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Seasonal plasticity in sympatric *Bicyclus* butterflies in a tropical forest where temperature does not predict rainfall

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Abstract

While variation in temperature appears to be the main environmental cue for plasticity in adult traits in many species of Mycalesina, relying on temperature would result in a mismatch between adult phenotype and environment in some regions. We measured phenotypes of six species of *Bicyclus* butterflies (Nymphalidae: Satyrinae: Mycalesina) in a humid tropical forest with two rainy seasons per year and modest unimodal seasonal temperature variation, such that temperature does not predict rainfall and butterflies can reproduce year-round. The butterflies showed subtle temporal variation in body size and relative eyespot size, while relative androconia length was robust to temporal environmental variation. After higher temperatures, body size tended be smaller, and relative eyespot size was larger for some species-eyespot combinations. This indicates that these butterflies follow the "hotter is smaller" rule, and show developmental plasticity in eyespot size that is typical in this clade. Eyespot sizes tended to be correlated with each other, except Cu1 in *B. auricruda* and some eyespots that always remained very small. Androconia length was not related to eyespot size. This pattern of correlations suggests conserved cue-use and shared mechanisms for eyespot size using both temperature and rainfall-related cues, with some exceptions.

Keywords: body size, climate, constraint, eyespots, phenotypic plasticity, developmental linkage, time-series.

1. INTRODUCTION

The tropics host a wide range of ecosystems, ranging from rainforests with minimal seasonality, to more open habitats that are typically characterized by strong seasonal fluctuations in rainfall and temperature. To cope with such environmental variation, many organisms show adaptive developmental plasticity, where a single genotype uses environmental cues during early stages to produce a phenotype that maximizes fitness in the environment experienced during later stages (West-Eberhard 1989, Pfennig 2021). While phenotypic plasticity is usually studied in the context of its adaptive value in current environments, the trait likely arose in an ancestor lineage and was shaped by past selection in ancestral environments (Ghalambor *et al.* 2007, Bhardwaj *et al.* 2020). Since there may be evolutionary conservatism in the use of particular cues and shapes of reaction norms, such conservatism may affect if and how species adapt to later changes in their environment (Ghalambor *et al.* 2007). Evolutionary conservatism in developmental plasticity thus affects how species respond to climate change and to changes in their geographic range (Oostra *et al.* 2018). Conserved developmental plasticity is likely to be present in many extant species of a lineage where it may have retained its adaptive value.

Adaptive developmental plasticity often involves a functional suite of traits linked by a shared underlying (hormonal) regulator (Whitman & Agrawal 2009, Mateus *et al.* 2014, Oostra *et al.* 2014b, Forsman 2015, Uller *et al.* 2018). Such linkage may constrain the independent evolution of plastic traits when lineages experience environmental shifts that exert opposing selection pressures on different traits within the functional suite. An additional indicator of the existence of common developmental mechanisms is congruence of responses of different traits to the same environmental cue (Mateus *et al.* 2014). Therefore, to gain insight into such linkage between traits, we can compare responses to cues for multiple traits within populations.

Tropical butterflies of the subtribe Mycalesina (Nymphalidae: Satyrinae: Mycalesina) are prominent examples of seasonal plasticity. Mycalesina species that fly in open and highly seasonal habitats often exhibit distinct wing patterns in wet and dry seasons which is most apparent in the size of the eyespots (Brakefield & Reitsma 1991, Windig *et al.* 1994, Halali *et al.* 2024). Many Mycalesina species have large ventral eyespots along the wing margins in the wet season, and strongly reduced eyespots in the dry season. Small eyespots are thought to be adaptive during the dry season because these brown butterflies probably spend most of their time resting on leaf litter and dry vegetation during this period (Brakefield & Larsen 1984, Brakefield & Frankino 2009). In contrast, the butterflies cannot rely on crypsis to avoid detection by predators in the wet season because the abundant growth of larval food resources (fresh grass) causes the background to be green. Instead, wet-season-form butterflies rely on marginally placed eyespots that deflect predator attacks away from the vital body parts (Lyytinen *et al.* 2004, Prudic *et al.* 2015, Ho *et al.* 2016, Halali *et al.* 2019, Chan *et al.* 2021). In addition to eyespot size, many other phenotypic traits also show distinct seasonal variation, including the colors of the eyespots and transversal bands (Mateus *et al.* 2014, Monteiro *et al.* 2015), life history traits such as body size and reproductive diapause (Pijpe *et al.* 2007, Halali *et al.* 2021), and behavioral traits such as mate choice and predator avoidance behavior (Prudic *et al.* 2011, van Bergen & Beldade 2019). Dry season forms are larger and have greater fat reserves, which is thought to improve their ability to survive dearth periods (Pijpe *et al.* 2007), while wet season forms may be smaller to reduce generation time (Kingsolver & Huey 2008). Notably, higher temperatures induce smaller body sizes in ectotherms (temperature-size rule a.k.a. "hotter is smaller"; Atkinson 1994), and this trait is thus not necessarily incorporated into the suite of linked traits. Seasonal forms also differ in pheromone production and the size of androconial patches (Dion *et al.* 2016, Balmer *et al.* 2018), and they might thus also differ in length of androconial brushes (hair-like scales that are involved in pheromonal communication; Bacquet *et al.*, 2015). Given their diversity, and seasonal plasticity of multiple traits, Mycalesina butterflies are excellent systems to understand evolutionary conservatism in developmental plasticity.

Even though rainfall is thought to be more important than temperature in determining the seasonal changes in coloration of the habitat (green versus brown) and resource availability (grasses for larvae to feed on; Valtonen *et al.* 2013), many Mycalesina butterflies use temperature as a cue to predict future conditions (Windig 1992, 1994a, Roskam & Brakefield 1996, Kooi & Brakefield 1999, Oostra *et al.* 2014a, van Bergen *et al.* 2017). This cue-use may be adaptive in large parts of the tropics where an increase in temperature predicts the onset of a period of increased rainfall (Brakefield & Reitsma 1991). In these environments, temperature may thus be used by caterpillars to predict the environmental conditions they will face during their adult life. Relative humidity does not appear to induce seasonal plasticity in Mycalesina butterflies (Brakefield & Reitsma 1991, Fischer *et al.* 2003), though it has been shown to play a role in determining pupal coloration in at least one species (Mayekar & Kodandaramaiah 2017). Indirect effects of rainfall on plant quality are predicted to be important because larvae that feed on grasses that cause slow growth tend to develop into dry season phenotypes (Kooi *et al.* 1996, Singh *et al.* 2020), and drought-stress in grasses – which is typical during dry seasons – tends to reduce larval growth rates in butterflies (Molleman *et al.* 2020). Photoperiod does not appear to be used as a cue in this clade (Brakefield & Mazzotta 1995). Therefore, dry-wet seasonal plasticity in Mycalesina butterflies seems to be regulated primarily by temperature, although we cannot rule out a role of other cues (Westneat *et al.* 2019, Halali *et al.* 2021, Rodrigues *et al.* 2021).

If developmental plasticity could evolve rapidly in Mycalesina butterflies, we would expect that temperature would not be used as a cue in regions where temperature does not predict rainfall (Roskam & Brakefield 1999, Halali *et al.* 2021). However, the use of temperature as a cue for developmental plasticity appears to be conserved across Mycalesina butterflies. When a Mycalesina species from a rainforest without strong seasonality was reared in the laboratory under a wide range of temperature conditions, it showed plasticity typical for species from seasonal environments, indicating that it had retained ancestral seasonal developmental plasticity (Oostra *et al.* 2014a). Furthermore, a comparison of responses to temperature among five Mycalesina species also suggested conserved reaction norms (van Bergen *et al.* 2017). Nevertheless, local adaption of developmental plasticity has been demonstrated within two species of *Bicyclus* (de Jong *et al.* 2010, Nokelainen *et al.* 2018), showing that reaction norms can evolve rapidly.

In the Mycalesina butterfly *B. anynana* (Butler, 1879), the thermal reaction norms of multiple traits – development time, adult mass, resting metabolic rate, and ventral eyespot size – have similar shapes, suggesting that the plastic responses of these traits are developmentally linked (Oostra *et al.* 2014a) and thus that their ability to evolve independently may be limited. However, the reaction norm of relative abdomen size differed from the other traits considered, suggesting that some traits can be uncoupled from others (Oostra *et al.* 2014a). Notably, in *B. anynana* ecdysteroid signalling is known to play a key role in regulating the life-history traits and wing-pattern elements whose reaction norms are coupled, whereas fat content does not appear to be controlled by this mechanism (Mateus *et al.* 2014, Oostra *et al.* 2014b). In addition, across five species, the shapes of the thermal reaction norm for development time, body mass, growth rate, and relative abdomen mass were generally similar across species, while relative fat content did not respond to variation in temperature in some species, again suggesting that this trait is uncoupled from the others (van Bergen *et al.* 2017). This indicates that traits involved in seasonal developmental plasticity in Mycalesina butterflies are usually linked, but that there are exceptions.

Here we investigate patterns of seasonal plasticity in six species of *Bicyclus* butterflies (Aduse-Poku *et al.* 2017) in a sub-montane tropical forest, by sampling periodically for 14 consecutive months, and quantifying body size, eyespot size, and the length of the androconia. Rainfall in this environment has on average a bimodal distribution with two dry and two wet seasons per year, while the daily maximum temperature follows a unimodal distribution (Valtonen *et al.* 2013, Molleman *et al.* 2022). Therefore, there is usually a cool dry season and a warm dry season. Firstly, this means that there would be selection against the typical thermal cue-use in Mycalesina, at least during part of the year. Secondly, the lack of correlation between temperature and rainfall makes this forest well suited to statistically tease apart the effects of these factors on butterfly phenotypes. We started by asking to what extent these butterflies exhibit seasonal plasticity in their natural habitat by testing for temporal autocorrelation in body size, relative eyespot size, and the length of androconial brushes. We then assessed to what extent temperature or rainfall could be used as cues for developmental plasticity, by determining if phenotypic traits are more often cross-correlated with temperature or with rainfall, also considering relative humidity as a possible cue. We further asked if cue-use and reaction norms are conserved for particular traits, by determining if each trait responds similarly to a given environmental cue across all species. Finally, we determined to what extent different plastic traits are linked within individual species.

2. METHODS

1. Data collection

Our study site was a sub-montane tropical forest near the Makerere University Biological Field Station (0°13’ - 0°41’N and 30°19’ - 30°32’E) in Kibale National Park, Western Uganda. In this region, there are two rainy seasons per year, while temperature has a unimodal distribution so that there is a warm and a cool dry season (Valtonen *et al.* 2013). Thus, unlike in study sites of previous studies (Oostra *et al.* 2014a, van Bergen *et al.* 2017), there is probably selection against developing a dry-season phenotype when temperatures are lower (and *vice versa*), at least during part of the year. During the study period, a data logger (Lascar EL-USB-2-LCD) was placed inside the forest, suspended 1 meter above the ground, and protected from direct rain and sunlight by a plate of roofing zinc. The logger recorded temperature, and relative humidity in half-hour intervals. In addition, daily rainfall and temperature data were obtained from a weather station at the field station (within 1km from all butterfly sampling locations; Chapman *et al.* 2018). To minimize the impact of our study on the local butterfly populations, we focussed our sampling efforts on male specimens. Up to ten individuals of the six most abundant *Bicyclus* species (*B. collinsi* (Hewitson, 1873); *B. mollitia* (Karsch, 1895); *B. smithi* (Aurivillius, 1899); *B. auricruda* (Butler, 1868); *B. golo* (Aurivillius, 1893), and *B. graueri* (Rebel, 1914)) were collected weekly from baited traps for 14 consecutive months (23rd July 2013 to the 26th of September 2014). All six species have eyespots in the distal region of the ventral wings (Figure 1). Whilst some species have multiple androconial brushes, all the investigated species have a prominent brush with its base located in the dorsal wing cell of the hindwing. This shared brush was selected as the androconial trait that could be compared across species.

