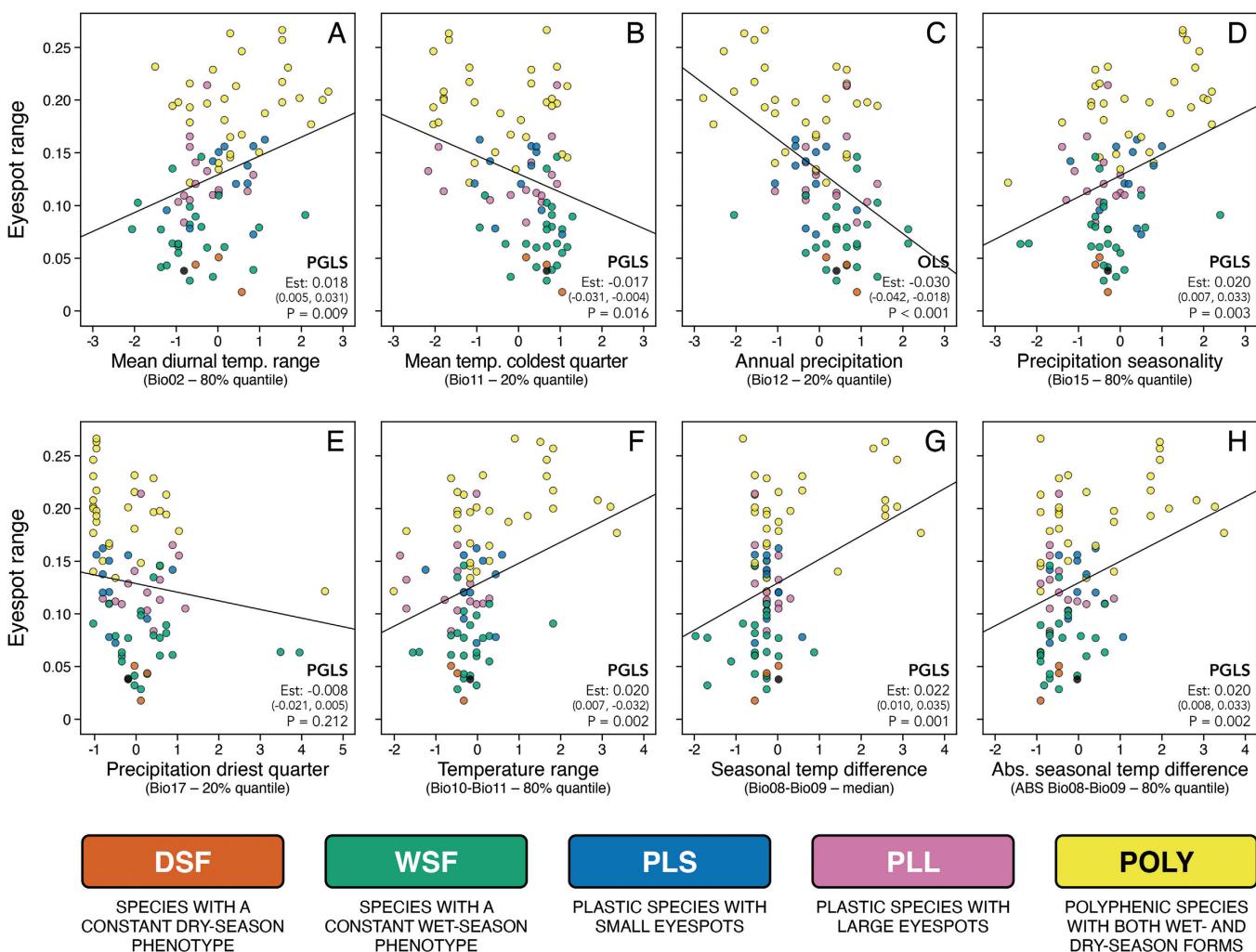


Figure 4. Regressions (A–H) showing the relationships between each of the environmental variables (standardized predicted niche occupancy [PNO] values with specific quantiles for eight climatic variables) and the eyespot range. The lower right corner inset for each plot indicates whether phylogenetic (PGLS) or ordinary least squares regression was used (see Methods for details) along with the estimated value, its confidence intervals, and *p* value. Points in scatter plots are colored according to the plasticity categories as depicted at the bottom.

plasticity may exist in nature (see below, also see Dongmo et al., 2018; Nokelainen et al., 2018). This aligns with the fact that phylogenetic signal in the eyespot range was moderate but significant (Pagel's lambda = 0.46 and up to 0.59 when an influential species is removed) despite strong niche conservatism, although we cannot entirely ignore the role of shared ancestry in the evolution of plasticity. We also acknowledge that we were not able to estimate error in our measurements, especially for the eyespot size plasticity, and thus not able to incorporate this error into regressions as would have been ideal. Overall, after taking all the caveats into account, we still found a strong support for a link between the degree of plasticity and climatic seasonality on a macroevolutionary scale.

