

Developmental plasticity for male secondary sexual traits in a group of polyphenic tropical butterflies

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ABSTRACT

Many organisms alter their investment in secondary sexual traits to optimise the fitness trade-off between reproduction and survival. Though seasonal variation in the expression of sexual traits is evident (e.g. conspicuous breeding plumage in birds), little attention has been given to short-lived organisms that inhabit relatively stable environments throughout their own lifetime but are exposed to strong environmental variation across generations. Some insects have evolved seasonal polyphenism to cope with intergenerational variation in environmental selection, yielding alternative phenotypes better suited for the environment in which they occur. Here we use mycalesine butterflies, which express distinct seasonal forms in response to wet-dry seasonal environments, to explore whether seasonal polyphenism extends to secondary sexual traits. Sex pheromones, which are produced by androconial patches on the wings of males, are a key determinant of mating success in these species. Through a series of field sampling, reaction norm experiments, pheromone titre quantification, and mate competition experiments, this study reveals that the size of the androconial patch on the forewing is mediated by the temperatures experienced during development, which correspond to the ecological conditions during the alternative wet and dry seasons in the wild. Furthermore, in one of the species, *Bicyclus anynana*, larger forewing patches are associated with both an increase in the production of pheromone components as well as the likelihood of mating. Taken together, these findings indicate that plasticity in androconial patches may be an adaptation to seasonal fluctuations in the environment as it favours reproductive success in the conditions in which each seasonal form flies. We discuss our findings in the context of seasonal differences in the optimal tradeoff between reproduction and survival.

Keywords: androconia, developmental plasticity, polyphenism, male sex pheromone, mating success, *Bicyclus anynana*

INTRODUCTION

In many seasonally breeding animals, secondary sexual traits, such as the ornamental plumage of birds and antlers of deer, only appear during seasons that are more favourable in terms of reproduction, when they play important roles in intra- and intersexual selection. These seasonal fluctuations in the expression of secondary sexual traits are closely correlated with fitness benefits during the breeding season and costs of trait expression throughout the rest of the year (Emlen & Nijhout 2000; Emlen 2001; Moczek 2010). The role of environmental conditions in regulating the expression of secondary sexual traits, as well as the proximate mechanisms underlying this phenomenon, have been the topic of numerous studies in the last decade, predominantly using long-lived systems which encounter multiple seasonal cycles throughout their lives (Robinson et al., 2008; Scordato et al., 2012; Winandy & Denoël, 2015). This body of work has shed light on how interactions between environmental and sexual selection, operating at different temporal and spatial scales, shape the evolution and seasonal expression of secondary sexual traits (see Cornwallis & Uller, 2010).

Animals with short generation times may be exposed to temporal and spatial fluctuations in the strength and direction of selection across generations (Hairston and Dillon 1990, Bergland et al. 2014). In such cases, generations occurring during seasons that are favourable for reproduction may invest more in the expression of secondary sexual traits, which are typically costly to produce or maintain. In contrast, generations of short-lived animals that live in more stressful environments are expected to reduce the costs of secondary sexual trait expression to optimize the trade-off between reproduction and survival. We explore this hypothesis using mycalesine butterflies; a group of polyphenic insects that shows a striking diversity in the number, size and position of androconia, which are small secondary sexual organs that are found on the wings of males (Condamin 1973; Costanzo & Monteiro 2007; Bacquet et al., 2015).

Species of mycalesine butterflies that inhabit wet-dry seasonal environments in the Old World tropics exhibit seasonal polyphenism with alternative wet and dry seasonal forms (Roskam & Brakefield 1999; Oostra et al., 2011). Long-term surveys have shown that generations that fly in the favourable, wet season are characterised by large marginal eyespots on their ventral wing surface, whereas these conspicuous wing patterns are dramatically reduced in generations that occur during the harsh, dry season (Windig et al. 1994, Brakefield et al. 2007). The discrete wing patterns of the seasonal forms are an adaptation to the alternative ecologies and predation pressures of the wet and dry seasons, and the different forms have the highest survival rates in the environment in which they typically occur (Lyytinen et al. 2003, Brakefield and Frankino 2009, Prudic et al. 2015). Temperature experienced during the late larval stage is an important environmental inducer of the seasonal forms both in the