All four wings of collected butterflies were placed on a Nikon grey card which was placed on graph paper (Figure 1) and photographed using a Nikon D7000 camera in a custom-made studio with constant light conditions (luminance and intensity) and the same manual settings of 1/125 shutter speed and F14 aperture for all photographs. We then used a macro in ImageJ to measure a proxy of the wing area of each wing, the area covered by four ventral eyespots, and the length of the basal hindwing androconial brush (Figure 2).

2. Data analysis

To obtain the average temperature for each day, we averaged the maximum and minimum temperatures provided by Chapman *et al.* (2018) rather than our own measurements as these also cover the months before butterflies were collected (necessary for cross-correlation analyses). We averaged rainfall and humidity by two-week period and month. As a proxy of body size, we averaged the area of triangles measured from forewings and hindwings (Figure 2). In general, wing area is a well-established proxy for body size in these butterflies (e.g. Bergen *et al.* 2024). We calculated relative eyespot size as eyespot area divided by the body size proxy. Relative androconia length was calculated as the length of androconia divided by wing length. To avoid periods with missing data due to low abundance of butterflies, species' averages of traits were calculated per two weeks or four weeks, depending on species abundance. The few remaining missing data points (5 out of 135) were replaced by the average of data points from two weeks before and two weeks after (for biweekly data) or four weeks before and four weeks after (for four-weekly data).

To assess whether butterflies showed seasonal dimorphism (distinct wet season and dry season morphs), we generated density plots of body size, eyespot size, and androconia length for each species using the R function *geom_density* from the package *ggplot2* (Wickham 2016, R_Core_Team 2024). We visualized temporal trends using the *loess* function in R (R_Core_Team 2024). To estimate whether traits were linked to habitat seasonality (seasonal changes in average phenotype), we performed autocorrelation analyses using the function *ggAcf* in the R package *ggplot2* (Hyndman & Khandakar 2008). Autocorrelation analysis tests whether within a single time series, there are correlations between data points that are a particular time lag apart, as would be the case with seasonal patterns (e.g. correlation between data points that are six months apart). To focus on seasonality, for each autocorrelation, we visually identified the lag with the most negative correlation coefficient and determined whether it was statistically significant, thus ignoring lags next to zero that typically show positive correlation coefficients. To test if temperature and rainfall drive temporal variation in butterfly traits, we performed cross-correlation analyses using the function *ggCcf* in the R package *ggplot2* (Wickham, 2016). In addition, we performed such analyses for relative humidity (related to both rainfall and temperature). Cross-correlation analysis tests whether two time-series are correlated with each other with a certain lag. We expected the lag to range within the length of development time, i.e. time between egg hatching and adult eclosion (about 6 weeks; Molleman *et al.* 2016, van Bergen *et al.* 2017) and development time with an added month to account for adult life span (Molleman *et al.* 2007) and an extra month if the effect is mediated by host-plant growth (Valtonen *et al.*, 2013; total 4 months lag). For each cross-correlation, we visually identified the lag with the highest correlation coefficient and noted the sign and whether it was statistically significant, focusing on lags of less than six months. Among butterfly species, we tested for correlation within traits (no lags), which may indicate an evolutionarily conserved mechanism across species. Within species, we tested for the degree of correlation among traits, which would indicate a linked developmental mechanism. Given the low number of species included in our sampling, we did not account for phylogenetic non-independence among species in our cross-correlation analyses.

3. RESULTS

1. Weather

During the study period, the maximum difference between the minimum and maximum weekly average temperatures was 4.3 °C. Temperature showed a unimodal distribution during the study period (Figure 3a). The weekly average daily rainfall ranged between 0 and 22 mm per day. Rainfall and relative humidity did not show the typical bimodal distribution pattern normally recorded for the site (Valtonen *et al.* 2013) due to unseasonal rain during a dry season (Figure 3b, c). Only relative humidity showed autocorrelation. There were no significant cross-correlations between temperature and rainfall or relative humidity (also for a longer period; Molleman *et al.* 2022).

2. Seasonality in phenotypes

If species show seasonal dimorphism (dry and wet season forms, but rarely intermediate forms), we would expect traits to have bimodal distributions, but all measured traits had a unimodal distribution in all studied species (Appendix 1, Fig. A1.1). There was significant seasonality in body size of *B. mollitia* (Table 1; Appendix 1, Fig. A1.2 and A1.3), and similar (but not significant) patterns in the other species (Appendix 1). Notably, the lag durations for body size were between two and four months, which corresponds more closely to the bimodal distribution of rainfall (three-month lag), than to the unimodal pattern of temperature (six-month lag). There was significant seasonality in the relative size of the forewing eyespot M1, the hindwing eyespot Cu1 of *B. mollitia,* the hindwing M1 of *B. collinsi* (Table 1; Appendix 1, Fig. A1.2 and A1.3), and similar (but not significant) seasonality in many of the other eyespots-species combinations (15 out of 24 species-eyespot combinations appear seasonal; Table 1). Notably, the lag durations for eyespot size were about six months, which corresponds more closely to the unimodal distribution of temperature than to the bimodal pattern of rainfall. Relative androconia length did not show clear seasonality (Table 1, Appendix 1 Fig A1.3).

3. Identifying drivers

For the three species with the highest sample size, we found 12 cross-correlations with temperature, 5 with rainfall and 10 with relative humidity (Table 2, see Appendix 1, A1.4a for cross-correlation plots). Butterflies tended to be smaller one to two months after higher temperatures, and larger one to two months after more rainfall and higher humidity (Table 2a). Eyespots tended to be larger two to five months after higher temperatures (five significant cross-correlations) and smaller two to five months after elevated humidity (four significant cross-correlations Table 2b-e). Androconia length showed no response to temperature or rainfall (Table 2f, Appendix 1, Figure A1.4f). Since androconia length hardly varied, its variation in relative length was driven by variation in body size.

Shared environmental responses among species

If species respond similarly to their environment, we expect within-trait correlations among species without a lag. For body size, we found that ten out of fifteen correlation coefficients were positive (i.e. butterflies of most species were relatively large at the same time, and *vice versa*), and four of these positive correlations were significant (Table 3a). Among eyespots, there were mainly positive correlation coefficients, and for each eyespot between five and eight of the fifteen possible correlations were significant (Table 3b-e), indicating that most species had relatively large eyespots at the same time, and *vice versa*. For example, for forewing eyespot Cu1, all fifteen correlation coefficients were positive and five were significant (Table 3b). Notably, the only negative correlation coefficients among eyespots were with M1 eyespots of *B. graueri* (both forewing and hindwing), and the eyespot sizes of this species never correlated with those of other species (Table 3b-e). For relative androconia length, all correlation coefficients between *B. collinsi*, *B. mollitia*, *B. smithi* and *B. golo* were positive, with seven being significant (Table 3f).

Shared mechanisms among traits within species

If developmental plasticity of different traits is regulated by a shared mechanism, we expect strong correlations between traits within species. Correlations between body size and eyespot size were infrequent (two out of 24 marginally significant positive correlations), while those between the different eyespots were common (26 out of 36), positive, and often significant. However, the hindwing eyespot M1 – which is very small in all species – was never significantly correlated with any of the other eyespots in any species, and no significant eyespot correlations were detected for *B. graueri.* Interestingly, in *B. auricruda*, the size of the forewing Cu1 eyespot was not correlated with any other eyespot. Relative androconia length was negatively correlated with body size in the three most common species, *B. collinsi, B. mollitia,* and *B. smithi,* and the correlation coefficient was also negative in the other species. This may be because androconia tend to be only slightly longer in larger individuals, so that relative androconia length decreases with body size. Notably, relative androconia length was not correlated to variation in relative eyespot size in any species (Table 4).

4. DISCUSSION

We measured body size, eyespot size, and androconia length of six species of *Bicyclus* butterflies for fourteen months in a tropical forest where temperature does not predict rainfall. Despite limited annual variation in thermal conditions, most species showed seasonal variation in the traits quantified. Body size showed seasonality linked to the bimodal distribution of rainfall, being larger after periods of higher humidity, and responded to temperature by following the "hotter is smaller" temperature-size rule (Atkinson 1994). Eyespots tended to be larger after higher temperatures, and smaller after increased humidity. The size of particular eyespots across time tended to be correlated across species, which suggests a common use of environmental cues and conserved mechanisms for eyespot plasticity across species. Within species, eyespot sizes tended to be correlated with each other, as expected when different eyespots in the same butterfly are linked through the shared developmental mechanisms. However, there were exception to these general findings.

Given that our study spanned only fourteen months, any correlation must be interpreted with caution. Furthermore, the range of average daily temperatures experienced was only 4.3 degrees Celsius and there was unusual rainfall during a dry season. More phenotypic variation may be observed when conditions are varied more in an experimental setting (e.g. van Bergen *et al.* 2017), but forest *Bicyclus* are notoriously difficult to rear in captivity (FM and OB personal observations). Furthermore, weather is multidimensional and may affect butterflies in a non-linear fashion (Roland & Matter 2016, Rodrigues *et al.* 2021). For example, vegetation greenness does not differ between wet seasons with modest rainfall and with heavy rainfall (Valtonen *et al.*, 2013), so that rainfall may also only affect butterfly phenotypes up to a certain threshold. Nevertheless, our results do suggest use of multiple cues, making the mechanism of seasonal polyphenism less clear cut than most lab-based studies have proposed. We further assume that in Mycalesina butterflies temperature is used as a cue for predicting rainfall, because rainfall affects resource availability and background coloration, and this is thought to affect the relative fitness of wet and dry phenotypes in the seasons. This indirect cue-use hypothesis is plausible and is commonly assumed to be correct (Brakefield & Reitsma, 1991; van Bergen & Beldade, 2019; Chan *et al.*, 2021; Halali *et al.*, 2021a). However, this has not been proven unequivocally. It is possible that temperature itself is the key variable, for example, because at higher temperatures satyrines may shift their activity to cooler times of the day, and thus are active at lower light intensities under which eyespots are more effective (Olofsson *et al.* 2010). We further assume that using mainly higher temperatures during immature stages as a cue for more wet-season phenotypes in the adult stage is the ancestral state in Mycalesina butterflies (Windig 1992, 1994b, Roskam & Brakefield 1996, Kooi & Brakefield 1999, Oostra *et al.* 2014a, van Bergen *et al.* 2017). However, other potential cues have rarely been investigated (Kooi *et al.* 1996, Singh *et al.* 2020, Rodrigues *et al.* 2021) and a full ancestral state reconstruction has not been attempted (but see Bhardwaj *et al.* 2020).

Cue-use and reaction norms appeared to be shared among most species. Five of the six species seem to follow the "hotter is smaller" rule (Atkinson 1994), being significant in *B. mollitia* and *B. smithi* (Table 2, Appendix 1 Figure A1.4). Higher temperatures appear to also induce larger eyespots in at least four species. In addition, humidity and rainfall also affect body size and eyespot size in some cases. More rainfall (or higher humidity) seems to increase body size and to decrease relative eyespot size. Moreover, auto-correlations within species often had a lag of about three months, which corresponds to seasonal variation in rainfall rather than in temperature. That the best cross-correlations were often at lags of three to five months may indicate that the effect of rainfall, and perhaps also temperature, may act via host plant quality. We would expect higher humidity to lead to higher host-plant quality which tends to lead to larger eyespots (Kooi *et al.* 1996), but we found the opposite. That larger eyespots are associated with larger body size, rather than the typically small-bodied wet season forms with large eyespots, may reflect the use of these multiple cues. At our study site, temperature is not a reliable predictor of rainfall (Valtonen *et al.* 2013), and we thus argue that there is selection against using temperature as the main cue for seasonal plasticity. Our data suggest that the response to temperature is to some extent conserved for most species, but that cues related to rainfall and/or humidity may be of similar importance. As effects of weather on butterfly phenotypes may be mediated by larval host plants, future studies could compare butterfly phenotypes with time series of host-plant quality.