An ecological framework for explaining the diversity of eyespot plasticity

Support for the hypothesis that both large (WSF) and small eyespots (DSF) can be adaptive, but only under specific conditions, is provided by both field and laboratory experiments. By releasing laboratory-reared WSFs and DSFs in the field during both the wet and dry seasons, using a mark–recapture

method Brakefield and Frankino (2009) demonstrated that DSFs have higher survival rates than WSFs in the dry season, but both forms have equal survival rates in the wet season. This indicates that there is an asymmetry in the strength of selection; exhibiting a WSF in the dry season is costlier than exhibiting a DSF in the wet season. Furthermore, Lyttinen et al. (2004) experimentally showed that WSFs are conspicuous on a background of brown leaf litter, and therefore, experience higher predation than DSFs in aviary experiments with Pied Flycatchers (*Ficedula hypoleuca*) used as predators. Prudic et al. (2015) then showed that Praying Mantises (*Tenodera sinensis*) were able to detect stationary WSFs much quicker than DSFs. However, WSFs were much more likely to survive an attack as their larger eyespots aid in deflecting attacks away from vital body parts (also see Chan et al., 2021; Ho et al., 2016).

These experiments provide support for a hypothesis that during increased activity (e.g., mate searching, courtship displays, egg laying, and feeding), adult individuals will benefit from having larger eyespots (i.e., WSFs), while during periods of lower activity, individuals will instead benefit from having smaller or no eyespots and being generally more cryptic (i.e.,