field and in the laboratory; lower or higher temperatures act as a cue for forthcoming dry or wet seasons, respectively (Kooi and Brakefield 1999, Brakefield et al. 2007). Moreover, developmental plasticity in mycalesine butterflies involves coordinated responses of morphological, physiological, behavioural and life history traits which together provide an adaptive response to climate instability (Oostra et al. 2014, Mateus et al. 2014, Nokelainen et al. 2018, van Bergen and Beldade 2018).

The wings of the mycalesine males typically carry one or several androconia which are small patches of differentiated scales and hair pencils that are involved in the production and distribution of male sex pheromones (MSP). The ultrastructure of these androconia (Dion et al., 2016) and the composition of MSP (Nieberding et al. 2008) have been thoroughly investigated using *Bicyclus anynana*, an African mycalesine butterfly that has become an important model system in the field of Eco-Evo-Devo (Brakefield et al. 2009). The pheromone blend of this species contains three components, Z9-14:OH (MSP1), 16:Ald (MSP2) and 6,10,14-trime-15-2-ol (MSP3), and variation in titres and ratios is used by females during courtship to assess male quality. In *B. anynana*, MSP production is a reliable indicator of male age (Nieberding et al., 2012) and inbreeding status (van Bergen et al., 2013) and expected to be costly as males of this species produce significantly less MSP when exposed to environmental conditions that mimic the harsh, dry season (Dion et al. 2016). The composition of the MSP and the production thereof in the two other species included in this study, *Bicyclus safitza* and *Mycalesis perseoides*, is less well understood. Bacquet and colleagues (Bacquet et al. 2015) used a selection method, targeting the most abundant and male-specific volatile compounds found in the androconia of 32 African mycalesine species, and detected two compounds in males of *B. safitza* that are likely to make up the MSP; TMP-2-ol and Z11-18:Ald. A similar approach, using mycalesine species from Asia, revealed two putative MSP compounds in *M. perseoides* (O. Brattström, unpublished data).

Here we use a series of approaches and experiments to examine whether the seasonal polyphenism in mycalesine butterflies extends to involve the size of androconial patches in males, and how this could be associated with seasonal changes in sexual selection. Our analyses of daily field samples of *B. safitza* collected over two annual seasonal cycles at a locality in Malawi show that males of the dry season form have smaller androconia on the forewing, but not the hindwing, than those of the wet season form. We then conducted laboratory experiments, using stocks of three species of mycalesine butterflies, *B. anynana*, *B. safitza*, and *M. perseoides*, to quantify the reaction norms of androconia size. We demonstrate that all species show a pattern of reduced size of forewing androconia in males reared at lower temperatures that induce the dry season form, while the rearing temperature did not affect the size of the hindwing androconia. Re-analysis of samples of male *B. anynana* reared in earlier

experiments confirm that there is a positive correlation between androconia size on the forewing and the amount of two components of the MSP previously demonstrated to be involved in female mate choice in this species. Finally, using male competition experiments we demonstrate forewing androconia size is associated with increased mating success in *B. anynana*, but does not affect the likelihood of mating in *B. safitza*.

MATERIALS AND METHODS

Specimens of mycalesine butterflies

We took advantage of the availability of previously collected specimens of mycalesine butterflies which were originally collected for studies on i) seasonal polyphenism in the wild, ii) developmental plasticity in mycalesine butterflies and iii) the effect of MSP production on mating success. For this study we re-imaged these specimens and quantified the size of the androconia. Subsequently, we conducted (iv) competition experiments, using 4- to 6-day-old males, to test whether mating success was affected by androconial size.

