

Seasonal Polyphenism in *Bicyclus dorothea* (Lepidoptera: Nymphalidae) Across Different Habitats in Cameroon

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Abstract

Many organisms exhibit changes in phenotypic traits as a response to seasonal environmental variation. We investigated the role of habitat in generating seasonal polyphenism in different populations of the light bush brown butterfly *Bicyclus dorothea* (Cramer, 1779) (Lepidoptera: Nymphalidae) in Cameroon. Butterflies were caught during the wet and dry seasons across four localities representing two distinct habitats, namely forest and ecotone (forest–savanna transition zone) over a 2-yr period (2015–2016). We found distinct variation in the wing pattern characteristics of butterflies in response to seasonality and habitat. Specifically we observed that: 1) all wing characters are not seasonally plastic in *B. dorothea*; 2) populations from ecotone tend to be more variable, with individuals exhibiting wings with large spots during the wet season and very reduced spots in the dry season while in forest populations, individuals exhibit wings with large spots during the wet season, but in the dry season, spots are not as greatly reduced as their ecotone counterparts; 3) this polyphenism in *B. dorothea* alternated consistently during the wet and dry seasons over the 2 yr of sampling. *Bicyclus* species have become a textbook example of seasonal polyphenism while this study extends this model system to the unique forest–ecotone gradient of Central Africa and demonstrates the complexity of seasonal forms in different habitats.

Key words: seasonal polyphenism, *Bicyclus dorothea*, ecotone, forest

Polyphenism is a phenomenon characterized by the production of two or more distinct phenotypes from a single genotype (Shapiro 1976). This phenomenon is common in many insect species who exhibit this feature as a response to variation in an environmental cue (Simpson et al. 2011). In a seasonal environment, polyphenism will be the result of contrasting but predictable cues, leading to different morphologies, life history traits or behaviors expressed in each season and allowing species to persist in a given environment (David et al. 1997, Oostra et al. 2011). These environmental cues can be temporal, spatial, biotic or abiotic (Whitman and Agrawal 2009). Polyphenism is thought to be an adaptive response and potentially a determining factor in the evolutionary success of insects (Simpson et al. 2011).

In recent decades, multiple aspects of polyphenism in insects have been studied (Capy 1993, David et al. 1997, Ribeiro and Freitas 2011). Though attention has been paid to a range of Lepidoptera species such as *Melanitis leda* (Brakefield and Larsen 1984, Brakefield 1987), *Manduca sexta* (Kingsolver et al. 2009), and *Automeris io* (Sourakov 2014), species belonging to the subtribe Mycalesina and the genus *Bicyclus* (Kirby, 1871) represent the best-studied taxa with respect to phenotypic plasticity in the field (Brakefield and Reitsma 1991, Windig et al. 1994, Brakefield and Frankino 2009,

Brakefield and Zwaan, 2011). Studies in phenotypic plasticity have used many *Bicyclus* species such as *Bicyclus anynana* (De Jong et al. 2010), *B. cottrelli*, *B. safitza*, *B. ena*, *B. vulgaris* and *B. vansoni* (Windig et al. 1994, Roskam and Brakefield 1996), and *B. sanaos* (Oostra et al. 2014, van Bergen et al. 2017). Studies conducted in Malawi have shown that during the dry season (characterized by scarce nutritive resources and a drop of monthly mean temperatures) adult *B. anynana* typically express wings with small cryptic eyespots (Brakefield and Reitsma 1991, Brakefield 1997). Moreover, apart from wing patterns, many other life history traits are also affected such as reproductive diapause, fat content, longevity, weight, development time of immature stages, and predator avoidance (Kooi et al. 1997, Zwaan et al. 2001, Zijlstra et al. 2002, Lytinen et al. 2004, Westerman and Monteiro 2016). During the wet season, resources are abundant and adult butterflies display wings with prominent and concentric eyespots along the distal margin. These favorable conditions allow butterflies to develop faster and produce two or three generations before the onset of the dry season. This polyphenism in *B. anynana* wing patterns appears to be mainly induced by temperature variation over wet (high temperature) and dry (low temperature) seasons in their natural habitat in Malawi (Brakefield et al. 2007).

In fact, *B. anynana* cohorts reared at 27°C in the laboratory exhibit wet season forms with prominent eyespots while those reared at temperatures ranging between 17 and 20°C exhibit dry season forms with cryptic eyespots (De Jong et al. 2010).

Central Africa is dominated by Congolese forest habitats that cover most of equatorial Africa. However, there is also considerable habitat variation throughout the biome where forests meet with other ecological communities, ecosystems or ecological regions at their boundary forming a community mosaic termed the ‘ecotone’ (McArthur and Sanderson 1999, Kark and Van Rensburg 2006). Ecotones occur at multiple spatial scales and range from natural boundaries to human-generated ecotones. These transition zones have also been highlighted as high speciation centers and hence many researchers have noted their importance for long-term conservation (Smith et al. 1997, Walker et al. 2003, Kark 2013, Senft 2009). In Cameroon, forest–savanna ecotones are present and represent an opportunity to address questions regarding habitat influence on seasonal polyphenism. Forest and ecotone habitats may present fluctuations in many climate components such as annual rainfall, monthly mean temperature, and vegetation composition which may exert influence on phenotypic plasticity in insects.