Even though we find overall similarities among species across traits and within species among traits, there are notable exceptions that show that plasticity of the functional suite of traits is not fully conserved, and that certain traits may be decoupled. This evolvability of reaction norms across species is consistent with within-species adaptation to local climates (de Jong *et al.* 2010, Nokelainen *et al.* 2018). The decoupling of traits may be similar to the decoupling of a set of traits including eyespot and body size, and fat content in *B. anynana* (Oostra *et al.* 2014a, van Bergen *et al.* 2017). The decoupling of traits that we found in our study species seems to pertain mainly to keeping certain eyespots small under all circumstances. Most notably, *B. graueri* did not show similar cue use as other species and correlations among its traits were absent, Additionally, all its eyespots are typically tiny, so that there is little scope for temporal patterns. The difference between this species and the rest in developmental plasticity may be explained by it being relatively distantly related to the others in this study (Aduse-Poku *et al.* 2021). In addition, *B. graueri* larvae feed on gingers (Zingiberaceae; FM Pers. Obs.) while the others feed on grasses, and gingers may be less susceptible to drought conditions and grow in more sheltered habitats. A further notable exception is the eyespot Cu1 in *B. auricruda*. We might expect the forewing Cu1 eyespot in *B. auricruda* and *B. collinsi* to not respond to cues. This is a large eyespot that butterflies can hide behind the hindwing (avoiding cost of apparency), and probably serves to intimidate predators rather than to deflect attacks, so that reducing its size during the dry season has no obvious adaptive advantage.

Androconia length varied little across seasons and was not correlated with eyespot size. This indicates that there is little selection for differential pheromone production during different seasons. This may be because these species probably reproduce throughout the year, with overlapping generations, such that selection on male courtship is not seasonal (Valtonen *et al.* 2013), so that selection on male courtship would not be seasonal. Perhaps androconia length is seasonal in populations with more seasonal reproduction.

In conclusion, we show that six species of *Bicyclus* in a tropical forest in Uganda have modest temporal variation in body and eyespot size, and little variation in length of androconia, without clear seasonal dimorphism. Body size and eyespot size appear to respond both to temperature and to rainfall/humidity. The response to temperature followed the temperature-size rule "the hotter is smaller" and higher temperatures led to larger eyespots, as is suggested to be the conserved response in Mycalesina butterflies. Rainfall appears to increase host-plant quality which leads to larger butterflies with smaller eyespots. The lag duration of autocorrelations suggests that cues related to rainy versus dry seasons play a dominant role. Eyespots seem to be developmentally linked, with the exception of eyespots that tend to remain tiny and the large Cu1 eyespot in *B. auricruda*.

Taken together, our results underline that tropical insects can adapt to regional climates in flexible ways: some plastic traits show conserved responses to environmental cues across species, while the environmental responsiveness of other traits can evolve independently. Species are likely to arrive at different solutions to thrive in regions with various levels of seasonality and relationships between environmental cues. Therefore, the evolution of phenotypic plasticity is an important component of the evolutionary histories of lineages that inhabit the diverse environments found within the tropics. Moreover, cue-use may be vulnerable to climate change which not only affects average weather conditions, but also the predictive nature of environmental cues.

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Data Availability Statement

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.rjdfn2zm7

Tables

Table 1: Seasonality of phenotypic traits in six *Bicyclus* species in Kibale National Park, Uganda with lags in months (see Appendix 1, Figure A1.3 for auto-correlation plots). Numbers indicate the lag duration with the strongest cross correlation and the - indicates a negative correlation. Correlations at $p < 0.05$ are indicated in bold with *.

Species:		*B. collinsi*	*B. mollitia*	*B. smithi*	*B. auricruda*	*B. golo*	*B. graueri*
N individuals:		243	302	329	86	70	60
Body Size		2 -	**4 - ***	4 -	no	2 -	3 -
Eyespot Forewing	Cu1	9-	7-	no	6 -	10 -	no
	M1	6 -	**6 - (*)**	no	no	10 -	no
Hindwing	Cu1	no	**6 - ***	no	6 -	9 -	no
	M1	**6 - ***	6 -	9 -	4 -	11 -	no
Androconia Length		9 -	6 -	no	no	no	no

Table 2. Overview of cross-correlations between environmental parameters and phenotypic traits of *Bicyclus* butterflies in Kibale National Park, Uganda with lags in months and direction of effects. VFW = ventral forewing, VHW = ventral hindwing. Numbers indicate the lag duration with the strongest cross correlation and the - indicates a negative correlation and + a positive correlation. Significant cross-correlations are indicated in bold font with * at p < 0.05. See cross-correlation plots in Appendix 1, Fig. A1.4). Sample sizes per species are given in Table 4.

Species	*B. collinsi*	*B. mollitia*	*B. smithi*	*B. auricruda*	*B. golo*	*B. graueri*
(a) Body Size						
Temperature	1 -	**2 -***	**2 -***	1 -	no	2 -
Rainfall	1 +	**1 +***	1 +	no	no	no
Humidity	**1 +***	**2 +***	**1 +***	no	no	no
(b) Eyespot VFW Cu1						
Temperature	no	**4 +***	**5 +***	2 +	no	no
Rainfall	no	no	no	no	no	no
Humidity	no	no	2 +	no	no	no
(c) Eyespot VFW M1						
Temperature	2 -	3 +	**5 +***	2 +	no	no
Rainfall	no	no	no	1 +	no	no
Humidity	no	**3 -***	**5 -***	1 +	1 +	no
(d) Eyespot VHW Cu1						
Temperature	no	**2 +***	**5 +***	3 +	4 +	no
Rainfall	**2 -***	no	no	no	no	no
Humidity	2 -	**3 -***	**5 -***	3 -	no	no
(e) Eyespot VHW M1						
Temperature	no	**3 +***	**4 +***	**3 +**	no	no
Rainfall	no	no	no	no	no	no
Humidity	no	no	no	no	no	no
(f) Androconia length						
Temperature	**1 +***	**1 +***	**1 +***	1 +	no	no
Rainfall	**1 -***	**1 -***	**1 -***	no	no	no
Humidity	**1 -***	**1 -***	**1 -***	no	no	no

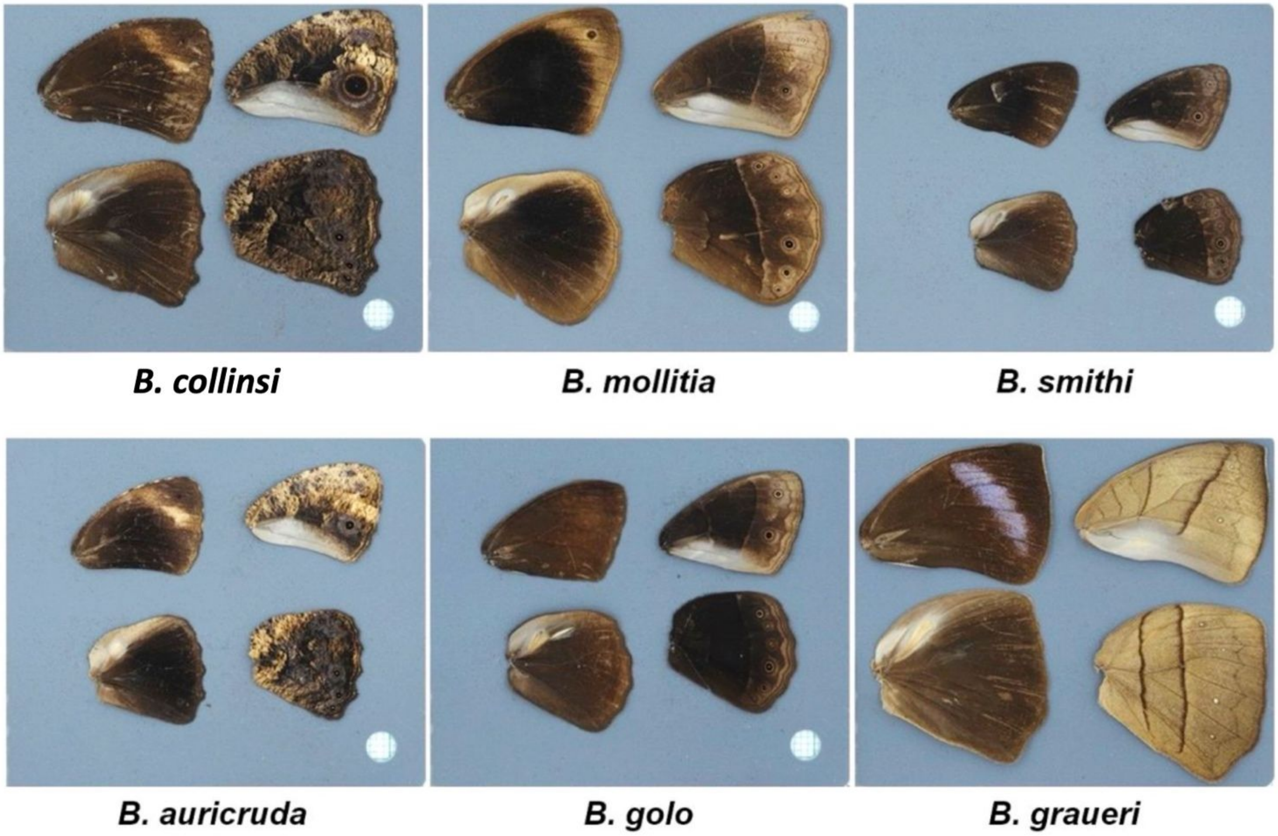
Table 3. Correlations within traits among species of *Bicyclus* butterflies in Kibale National Park where correlation coefficient are at the top right and p-values at the bottom left. Correlation coefficients with * are significant at $p < 0.05$, and in bold font also those $p < 0.1$. VFW = ventral forewing, VHW = ventral hindwing. Body size is an index of wing area, eyespot size is eyespot area divided by the index of wing area, and androconia length is relative to wing length. Sample sizes per species are given in Table 4.