i) Seasonal polyphenism in the wild

Specimens of *B. safitza*, derived from a longitudinal sampling of *Bicyclus* butterflies (see van Bergen et al., 2016), were used to explore whether variation in androconial patch size is associated to patterns of seasonality in the natural environment. Butterflies were caught on a daily basis between January 1996 and December 1997 in a seasonal open habitat in Zomba, Malawi (15°22'S, 35°19'E) and stored in entomological envelopes (N=250). Specimens remained refrigerated ($\pm 5^{\circ}\text{C}$) until they were identified to species level and processed in 2013. Climatic data were obtained from a weather station at Chancellor College, which is located about 2 km from the trapping site.

ii) Developmental plasticity in three species of mycalesine butterflies

Specimens of *B. anynana* (N=137), *B. safitza* (N=163) and *M. perseoides* (N=181) were used to investigate whether mycalesine butterflies exhibit temperature-induced developmental plasticity for androconial patch size. The samples used here were obtained from two temperature reaction norm experiments using mycalesine butterflies by Oostra et al. (2011) and van Bergen et al. (2017). In both studies young larvae were randomly divided over four climate-controlled chambers (21°C, 23°C, 25°C and 27°C, 70% R.H. and L12:D12), within one day after hatching and reared in sleeves of gauze-like material. All individuals from *B. anynana* were reared on young maize plants (Oostra et al., 2011) while individuals from *B. safitza* and *M. perseoides* were reared on

wheat plants as development was poor on maize (van Bergen et al., 2017). The resulting pupae were placed in individual transparent pots and kept at the same temperature treatment. For all species, development in cooler temperatures resulted in dry season form individuals while warmer temperatures induced wet season form individuals (for details see van Bergen et al. 2017). One day after eclosion, the adults were euthanized by freezing at -20°C and stored in individual entomological envelopes until dissection and imaging.

iii) Production of male sex pheromones

To examine whether the production of MSP is correlated with the size of the androconial patch we used samples and MSP data from van Bergen et al., 2013. Here, ten outbred families of *B. anynana* were generated and reared in individual cages at 27°C, 70% R.H. and L12:D12, generating wet season form individuals. Several males from each family were sacrificed and the three MSP components were extracted by soaking one fore- and one hind-wing of each individual in 500 µl of hexane. The amount of each component of the MSP, namely MSP1, MSP2 and MSP3, was quantified using gas-chromatography (see van Bergen et al., 2013 for details) and the remaining wings from each sample were stored in entomological envelopes at -20°C. Here we quantified the size of the androconial patches of these samples (N=67) using the procedure described below.

iv) Competition experiments

We performed replicated competition experiments, using the phenotypic variation expressed by males of *B. anynana* and *B. safitza* within a single thermal environment, to study the association between the size of the androconial patches and male mating success. Semi-natural conditions were provided through the use a large flight cage, with the availability of both host plants and adult food, thus allowing males to express natural mate location and courtship behaviours. Both insect rearing and competition experiments were conducted under controlled laboratory conditions with 27°C, 70% R.H. and L12:D12. Several hundred larvae of *B. anynana* and *B. safitza* were reared and the resulting pupae were sexed and placed in separate cylindrical hanging cages to avoid mating prior to the competition experiments (details in Brakefield et al. 2009). Subsequently, thirty 4- to 6-day-old wet season form adults were released in a large flight cage (1.45m x 1.15m x 1.6m) at a 2:1 male-to-female ratio, exactly 3.5 hours after lights on. The flight cage was monitored at intervals of 10 minutes for 8 hours during which mating pairs were removed and replaced by new individuals. Males were recorded as successful or unsuccessful depending on whether or not they mated before the end of the experiment. All individuals were euthanised immediately after the experiment and were not reused in other mating trials.

Individuals were provided with young maize plants and sliced banana on wet cotton throughout the experiment. Replicate competition experiments were conducted for both *B. anynana* (N=3) and *B. safitza* (N=4).