This study investigates seasonal variation in the morphological traits of *Bicyclus dorothea* (Cramer, 1779) (Lepidoptera: Nymphalidae), congener of *B. anynana*, across different habitats (forest and ecotone) of Cameroon. Physiognomic and climatic variation between forest and ecotone habitats may drive divergence in morphological responses to seasonality in *B. dorothea*. In this context, we expected populations from climatically less variable forest habitats to show lower variation in morphological features compared to populations from the more variable ecotone habitats. In this study, we first determined 1) which morphological features are seasonally plastic in *B. dorothea* by measuring wing features on individuals caught in each habitat over time across a year. We then examined 2) the extent to which forest and ecotone habitats differ from one another in structuring morphological variation of *B. dorothea* across seasons.

Material and Methods

Species

B. dorothea is a tropical fruit-feeding butterfly found in West and Central Africa (Condamin 1973). It is found in the whole West African forest zone. Its distribution extends to southern Cameroon, the northern part of the Republic of Congo, Democratic Republic of Congo, Central African Republic and reaches the Semuliki forest at the border with Uganda (Aduse-Poku et al. 2017). Where it occurs, *B. dorothea* seems to prefer low- to mid-altitude habitats. In Cameroon, *B. dorothea* is found widely in mid-altitude forests and across many forest–savanna ecotone transition areas. It is uncommon or absent in highland and dry savanna habitats (Dongmo et al. 2017). Up to now, no studies have investigated the dispersal ability or behavior of *B. dorothea* adults. However, from our observations in the field, adults appear to be relatively sedentary and are generally found reliably and restricted locally to sites where larval host plants and adult food resources are abundant.

Study Sites and Sample Collections

Cameroon is a central African country with highly variable topography and associated vegetation and climatic parameters across large altitudinal gradients. The south of the country consists of tropical rainforest while the north and far north is made up mainly of lowland savanna. We used four sites in Cameroon to determine the relationship of vegetation and climate on polyphenism in *B. dorothea*—two in the tropical rain forest, Mbalmayo (N 3.388, E 11.47, 768 masl) characterized by a degraded forest and Somalomo (N 3.37405, E 12.7332, 638 masl), a primary forest in the Dja wildlife reserve. The two other sites, a woodland forest–savanna ecotone at Ako (N 6.68783, E 10.70687, 706 masl) and a forest–savanna ecotone at Ndikiniméki (N 4.76986, E 12.7332, 812 masl) were also sampled, representing ecotone habitat (Fig. 1). We used Thermochron iButton data loggers (model: DS1922) in each site to record ambient temperature. Data loggers were suspended at 2 m from the ground and protected from direct sun. Rainfall data were obtained from the Mbalmayo weather station and approximated for the remaining sites—Bafia (50 Km from Ndikiniméki),

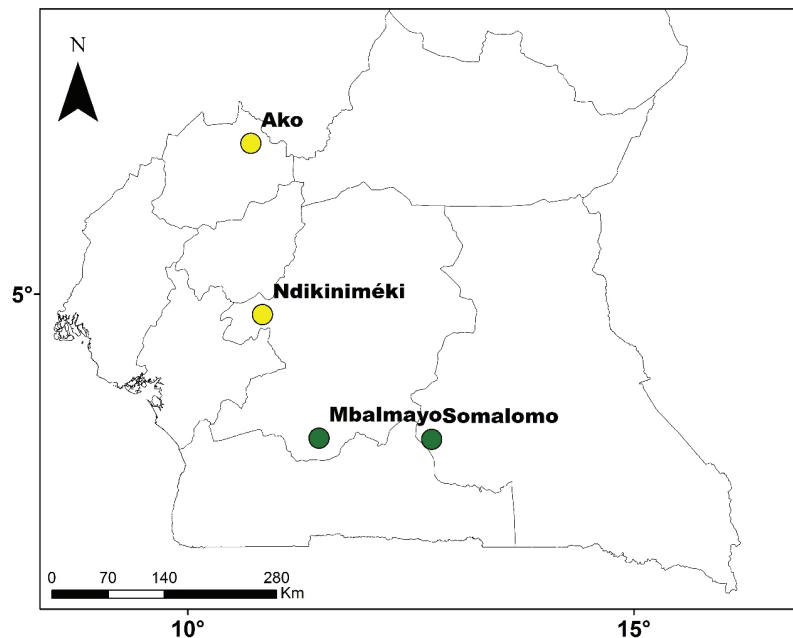


Fig. 1. Map showing sampling sites in Cameroon. Yellow circles represent ecotone sites while green circles represent forest.