	B. collinsi	*B. mollitia*	*B. smithi*	*B. auricruda*	*B. golo*	*B. graueri*
(a) Body size						
B. collinsi		**0.529***	0.221	0.186	-0.026	0.077
B. mollitia	**0.005***		**0.436***	0.197	-0.302	0.083
B. smithi	0.268	**0.016***		-0.053	-0.072	0.346
B. auricruda	0.524	0.499	0.858		**0.571***	-0.050
B. golo	0.930	0.293	0.806	**0.042***		**0.685***
B. graueri	0.784	0.770	0.206	0.866	**0.007***	
(b) Eyespot VFW Cu1						
B. collinsi		0.176	**0.374**	0.208	**0.727***	0.071
B. mollitia	0.380		**0.477***	**0.781***	0.231	0.172
B. smithi	**0.055**	**0.008***		0.217	**0.477**	0.148
B. auricruda	0.475	**0.001***	0.457		0.330	0.392
B. golo	**0.003***	0.426	**0.085**	0.271		0.270
B. graueri	0.803	0.539	0.600	0.166	0.351	
(c) Eyespot VFW M1						
B. collinsi		**0.399***	**0.549***	**0.505**	**0.657***	-0.101
B. mollitia	**0.039***		**0.492***	0.452	**0.499**	-0.197
B. smithi	**0.003***	**0.006***		0.315	**0.752***	-0.005
B. auricruda	**0.065**	0.105	0.272		0.351	0.161
B. golo	**0.011***	**0.069**	**0.002***	0.239		0.094
B. graueri	0.720	0.483	0.986	0.582	0.750	
(d) Eyespot VHW Cu1						
B. collinsi		**0.673***	**0.540***	0.380	**0.602***	0.348
B. mollitia	**<0.001***		**0.588***	**0.522**	0.381	0.133
B. smithi	**0.004***	**<0.001***		0.345	**0.628***	0.222
B. auricruda	0.180	**0.055**	0.228		0.311	0.268
B. golo	**0.023***	0.179	**0.016***	0.301		0.064
B. graueri	0.204	0.637	0.427	0.355	0.828	
(e) Eyespot VHW M1						
B. collinsi		**0.569***	**0.331**	0.209	0.117	-0.351
B. mollitia	**0.002***		**0.567***	0.365	**0.547***	**-0.517***
B. smithi	**0.091**	**0.001***		0.414	**0.751***	**-0.466**
B. auricruda	0.473	0.199	0.141		0.307	**-0.475**
B. golo	0.691	**0.043***	**0.002***	0.307		-0.107
B. graueri	0.200	**0.048***	**0.080**	**0.086**	0.715	
(f) Androconia length						
B. collinsi		**0.684***	**0.449***	-0.044	**0.738***	**0.522***
B. mollitia	**<0.001***		**0.812***	-0.034	**0.488**	-0.059
B. smithi	**0.019**	**<0.001***		-0.007	0.288	-0.143
B. auricruda	0.881	0.909	0.982		0.023	0.067
B. golo	**0.003***	**0.077**	0.319	0.941		**0.557***
B. graueri	**0.046***	0.835	0.612	0.820	**0.039***	

Table 4. Correlations between traits within species of *Bicyclus* butterflies in Kibale National Park where correlation coefficients are at the top right and p-values at the bottom left. Sample sizes n are for two-week periods of *B. collinsi*, *B. mollitia* and *B. smithi* and the other species one-month periods. Correlation coefficients with * are significant at $p < 0.05$, and in bold font also those $p < 0.1$. VFW = ventral forewing, VHW = ventral hindwing. Body size is an index of wing area, eyespot size is eyespot area divided by the index of wing area, and androconia length is relative to wing length.

	Body size	VFW Cu1	VFW M1	VHW Cu 1	VHW M1	Androconia
***B. collinsi* n = 27**						
Body size		0.195	0.290	0.139	0.066	**-0.545***
Eyespot VFW Cu1	0.331		**0.719***	**0.657***	0.228	0.184
M1	0.142	**<0.001***		**0.539***	**0.502***	0.016
VHW Cu1	0.489	**<0.001***	**0.004***		**0.436***	0.194
M1	0.745	0.252	**0.008***	**0.023***		0.038
Androconia length	**0.003***	0.357	0.937	0.333	0.852	
***B. mollitia* n = 30**						
Body size		-0.008	0.166	-0.079	0.002	**-0.604***
Eyespot VFW Cu1	0.965		**0.815***	**0.794***	**0.840***	0.300
M1	0.381	**<0.001***		**0.779***	**0.877***	0.045
VHW Cu1	0.680	**<0.001***	**<0.001***		**0.844***	0.304
M1	0.991	**<0.001***	**<0.001***	**<0.001***		0.202
Androconia length	**<0.001***	0.107	0.812	0.103	0.284	
***B. smithi* n = 30**						
Body size		**0.347**	0.288	0.302	0.204	**-0.336**
Eyespot VFW Cu1	**0.060**		**0.829***	**0.849***	**0.749***	-0.017
M1	0.122	**<0.001***		**0.859***	**0.849***	0.022
VHW Cu1	0.105	**<0.001***	**<0.001***		**0.868***	0.222
M1	0.280	**<0.001***	**<0.001***	**<0.001***		0.252
Androconia length	**0.070**	0.928	0.910	0.238	0.179	
***B. auricruda* n = 14**						
Body size		0.018	0.029	**0.488**	0.380	-0.353
Eyespot VFW Cu1	0.952		0.376	0.396	0.396	0.299
M1	0.923	0.185		**0.690***	**0.702***	0.162
VHW Cu1	**0.077**	0.161	**0.006***		**0.799***	0.172
M1	0.180	0.161	**0.005***	**<0.001***		-0.147
Androconia length	0.216	0.299	0.581	0.557	0.616	
***B. golo* n = 14**						
Body size		0.090	0.039	0.078	-0.033	-0.386
Eyespot VFW Cu1	0.759		**0.871***	**0.917***	**0.919***	0.448
M1	0.895	**<0.001***		**0.755***	**0.899***	0.385
VHW Cu1	0.791	**<0.001***	**0.002***		**0.828***	0.439
M1	0.912	**<0.001***	**<0.001***	**<0.001***		0.433
Androconia length	0.173	0.108	0.175	0.117	0.122	
***B. graueri* n = 15**						
Body size		-0.112	-0.111	-0.182	0.277	-0.341
Eyespot VFW Cu1	0.692		0.269	0.000	-0.322	0.395
M1	0.695	0.332		-0.013	-0.164	0.037
VHW Cu1	0.516	1.000	0.963		-0.023	0.259
M1	0.318	0.242	0.559	0.934		-0.302
Androconia length	0.213	0.145	0.895	0.352	0.275	

Figures



B. collinsi **B. mollitia** **B. smithi**

B. auricruda **B. golo** **B. graueri**

Figure 1. Examples of wings of the six studied species of *Bicyclus* butterflies with on each grey card in top row forewings and bottom row hindwings, while dorsal sides are on the left and ventral sides on the right. Average forewing length of *B. collinsi* = 2.61, *B. mollitia* = 2.77 cm, , *B. smithi* = 2.21, *B. auricruda* = 2.33, *B. golo* = 2.41, and *B. graueri* = 3.18.

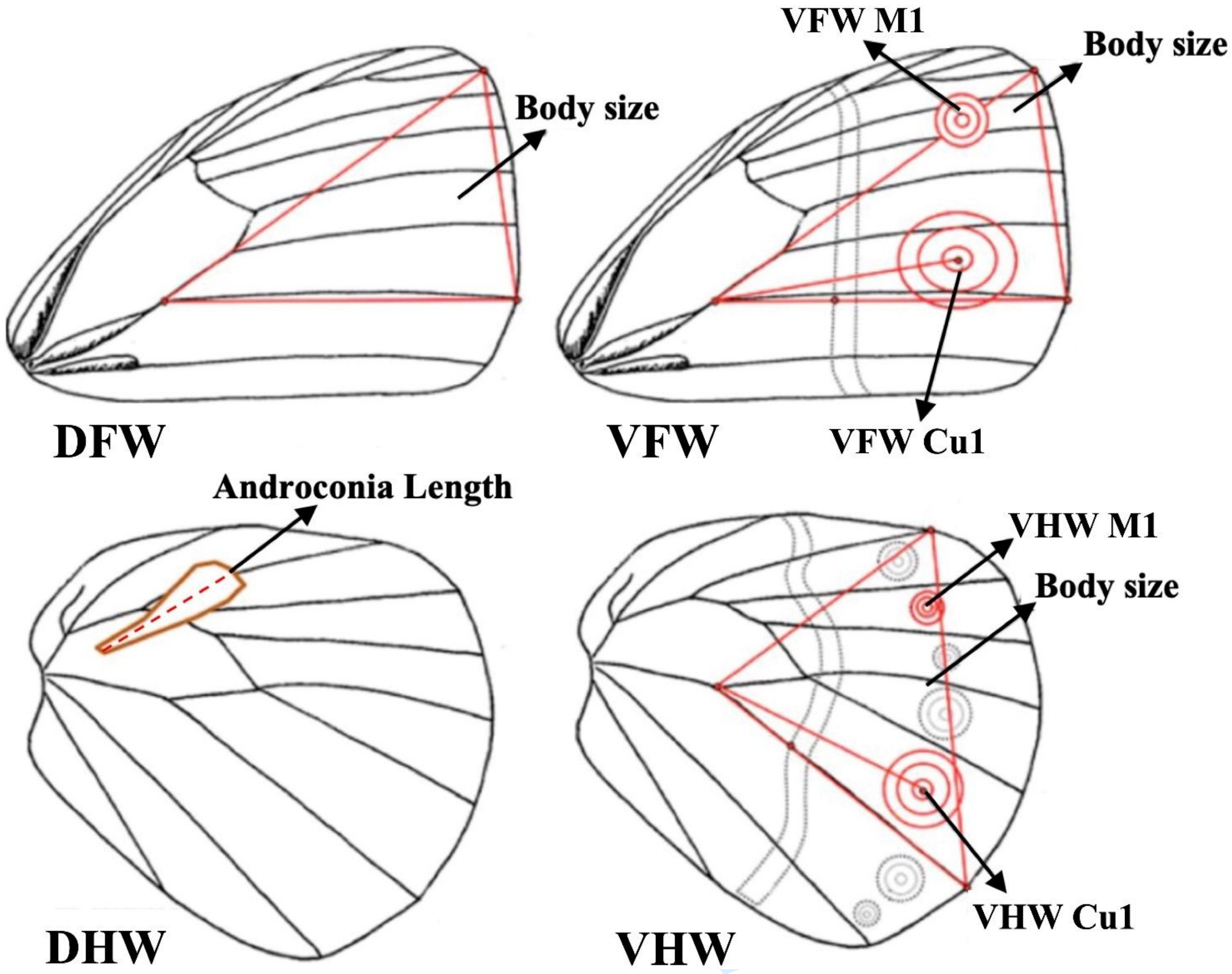


Figure 2. Schematic drawing of *Bicyclus* wings indicating the traits measured in this study in red. The wing area index (body size) is the area of the triangle, the eyespot in forewing cell 2 is VFW Cu1, and in cell 5 is VFW M1, in the hindwing they are VHW Cu1 and VHW M1. Length of the androconial brush was measured as a straight line from base to tip, and wing length as the lowest side of the triangle on the hindwing.