Imaging and image processing

All specimens used in the study were carefully dissected, initially by removing one fore- and one hindwing. Subsequently, the inner hairpencil on the dorsal hindwing was removed to reveal the androconial patch located below (see Figure 1). The ventral wing surfaces were imaged using a Leica DFC495 digital camera coupled to a Leica M125 stereomicroscope using Leica Application Software (LAS) Ver. 3.8.0. Subsequently more detailed images were obtained from the androconial patches on the ventral forewing and the dorsal hindwing. All photographs were analysed using Fiji image processing software (Schindelin et al., 2012) and the size of the androconial patches was measured as the area within a polygon formed by a series of landmarks (see Figure 1A). For the field specimens, eyespot areas were measured on both the ventral fore- and hindwing by fitting an ellipse to the black inner-disc and the yellow outer ring of the large eyespot in cell Cu1. An area enclosed by three clear landmarks was used as a proxy of wing size. Repeatability of image analysis was assessed using two independent measures of the same specimen (N = 30) and was high for the androconia on both the forewing ($R^2 = 0.79$) and hindwing ($R^2 = 0.75$) for *B. anynana*.

Statistical analyses

The four wing-pattern measurements of the specimens from the wild were corrected for wing area and subsequently reduced into a single variable using a principal component analysis. The first principal component (PC1), which reflects an index of seasonal form, was transformed into a categorical variable (DSF or WSF, see supplementary table 1) and used as independent variable in a one-way ANOVA test to determine the effect of the seasonal form on androconial sizes. Reaction norm data was also analysed using one-way ANOVAs, with the developmental temperature as the independent variable. Post hoc comparisons were performed, using Tukey's honest significant differences (HSD) tests, to specify significant differences between the temperature treatments. To test whether MSP production was correlated to the size of the androconial patches we performed a multivariate analysis of covariance (MANCOVA) with MSP1, MSP2 and MSP3 titres as dependent variables and the family and size of the androconia as fixed variables, testing for the hind- and forewing independently. The results of the univariate ANCOVAs were used to specify the relationship between the size of the androconia and each individual MSP component. Finally, we used a general linear mixed model with a binomial distribution and a logit link to examine whether male androconia size could predict mating success (1 or 0). Experimental replicates were included as random effects in the models. The measures of androconial patch size presented here

are absolute areas, tests were also conducted using the relative patch size (androconia size divided by wing area) and these gave similar results (see supplementary information). All statistical analyses were performed with the R Statistical Package v 3.1.2 (R Development Core Team 2017).

RESULTS

Seasonal variation in the expression of secondary sexual characters (B. safitza)

PC1 explained 75.8% of the variation and its distinct bimodal distribution was used to classify individuals to seasonal form (Figure 2B and supplementary information). We confirm that wet season form individuals in Malawi only occur between January and May, the period that is characterized by high rainfall and high temperatures (Figure 2A). Seasonal form had a significant effect on the forewing androconia size of *B. safitza* males (one-way ANOVA; $F_{1,248} = 468.3$, $p < 0.001$) with wet season form individuals carrying larger androconia than dry season form individuals (2D). In contrast, there was no difference (one-way ANOVA; $F_{1,248} = 0.583$, $p = 0.446$) in hindwing androconia size between wet and dry seasonal forms (Figure 2F).

Reaction norms for secondary sexual characters (B. anynana, B. safitza and M. perseoides)

We found a significant effect of developmental temperature on the androconia size of the forewing in *B. anynana* (one-way ANOVA; $F_{3,133} = 14.137$, $p < 0.001$), *B. safitza* (one-way ANOVA; $F_{3,159} = 133.380$, $p < 0.001$) and *M. perseoides* (one-way ANOVA; $F_{3,177} = 34.059$, $p < 0.001$). Individuals reared at higher temperatures developed significantly larger androconia on the ventral forewing and this effect of developmental temperature was most pronounced in *B. safitza* (Figure 3). Furthermore, in *M. perseoides* the expression of the secondary sexual traits changed gradually and linearly along the temperature gradient, while the androconia size of both *Bicyclus* species showed a discontinuous change to the temperature gradient, with the two higher temperatures yielding significantly larger patches than the two lower temperatures. Developmental temperature had no effect on hindwing androconia size in any of the three species (Figure 3).