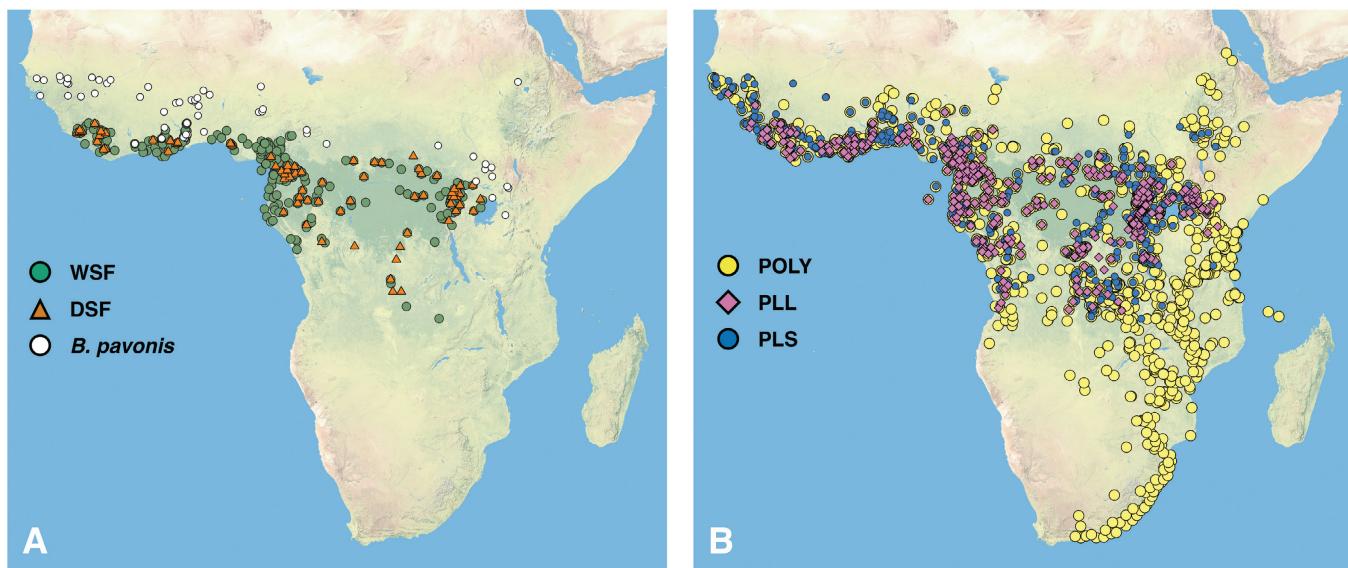


Figure 5. Plots of species occurrences on the ESRI World Physical Map layer according to their assigned plasticity category (see *Methods*). Each point on the map represents a unique location where one or more species within a specific plasticity category are documented. Briefly, WSF and DSF categories represent species with either nonplastic wet-season or dry-season form-like phenotypes in nature. Plasticity small (PLS) and plasticity large (PLL) categories exhibit a moderate to high degree of plasticity, but none of the species ever show more than one of the extreme phenotypes (wet or dry). Species in the potentially polyphenic (POLY) category typically exhibit the highest degree of plasticity in the eyespot size, with each species being capable of developing both wet and dry season form phenotypes.

DSFs). While the adaptive explanations mentioned above to some extent explain the maintenance of polyphenism in species in seasonal environments or savannahs that have received most of the attention (but see Halali et al., (2021a)), we currently lack hypotheses for the evolution of diverse plasticity patterns across species. Here we propose a new framework for the evolution of eyespot size in attempting to explain the diversity of plasticity patterns (including lack of plasticity) found across both species and habitats. Our framework incorporates habitat seasonality and time constraints, species life-history strategies, and the abundance of larval host plants to explain eyespot size variation. This framework is largely hypothetical but generates testable hypotheses for future studies.

As discussed above, the experimental evidence for the adaptive value of different eyespot sizes strongly suggests that the general activity level of a butterfly can be expected to match an optimal eyespot size. In our proposed framework (see Supplementary Figure S16 for the illustration), we therefore consistently expect larger eyespot sizes to be linked to an increase in activity levels, and that species activity levels are linked to different life-history strategies (i.e., slow to fast—Braby, 2002; Halali et al., 2021b).

In highly seasonal habitats, such as savannahs, there is a marked shift in host plant availability between seasons such that reproduction is only possible during the wet season. This imposes strong time constraints on reproduction, and in such environments, selection is expected to favor a faster pace of life (Braby, 2002; Halali et al., 2021b). We expect such a lifestyle including heightened courtship displays and matings, and a higher density of individuals to favor larger, more conspicuous, marginal ventral eyespots (as in WSFs), which can act as an antipredator device (Prudic et al., 2015). In contrast, the limited (or absent) food plants available in the dry season are expected to favor a slower pace of life, or even reproductive diapause (Braby, 2002; Halali et al., 2020,

2021a), and therefore smaller eyespots and cryptic wing patterns (as in DSFs). Taken together, we hypothesize that if eyespot size evolves as a correlated response to life-history strategy and some degree of environmentally induced plasticity is already present in a species, a stronger plasticity or even seasonal polyphenism is expected to evolve when colonizing such seasonal environments. The geographical distribution of potentially polyphenic species (category POLY) supports this scenario as they have the broadest distribution and occupy the driest savannah habitats and highly seasonal areas of southern Africa (Figure 5). A single unusual exception is *B. pavonis* (category WSF), a species that displays a constant WSF and extends further north into the Sahel region than any other *Bicyclus* species (Figure 5).

In less seasonal habitats, such as evergreen forests, canopy cover can buffer strong seasonal fluctuations and provide stable hostplant availabilities at various levels, hence enabling reproduction using various strategies throughout the year. Indeed, many forest species that breed throughout the year tend to exhibit a slower life-history strategy likely due to relaxed time constraints on reproduction compared to savannahs (Braby, 2002; Halali et al., 2020, 2021b). Furthermore, records of life span in the wild gathered from a mark–recapture study of butterflies in Kibale forest in Uganda included several forest species of *Bicyclus*. Many of these survived for at least 3 months as adults, and most of these long-lived species typically display small eyespots (category DSF and PLS) (Molleman et al., 2007). We hypothesize that in species with slower life-history strategies, having smaller eyespots or more cryptic wing patterns is likely to be highly beneficial to avoid detection from visually hunting predators. Alternatively, other species in the same habitat might adopt faster strategies or any range of strategies on the slow-fast continuum as species can reproduce throughout the year. In the scenario where a species is adopting a fast strategy (thus, similar to savannah species), having larger eyespots will be expected to

be beneficial. In summary and as proposed above, the diversity of eyespot size seen in stable environments may evolve as a correlated response to the broad range of possible underlying life-history strategies. Overall, we posit that the increased complexity of forests provides a range of micro-habitats that may act as “micro” selective regimes allowing the co-existence of several plasticity patterns within the same general habitat. The geographical distribution of plasticity patterns further complements our hypothesis. For example, species that exhibit nonplastic wing patterns (category WSF and DSF) and those that do not express polyphenism (category PLS and PLL) have the most forest-restricted distributions (Figure 5).