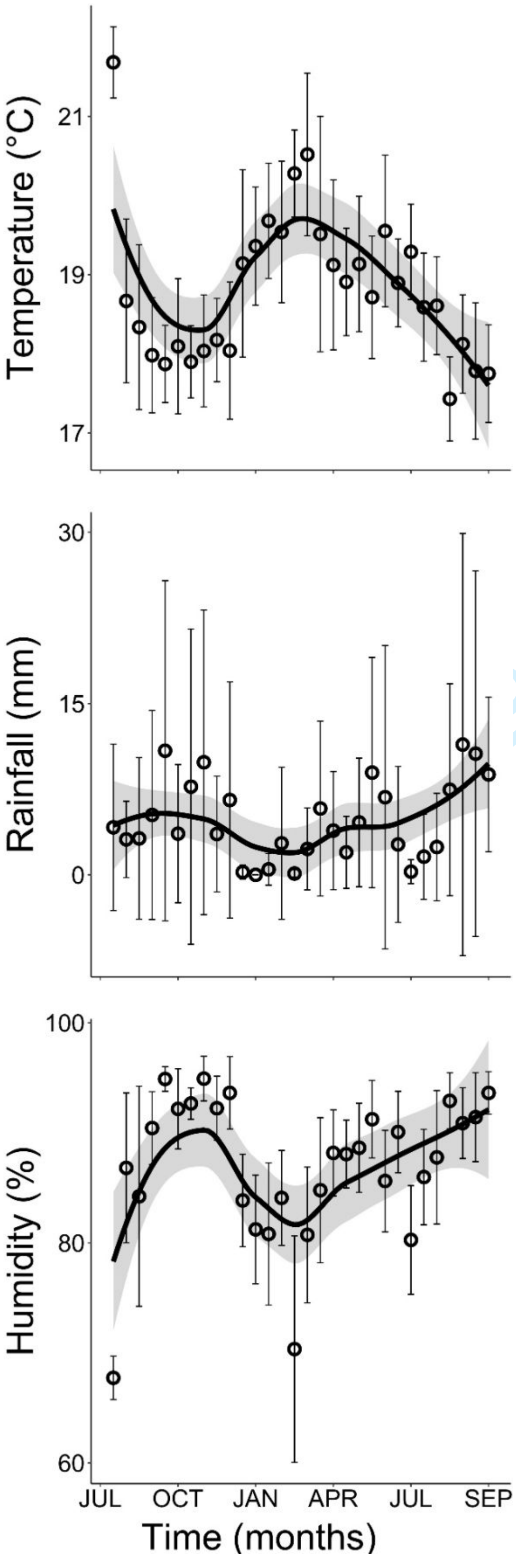


Figure 3. Temporal trends in environmental factors during the study period in Kibale National Park, Uganda; with temperature (°C), rainfall (mm), and relative humidity (%). The lines in the graph represent loess regressions with 95% confidence intervals.

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SUPPORTING INFORMATION

Seasonal plasticity in sympatric *Bicyclus* butterflies in a tropical forest where temperature does not predict rainfall

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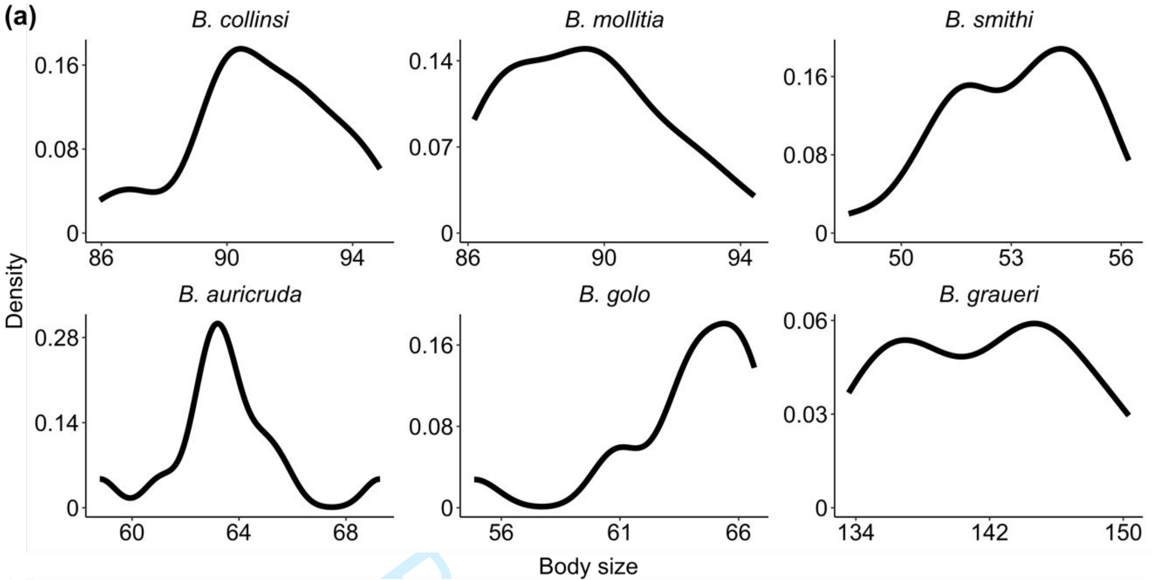
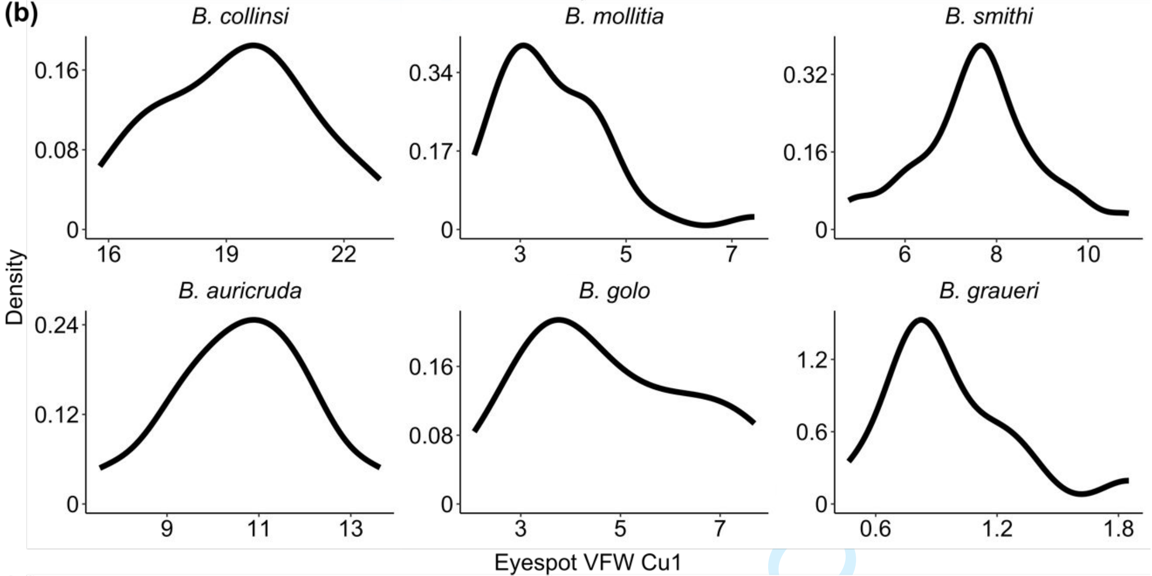
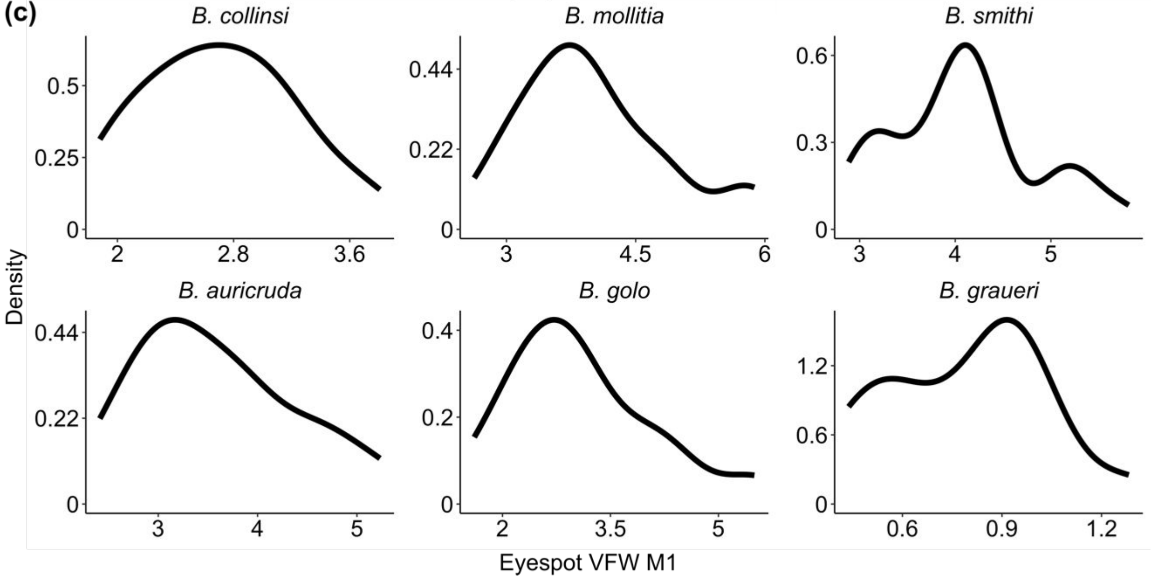
Appendix 1


(a)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Density
0.16
0.08
0
86
90
94
0.14
0.07
0
86
90
94
0.16
0.08
0
50
53
56
0.28
0.14
0
60
64
68
0.16
0.08
0
56
61
66
0.06
0.03
0
134
142
150
Body size


(b)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Density
0.16
0.08
0
16
19
22
0.34
0.17
0
3
5
7
0.32
0.16
0
6
8
10
0.24
0.12
0
9
11
13
0.16
0.08
0
3
5
7
1.2
0.6
0
0.6
1.2
1.8
Eyespot VFW Cu1


(c)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Density
0.5
0.25
0
2
2.8
3.6
0.44
0.22
0
3
4.5
6
0.6
0.3
0
3
4
5
0.44
0.22
0
3
4
5
0.4
0.2
0
2
3.5
5
1.2
0.6
0
0.6
0.9
1.2
Eyespot VFW M1