Correlations between androconia size and MSP production (B. anynana)

The correlation between the size of the androconia and the total production of MSP approached significance for the patch on the forewing (Wilks's $\lambda = 0.87$, $F_{3,53} = 2.71$, $p = 0.054$) while no significant correlation was found for the patch on the hindwing (Wilks's $\lambda = 0.92$, $F_{3,53} = 1.45$, $p = 0.239$). Univariate ANCOVAs revealed significant correlations between forewing androconia size and individual pheromone component titres of MSP1

(one-way ANCOVA; $F_{1, 55} = 8.156$, $p = 0.006$) and MSP3 (one-way ANCOVA; $F_{1, 55} = 4.879$, $p = 0.031$), with larger patches leading to larger quantities of these two MSP components (Table 1).

Competition experiments (B. anynana and B. safitza)

Forewing androconia size had a significant effect on the probability of mating in *B. anynana* ($z = 2.085$, $p = 0.037$), with males with larger forewing patches being more likely to mate, while such an association was not found for *B. safitza* (Figure 4). The likelihood of mating was not affected by the size of the hindwing patch in both species (see supplementary table 2). Compared to *B. anynana*, overall mating success was lower in males of *B. safitza* (Figure 4).

DISCUSSION

Sexual selection and the seasonal ecology of mycalesines

There is ample evidence to suggest that temporal fluctuations in the intensity and direction of environmental selection have driven the evolution of developmental phenotypic plasticity with alternative forms in mycalesine butterflies (Brakefield and Frankino 2009, van den Heuvel et al. 2013). Our results suggest that the discrete seasonal environments may impose similar fluctuations in sexual selection. The development of larger androconial patches in males is favoured during the wet season that is more favourable for reproduction, while smaller ones are favoured during the dry season when breeding conditions are not optimal. During the wet season mycalesine males spend much of their time chasing and courting females and their relatively large androconial patches may enable the production of larger quantities of MSP and lead to increased reproductive success. In contrast, males that eclose in the early dry season, when larval host plants are largely absent, have significantly reduced androconial patches on their forewings. The generation that occurs during this season remains mostly inactive as they have to survive for many months until they can mate and reproduce with the onset of the next rains and the regeneration of grasses (Brakefield and Reitsma 1991, Windig 1994). The significant reduction in androconial patch size – with dry season form males of *B. safitza* displaying androconia, on average, 61.5% smaller than wet season form males in the wild – may imply that the costs of possessing large androconia may exceed the potential fitness benefits during the harsh, dry season.

Moreover, recent experimental work using *B. anynana* has demonstrated that females reared under conditions that mimic the dry season initiate courtship and compete for mating opportunities (Prudic et al. 2011). The

authors of this study suggest that the males of *B. anynana* may be the choosy sex during the dry season and therefore invest less in the development of sexual traits. Another trait involved in mate choice in *B. anynana* – the size and UV-reflectance of the dorsal eyespot centres – is also significantly reduced in dry season form males (Bhardwaj et al. 2017) while other pigmentation patterns on the dorsal wing surface are typically not plastic in this species (Mateus et al. 2014). Taken together these results suggest that the complex seasonal environments in which mycalesine butterflies occur play an important role in the evolution of sexually selected traits in these species (see also Miller & Svensson 2014).

Developmental plasticity in secondary sexual traits.

The expression of secondary sexual traits is mediated by environmental conditions in many species. One of the most compelling examples of developmental plasticity of sexual traits is the development of eversible abdominal androconial organs in *Cretonotos* moths (Lepidoptera: Arctiidae) (Schneider et al., 1982). Males that are exposed to high concentrations of pyrrolizidine alkaloids – which are secondary metabolites produced by the host plant – during late larval development possess significantly larger scent organs and produce higher amounts of pheromones as adults (Boppré & Schneider 1985; Schmitz et al., 1989). Our results unambiguously demonstrate that forewing androconia size of mycalesine butterflies is strongly influenced by the temperatures experienced during larval development. Males of all three species develop significantly larger androconial patches on their forewing when reared at higher temperatures in the laboratory. These experimental results confirm our findings from the field, where individuals of *B. safitza* that were exposed to high ambient temperatures during development expressed significantly larger patch sizes. Furthermore, in *B. anynana* and *B. safitza* the response of the forewing patch to developmental temperature was discontinuous with a significant increase between 23°C and 25°C; a reaction norm shape that is typical of polyphenism.