Putative macroevolutionary pathways for the evolution of eyespot size plasticity

The macroevolutionary framework of this study allows us to propose new hypotheses and generate testable predictions regarding the evolution of polyphenism in *Bicyclus* and more broadly Mycalesina butterflies. We first propose that the evolution of eyespot size plasticity follows the plasticity-first hypothesis (Levis & Pfennig, 2016) such that moderate ancestral plasticity was refined by selection to increase or decrease, likely by altering the slope of the population reaction norm, and thus evolve into either true polyphenism or a more canalized response. There is some support for this hypothesis. For example, savannah species or populations tend to show steeper reaction norms or higher plasticity than moderately plastic forest species (e.g., Dongmo et al., 2018; Roskam & Brakefield, 1996; van Bergen et al., 2017; Zhen et al., 2023). In addition, we propose an alternative hypothesis where species are inherently plastic with similar slopes for their reaction norm but with a range of intercepts, and where the extent of variation along the environmental axis explains the degree of plasticity. Species in savannahs exhibit a higher degree of plasticity (or polyphenism) simply because this habitat is more temporally heterogeneous compared to forests (see de Jong et al., 2010; Nokelainen et al., 2018; Oostra et al., 2014).

Both hypotheses are mutually nonexclusive, and it is quite likely that the current diversity in the eyespot size and its plasticity is the combination of both processes. However, the common denominator is that both hypotheses predict ancestral species to be (moderately) plastic. We acknowledge that this study is based solely on the patterns in the wild and we are not certain whether those species that do not show plasticity in the wild are truly nonplastic. We believe that future common garden experiments, especially estimating reaction norms for forest species exhibiting strong or no apparent plasticity, will allow disentangling of the two hypotheses.

Conclusion

Phenotypic plasticity can be crucial during the early phases of niche shifts and can even give rise to evolutionary innovations (Levis & Pfennig, 2016; Mozeck et al., 2011; Price et al., 2003). Here, using the radiation of *Bicyclus* butterflies in sub-Saharan Africa, we first demonstrate that species in seasonal environments generally exhibit stronger phenotypic plasticity, suggesting that it may have played a key role in promoting colonization of seasonal savannahs. Moreover, we also show that species in less seasonal habitats show a variable degree of plasticity, ranging from highly plastic to an apparent lack of plasticity with fixed wet- and dry-season-like phenotypes (a similar pattern was also observed by

Brakefield & Frankino, 2009). We propose an evolutionary framework to explain the adaptive value of this high diversity of plasticity patterns and develop two hypotheses about how plasticity may have evolved within the genus. It is now clear that future common garden experiments, focusing on forest rather than savannah species as has generally been the case until now, will be crucial to empirically test the proposed hypotheses. Although we focused on *Bicyclus*, which is the main African Mycalesina radiation, related genera from both Asia and Madagascar also frequently exhibit seasonal polyphenism. The fact that geographically separate clades across the whole Mycalesina subtribe have independently colonized highly seasonal environments such as savannahs (Halali et al., 2021b) suggests that polyphenism has evolved convergently as a response to increasing seasonality. This pattern fits the flexible stem model (e.g., Schneider & Meyer, 2017; Wund et al., 2008), indicating that ancestral plasticity may have biased the direction of evolution resulting in similar solutions—the evolution of strong plasticity or polyphenism—to similar problems, here in seasonal environments. The spectacular radiations of Mycalesina butterflies of some 320 species, as well as in other groups of polyphenic butterflies distributed across the tropics and temperate regions, offer unique opportunities to test how phenotypic plasticity promotes successful invasions of new adaptive zones and subsequent adaptive radiations.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

Raw data and R scripts used in the analyses can be accessed through Dryad at <https://doi.org/doi:10.5061/dryad.3n5t-b2rrt>

Author contributions

All authors contributed to designing the project. O.B. photographed all the museum specimens and S.H. analyzed these images for quantifying traits of interest. O.B. collected all the location records and carried out environmental niche modeling. S.H. carried out phylogenetic analyses. Both S.H. and O.B. wrote the manuscript together, with inputs from P.M.B.

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Conflict of interest: The authors declare no conflict of interest.

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