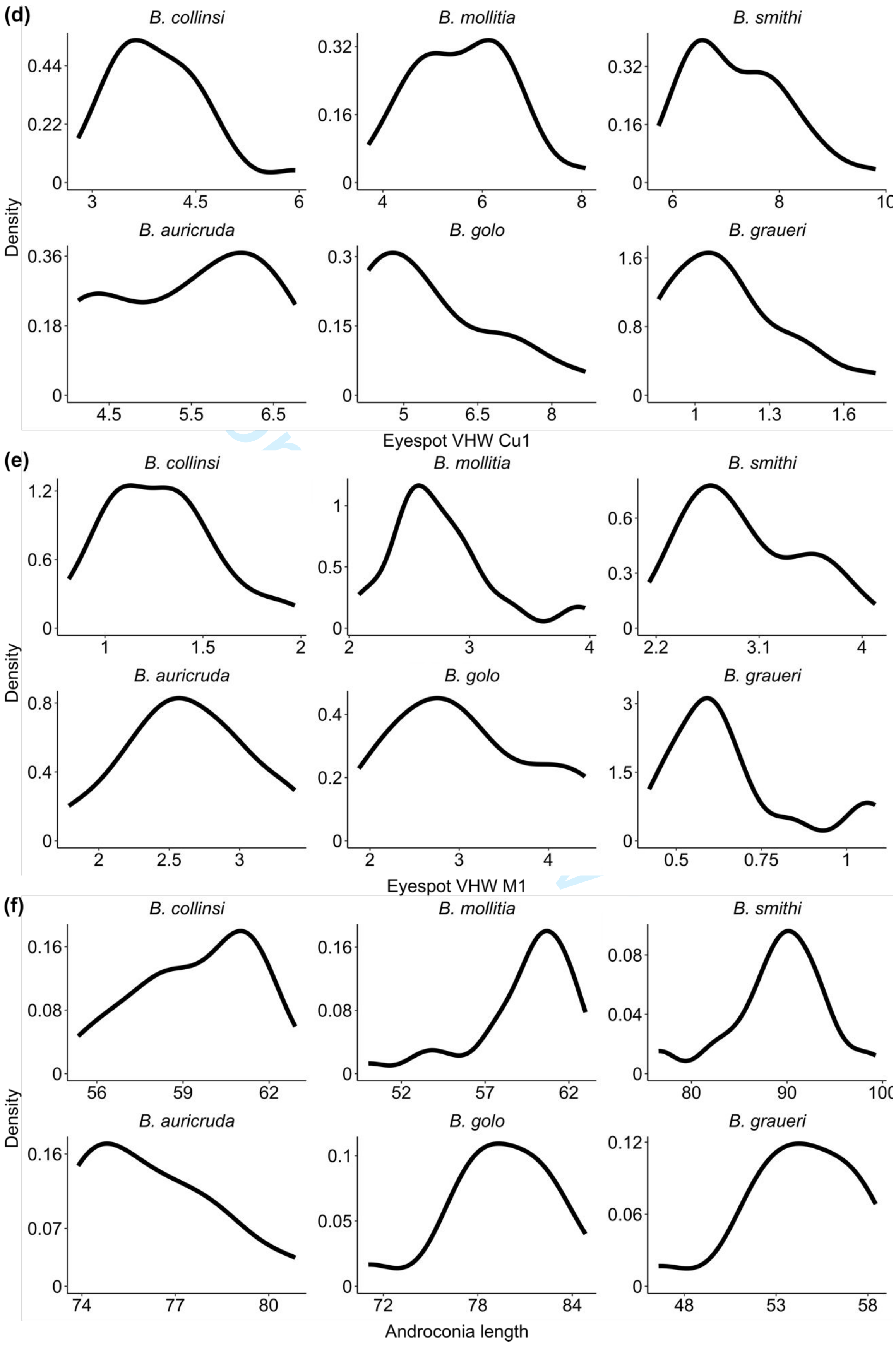
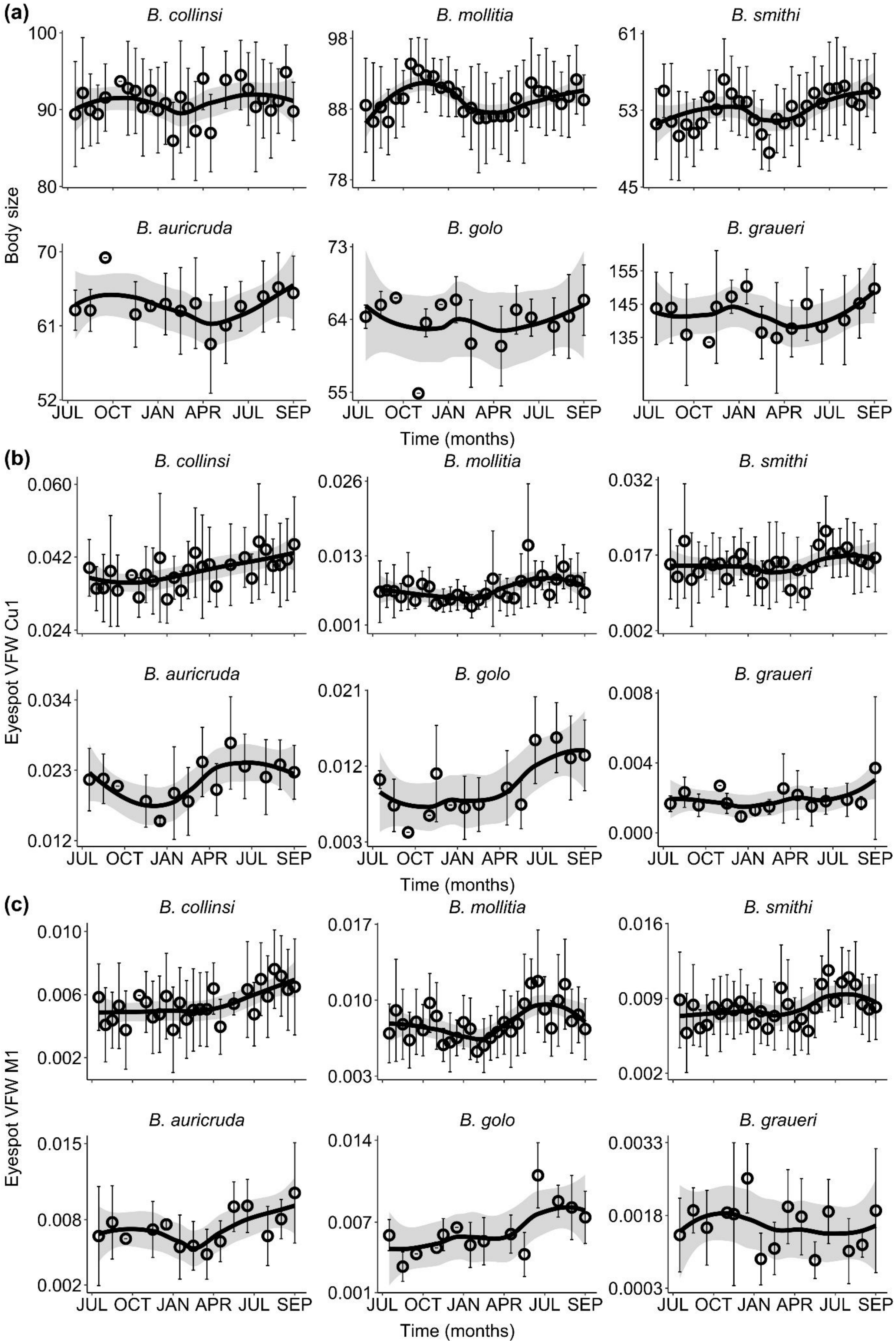


Figure A1.1. Density plots showing the distribution of traits within six species of *Bicyclus* in Kibale National Park, Uganda. VFW = ventral forewing, VHW = ventral hindwing.


(a)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Body size
100
90
80
98
88
78
61
53
45
70
61
52
73
64
55
155
145
135
JUL OCT JAN APR JUL SEP
Time (months)
(b)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Eyespot VFW Cu1
0.060
0.042
0.024
0.026
0.013
0.001
0.032
0.017
0.002
0.034
0.023
0.012
0.021
0.012
0.003
0.008
0.004
0.000
JUL OCT JAN APR JUL SEP
Time (months)
(c)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Eyespot VFW M1
0.010
0.006
0.002
0.017
0.010
0.003
0.016
0.009
0.002
0.015
0.008
0.002
0.014
0.007
0.001
0.0033
0.0018
0.0003
JUL OCT JAN APR JUL SEP
Time (months)

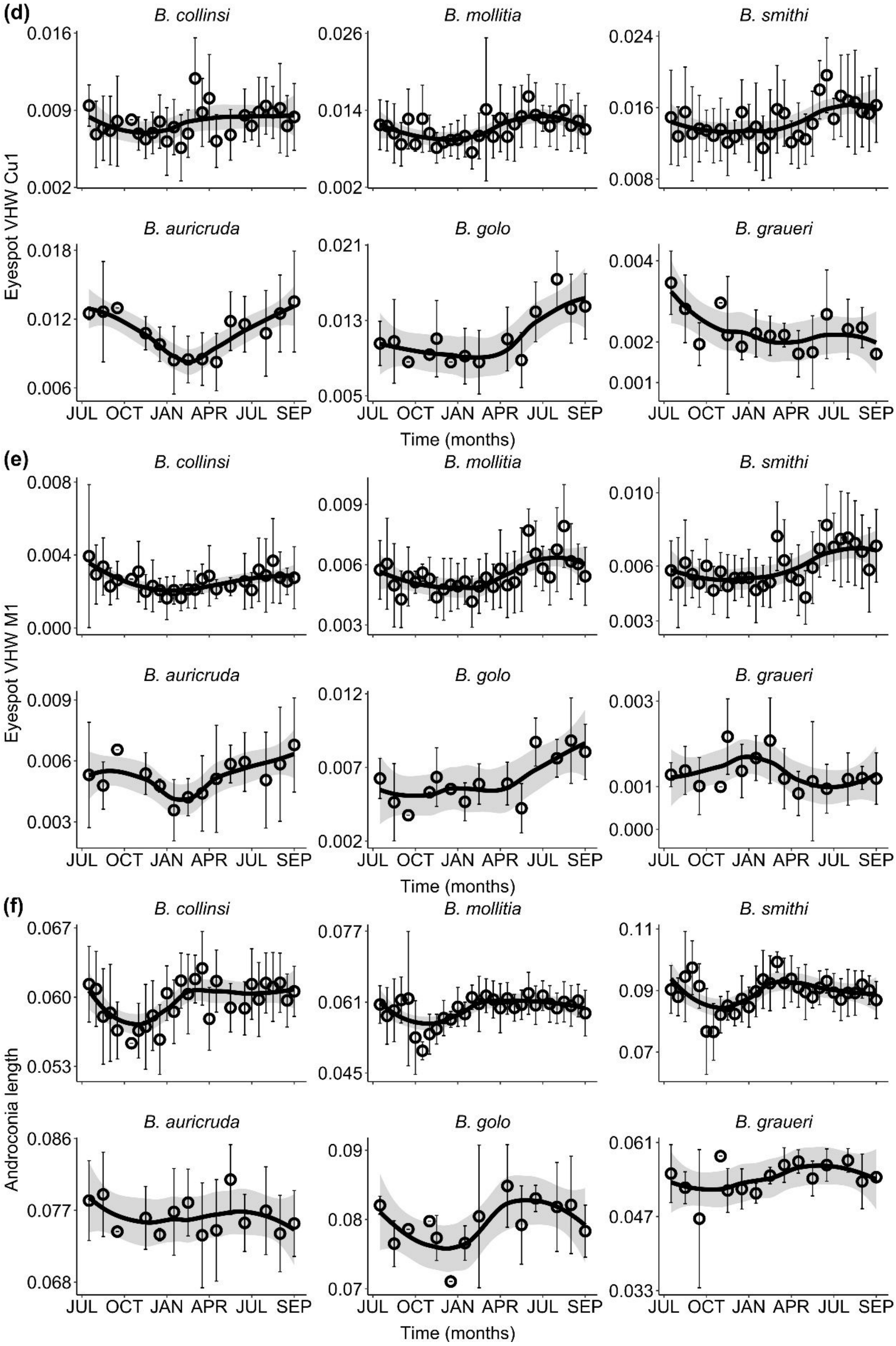


Figure A1.2 Temporal trends in traits in six species of *Bicyclus* butterflies in Kibale National Park, Uganda. VFW = ventral forewing, VHW = ventral hindwing. Eyespot size is relative to body size (eyespot area/average wing area), and androconia length to wing length. The lines in the graph represent loess regressions with 95% confidence intervals.