Interestingly, we find that, despite environmental perturbation, the development of androconia on the hindwing consistently results in patches of a similar size. This validates the idea that developing tissues – with similar properties in terms of their structure and function – can have distinct sensitivities and patterns of response to external environmental cues and corresponding internal signals (Mateus et al. 2014). The canalized response of androconial patches on the hindwing may reflect differences in importance of the MSP components and their localised production on the wings. In *B. anynana*, MSP2 is exclusively produced in the androconia on the hindwing while MSP1 and MSP3 are associated with both androconial patches (Bacquet et al. 2015, Dion et al. 2016). As the developmental plasticity of androconial sizes only extends to the forewing, the titres of MSP1 and MSP3 are expected to be produced in higher quantities when males are reared at higher temperatures while

levels of MSP2 should show a lack of developmental plasticity. Data from a recent study by Dion and colleagues provide conclusive evidence for this hypothesis (Dion et al. 2016). Here, the authors first demonstrate that the biosynthesis of MSP in *B. anynana* is temperature-dependent. Males produce larger amounts of MSP when exposed to higher temperatures as adults, which is likely associated with increased activity of the enzymes involved in pheromone biosynthesis. Importantly, their results also reveal that the production of MSP1 and MSP3, and not MSP2, is affected by the temperatures that males experience during larval development. Based on our results we postulate that the increased production of these two components observed in wet season form males is due to developmental plasticity of androconial sizes on the forewing.

Though each MSP component affects male mating success in *B. anynana*, MSP2 titers, and its relative ratio to other MSP components, are considered to be honest indicators of male quality (Nieberding et al. 2012, van Bergen et al. 2013). Recent work has shown that MSP2 titres are under strong sexual selection and depleted in additive genetic variation (Nieberding et al., 2012). Our data reveal that the androconial patch responsible for the production of this specific component of the MSP blend is non-plastic across seasonal environments, which could lead to maximized production independently of environmental conditions, enabling full production of MSP2 in the early wet season when the dry season specimens are thought to reproduce.

Androconial plasticity in mycalesine butterflies.

The more than 300 extant species of mycalesine butterflies show comparatively little morphological variation and are notoriously challenging to identify. Hence, the wide diversity in the number, size and position of androconia on the wings of males has become the key taxonomic trait used to distinguish these species (Condamin 1973, Brattström et al. 2016) Despite considerable periods of independent evolution (Aduse-Poku et al. 2015), the three species used in this study demonstrate strikingly similar patterns of plasticity for androconial structures; the scent organs on the hindwing are largely insensitive to thermal variation while the same external cue induces the development of larger sexual structures on the forewing. Furthermore, similar patterns of androconial plasticity have been observed in *Bicyclus sanaos* and *Mycalesis mineus*, two mycalesine butterflies from Africa and Asia, respectively (E. van Bergen, unpublished data) which suggest that this phenomenon is highly conserved across all species of mycalesine butterflies.

Finally, though these morphological structures play an important role in chemical communication, recent work, using 32 African mycalesine species, has revealed that the majority of MSP components are found exclusively in a single androconial unit in each species (Bacquet et al. 2015). The remaining androconial structures typically lack significant amounts of MSP components and are considered to have either lost their function in olfactory

communication or never had one. Instead, these elaborate morphological structures may be involved in other forms of intra- and intersexual communication. For example, the androconial hairs and forewing patches become clearly visible during courtship, when males are positioned close to the female and open and close their wings in rapid succession (Nieberding et al., 2008), which may suggest that these structures could play role in visual communication. Further studies are needed to improve our understanding of the role of androconia in multimodal communication in these seasonal polyphenic butterflies.

Conclusions

The aim of this study was to explore whether the size of androconial patches is under sexual selection in mycalesine butterflies and how the seasonal expression of these secondary sexual traits is induced in these polyphenic insects. Our results reveal extensive temperature-induced plasticity of the forewing androconial patch size in *B. safitza* individuals under field conditions, and in all three species under experimental conditions. Furthermore, in one of the species included in this study, *B. anynana*, larger forewing patches were correlated with increased production of two components of their MSP and increased male mating success. In *B. safitza*, the species that showed the strongest plastic response to developmental temperature, within-environment variation for the size of the forewing androconia was not associated with male mating success. However, in this species the total number of successful matings observed during the competition experiments was very low which suggests that the experimental conditions used may not have been optimal for this species. Hindwing androconial patch size demonstrated environmental canalization as this trait was insensitive to seasonal variation in *B. safitza* and developmental temperatures in all three species studied here. The size of the hindwing patch was not correlated with MSP production in *B. anynana* nor did it affect the likelihood of mating in *B. anynana* and *B. safitza*. Our findings underscore the importance of developmental plasticity to optimise the fitness trade-off between reproduction and survival in short-lived organisms that are exposed to strong environmental variation across generations. Furthermore, our work provides a framework within which the effects of fluctuating selection pressures on secondary sexual traits can be studied.

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FIGURE LEGENDS

Figure 1. Representation of morphological differences in androconial structures among the investigated species of mycalesine butterflies; **A)** *Bicyclus anynana*, **B)** *Bicyclus safitza*, and **C)** *Mycalesis perseoides*. Androconia are formed of differentiated scales and in mycalesine males they generally consist of linked patches and hair pencils found on both fore- and hindwings. The androconia are involved in the production and release of male

sex pheromones (MSP). Left side of each species panel shows the ventral forewing (mirrored) and dorsal hindwing, and the three smaller images to the right show the androconia in detailed view. The upper of the detailed images shows the main androconia on the ventral forewing, the middle shows the dorsal hairpencil(s) on the hindwing, and the lower shows the patch located below the inner hairpencil (hairs removed to aid visibility). The size of the androconial patches was measured as the area within a polygon formed by a series of landmarks (examples shown with black dots in panel A).



Figure 2: **A)** A schematic representation of temperature, relative humidity and daily rainfall through the fluctuating dry and wet seasons in Zomba, Malawi. Dark green dots are daily mean temperature measurements while daily measurements of relative humidity are given in blue. Dashed lines reflect the seasonal fluctuations in temperature (dark green) and relative humidity (blue) while the purple bars represent the daily rainfall in mm. The background colours illustrate the dry season (yellow) and wet season (light green). **B)** Bimodal distribution of wing pattern elements used to classify the distinct seasonal forms in *B. safitza*. Throughout the figure DSF individuals are represented by red symbols while WSF specimens are represented by black symbols. The androconial patch sizes of the specimens are given for the date of capture in panel **C** and **E**, representing the fore- and hindwing patches respectively. Patches on the forewing are represented by circles while the patches on the hindwing are represented by diamonds. Panel **D** and **F** demonstrate variation in fore- and hindwing androconial patch size, respectively, between seasonal forms in *B. safitza*. Statistical significance is indicated as ns (non-significant) = $p > 0.05$ and *** = $p < 0.001$.