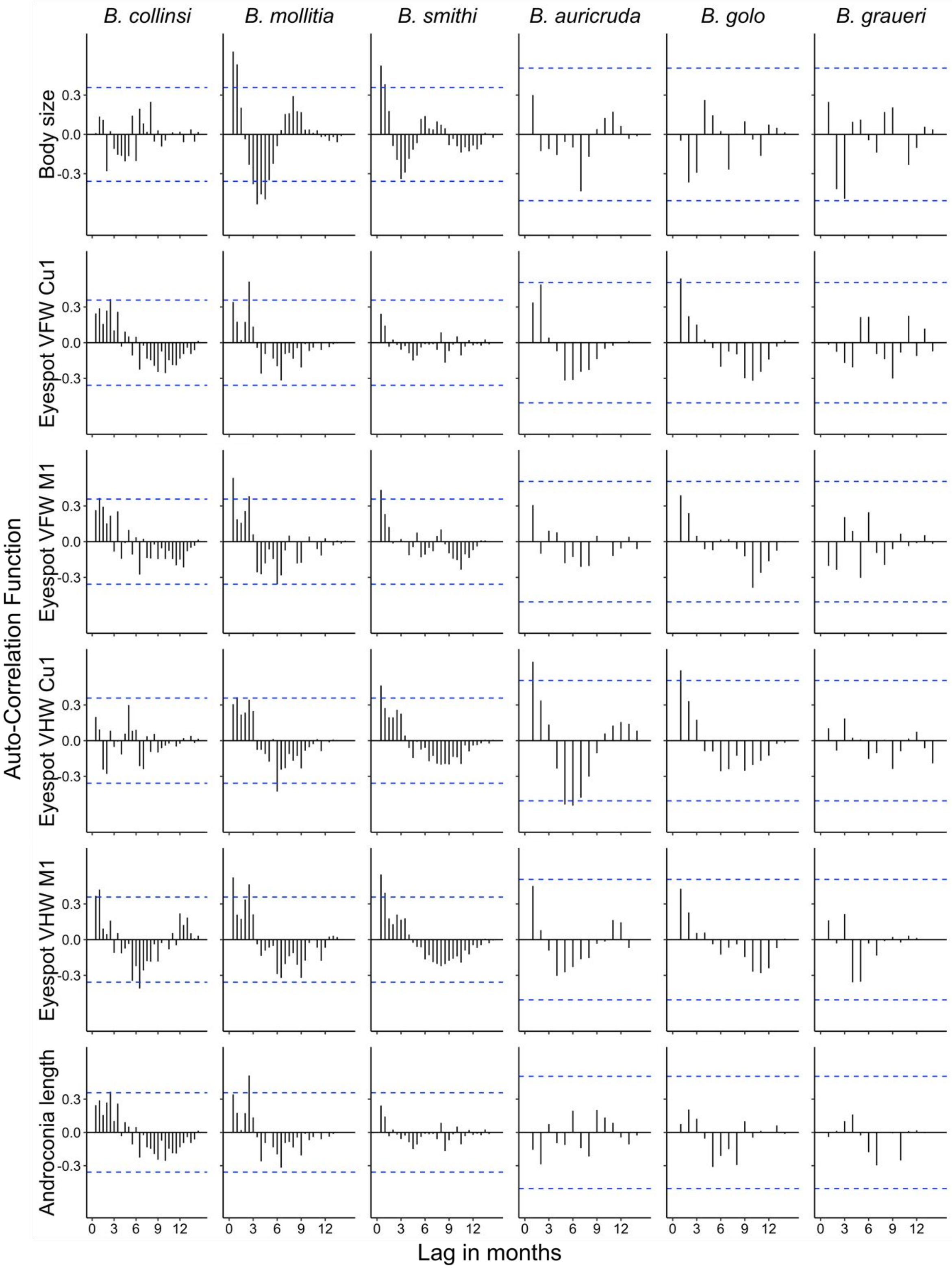
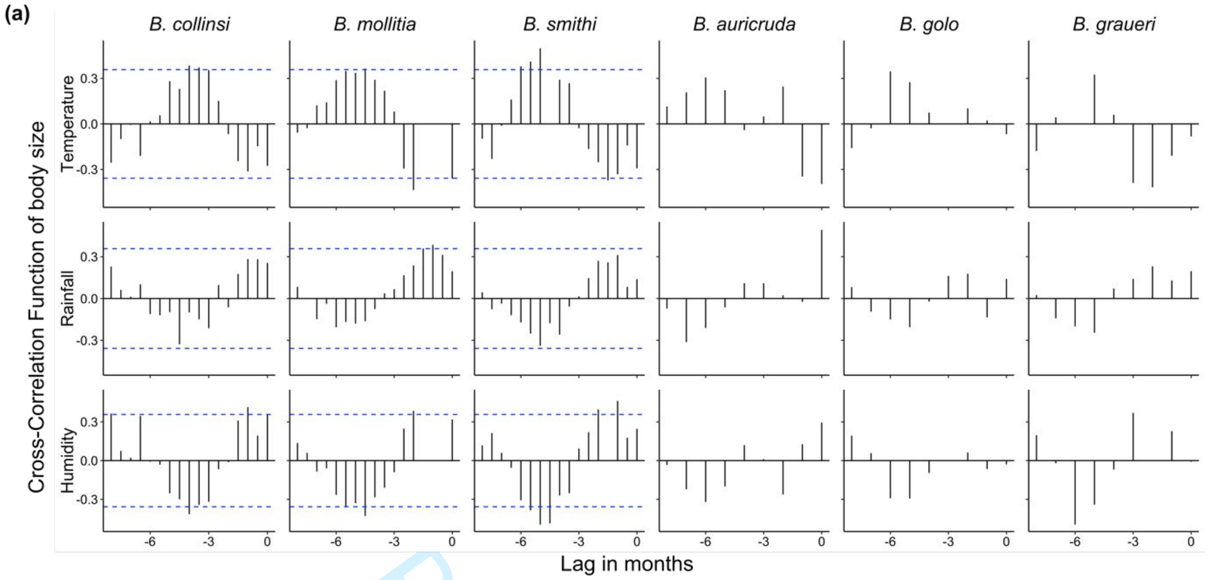
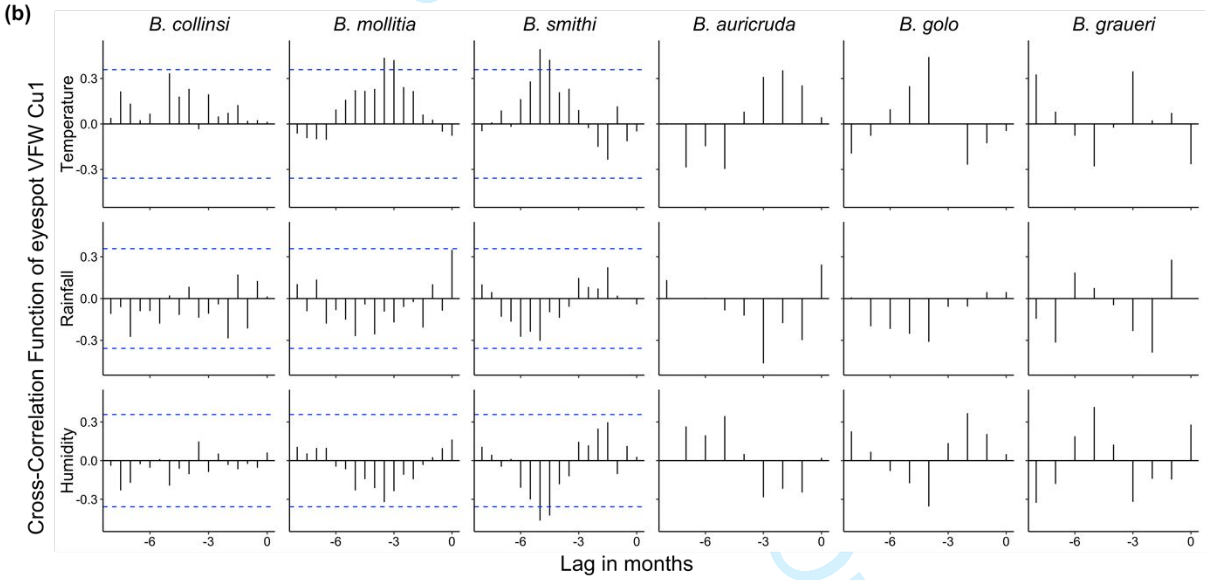
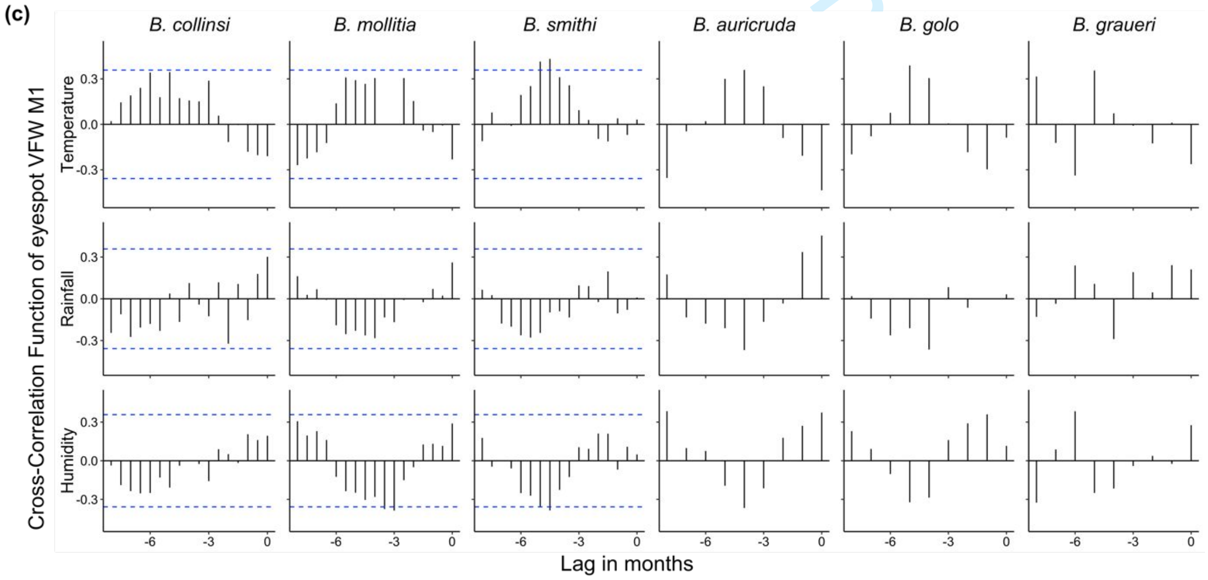


Figure A1.3. Autocorrelations for traits in three species of *Bicyclus* butterflies in Kibale National Park, Uganda. Autocorrelations are considered significant when they cross the dotted lines, representing p-critical of 0.05. This figure is summarised in Table 1 in main text.


(a)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Cross-Correlation Function of body size
Temperature
Rainfall
Humidity
0.3
0.0
-0.3
-6
-3
0
Lag in months


(b)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Cross-Correlation Function of eyespot VFW Cu1
Temperature
Rainfall
Humidity
0.3
0.0
-0.3
-6
-3
0
Lag in months


(c)
B. collinsi
B. mollitia
B. smithi
B. auricruda
B. golo
B. graueri
Cross-Correlation Function of eyespot VFW M1
Temperature
Rainfall
Humidity
0.3
0.0
-0.3
-6
-3
0
Lag in months