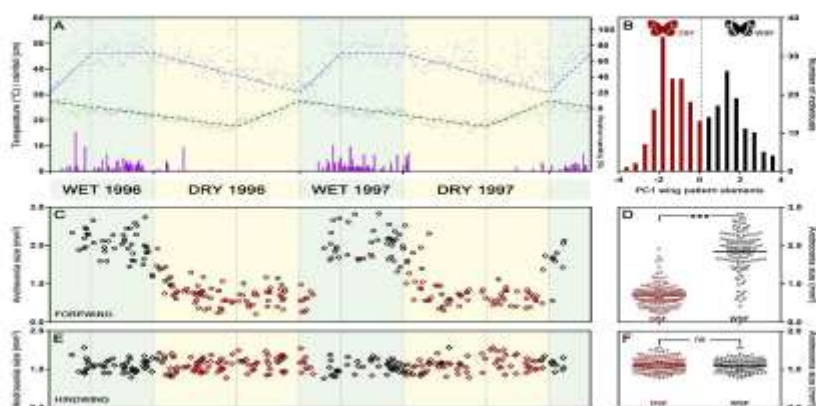


Figure 3. Effect of developmental temperature on the androconia size on the **A)** forewing and **B)** hindwing in *B. anynana* (red), *B. safitza* (blue), and *M. perseoides* (green). Letters represent differences between groups as reported by Tukey's post-hoc test, coding for each species separately, while the error bars represent 95% confidence intervals. Effects remained significant when testing the effect of temperature on androconial patch size expressed relative to wing size (see supplementary information).

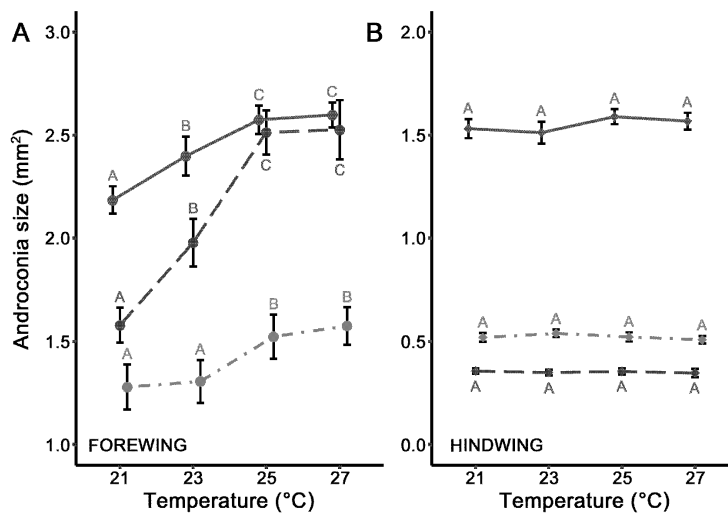


Figure 4. Estimated probability of mating as a function of forewing androconia size for **A)** *B. anynana* (N=157) and **B)** *B. safitza* (N=107). Lines represent the model prediction, while the grey area represents the 95% confidence interval of the prediction. Data points are represented by open circles.

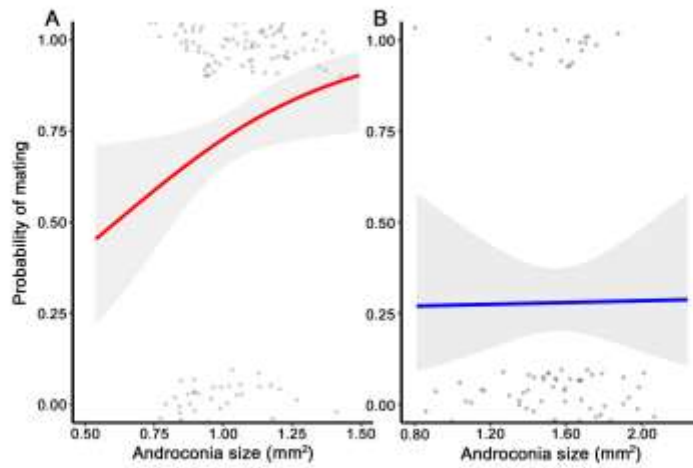


Table Legend

Table 1. Correlations between the sizes of the androconial patches and the production of MSP1, MSP2 and MSP3 in *B. anynana*, using variation among ‘families’ as a covariate (not shown). Only the production of MSP1 and MSP3 is positively correlated with the size of the androconial patch on the forewing (in bold).

Androconial patch	Pheromone component	F	df	p
Forewing	MSP1	8.156	1, 55	0.006
	MSP2	0.049	1, 55	0.825
	MSP3	4.879	1, 55	0.031
Hindwing	MSP1	1.086	1, 55	0.301
	MSP2	0.119	1, 55	0.730
	MSP3	3.369	1, 55	0.071