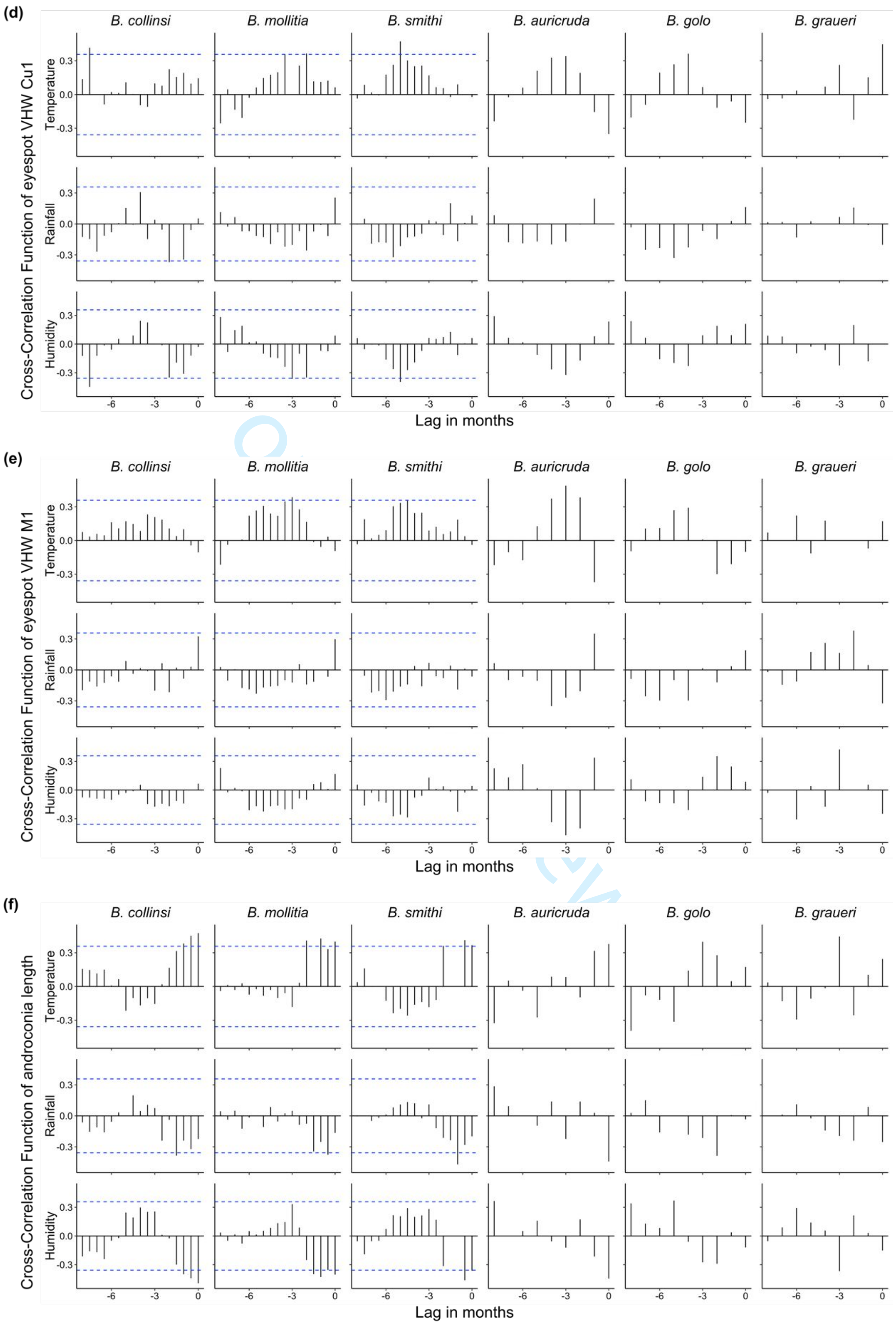
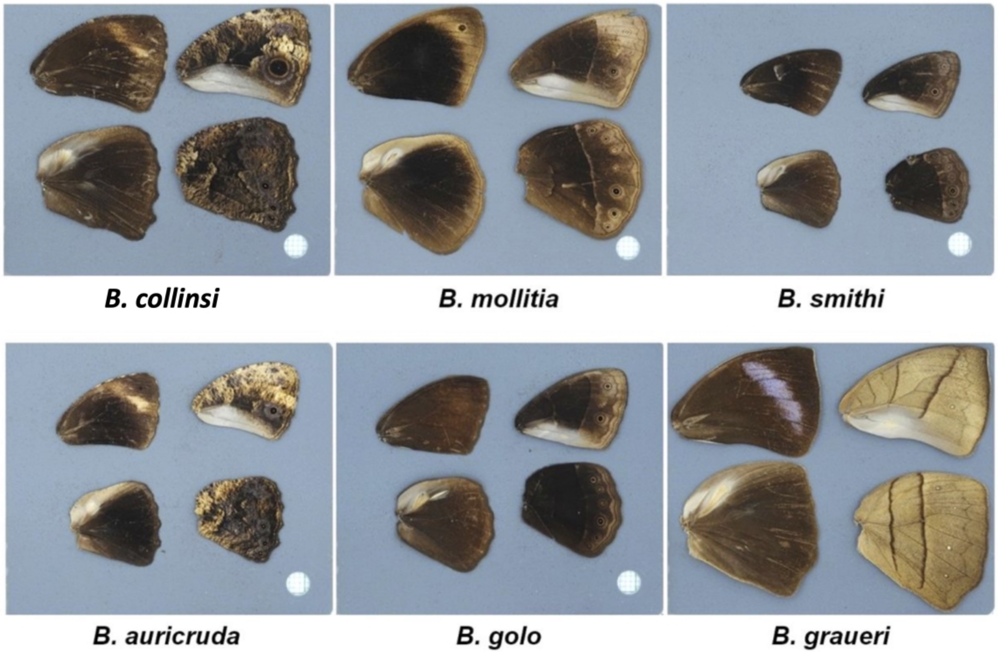
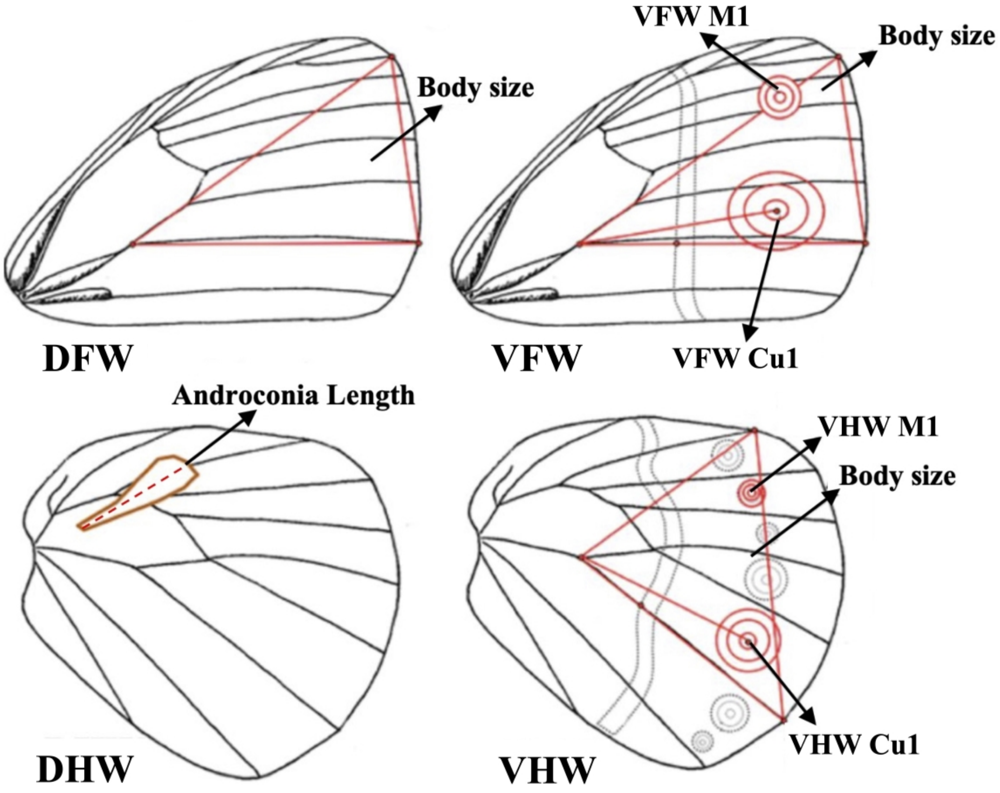


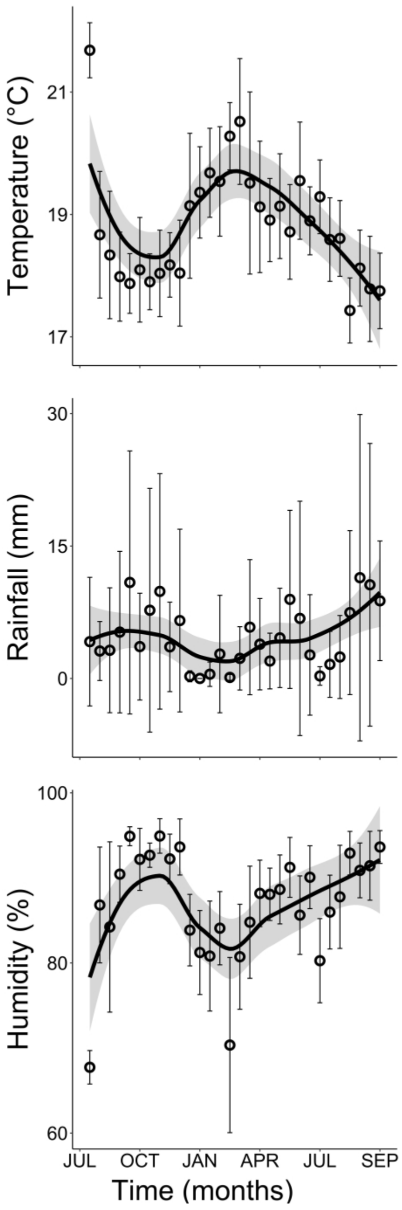
Figure A1.4. Cross-correlations between butterfly traits; A) body size, B) Relative eyespot size, and C) relative androconia length, and weather parameters for six species of *Bicyclus* in Kibale National Park, Uganda. Cross-correlations are considered significant when they cross the dotted lines, representing p-critical of 0.05. This figure illustrates Table 2 in the main text.



1193x781mm (72 x 72 DPI)

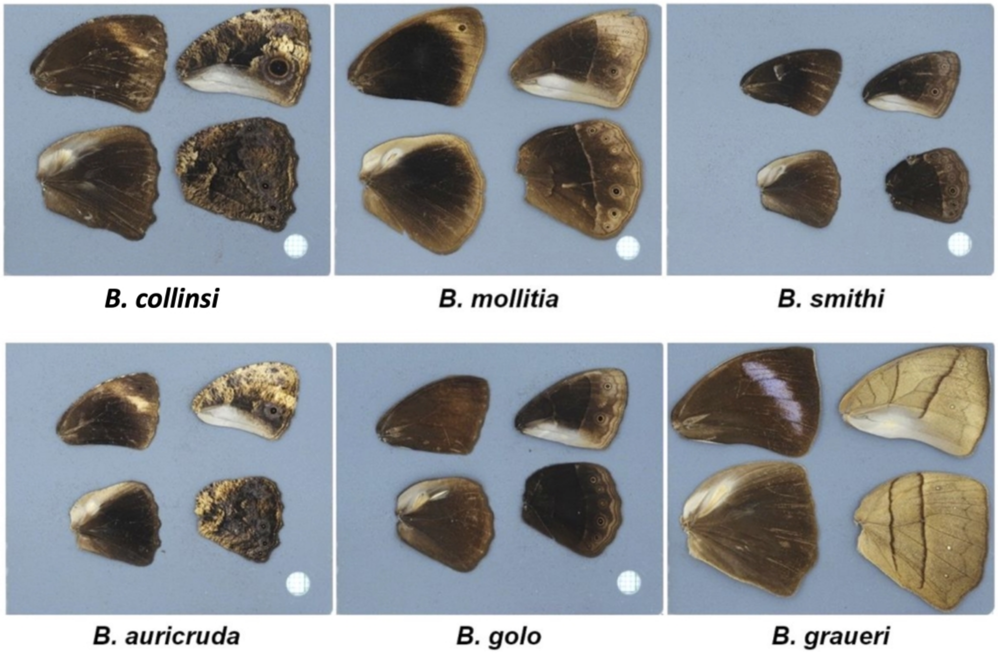


1788x1404mm (72 x 72 DPI)



820x2472mm (72 x 72 DPI)

In a tropical forest with limited temperature variation, Bicyclus butterflies demonstrate size changes in response to temperature but exhibit non-plastic eyespot sizes. Larger butterflies within species tend to have larger eyespots, revealing conserved cues and shared mechanisms, while exceptions exist for tiny eyespots unlinked to androconia length.



1193x781mm (72 x 72 DPI)