Akonolinga (41 Km from Somalomo), and Bambui (80 Km from Ako). In all study sites, seasonal timing is similar with the onset of the dry season in mid-November extending to early March. However, there are differences in the amount and variation in temperature and rainfall (Fig. 3a–d). The ecotone sites (Ako and Ndikiniméki) have a unimodal rainfall pattern with reduced precipitation from November to February (Fig. 3a and b) while in the forest sites, the rainfall pattern is bimodal with relatively high precipitation during the dry season (November to February) compared to ecotone locations (Fig. 3c and d).

At each site, individual butterflies were caught using sweep nets in addition to over-ripe banana-baited traps placed in road tracks or clearings in the forest, a preferred microhabitat of *B. dorothea* (Larsen 2005). Sampling was conducted two or three times during the dry (trapping from January to March) and wet (trapping from June to August) seasons in both 2015 and 2016 at each locality. Butterflies caught were brought to the entomology laboratory of the International Institute of Tropical Agriculture (IITA-Yaoundé, Cameroon) and kept in a room maintained at 26°C, 75% RH and 12:12 (L:D) h photoperiod and used for rearing study (for a related study not described here). After their death, wild-caught individuals were stored in small glassine envelopes for further morphological analysis.

Morphological Measurement

The wing spots of *B. dorothea* are similar in structure and position to most *Bicyclus* species; however, only ventral spots are present and dorsal spots are absent in this species. There are nine spots in total, two on the forewings and seven on the hindwings, though sometimes one can find three spots on the forewing and eight on the hindwing of some individuals. Eyespots are approximately circular in shape and

made of numerous rings: a white ring in the center of the eyespot, a black disc, a cream yellow disc and an outer gold disc (Brakefield and French 1997). Prior to measurement, fore and hindwings were separated from the body of dead butterflies. Thirteen wing pattern characters (Fig. 2) were measured. Since the spots are not perfectly circular, all measurements were done parallel to the vein of the wing. Moreover, measurements were done only on the left fore and hindwings. The right wings were used in cases where the left wings exhibited significant damage. To measure the diameter of the eyespot, the fore and hindwings were placed between two glass slides under a stereomicroscope fitted with a micrometer eyepiece at 6× magnification. Wing length, from the thorax to the apex and the width of the fore and hindwings were measured using a caliper (0.1 mm accuracy).

Statistical Analysis

To analyze the morphological variation in wing characters, a principal component analysis (PCA) was performed on data of each sex to reduce the wing pattern variability for forest and ecotone habitats. The first two components were used for further analysis (see results for further details). We then applied nested analysis of variance (ANOVA) models on the two retained principal components with season, habitat, and sex as fixed factors, and sampling sites nested in habitat. We constructed models with interaction terms between season and habitat, and with sites nested in habitat. Visual inspection of residual plots of each model did not reveal any obvious deviations from homoscedasticity or normality. *P*-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. All statistical operations were performed with R v 3.4.0 software (Team, R. C. 2017).



Fig. 2. Characters measured by the stereo microscope and caliper. Shown are one fore and one hind wing of a *B. dorothea* male. 1 = length of the fore wing; 2 = width of the fore wing; 3 = diameter of the first eyespot of the fore wing; 4 = diameter of the second eyespot of the fore wing; 5 = length of the hind wing; 6 = width of the hind wing; 7 = diameter of the first eyespot of the hind wing; 8 = diameter of the second eyespot of the hind wing; 9 = diameter of the third eyespot of the hind wing; 10 = diameter of the fourth eyespot of the hind wing; 11 = diameter of the fifth eyespot of the hind wing; 12 = diameter of the sixth eyespot of the hind wing; 13 = diameter of the seventh eyespot of the hind wing.

Table 1. Number of butterflies captured during 2 yr in each site

Sampling sites	Habitat	Sex	Seasons				Total
			Wet 2015	Dry 2015	Wet 2016	Dry 2016	
Ako	Ecotone	Males	83	69	44	38	369
		Females	31	26	50	28	
Ndikiniméki		Males	72	69	54	43	382
		Females	50	27	39	28	
Mbalmayo	Forest	Males	74	69	67	58	434
		Females	54	34	56	22	
Somalomo		Males	30	93	36	28	313
		Females	34	36	35	21	

Results

We recorded a total of 1,498 butterflies during 2 yr of sampling in four sites representing two distinct habitats (Table 1). PCA performed on our data recorded 74.4% of the total variation of the first two principal components (PC1 and PC2) in males and 75.5% in females. The

Table 2. Component weights and eigenvalues for the principal component analysis with data of all habitat and seasons pooled together in males and females

Traits	Males		Females	
	PC1	PC2	PC1	PC2
Length of the forewing	0.099	0.561	-0.150	0.506
Length of the hind wing	0.125	0.549	-0.153	0.481
Width of the forewing	-0.019	0.054	-0.163	0.406
Width of the hind wing	0.138	0.565	-0.176	0.485
Diameter first eyespot forewing	0.333	-0.097	-0.325	-0.128
Diameter second eyespot forewing	0.346	-0.053	-0.329	-0.116
Diameter first eyespot hind wing	0.293	-0.082	-0.287	-0.113
Diameter second eyespot hind wing	0.345	-0.106	-0.322	-0.135
Diameter third eyespot hind wing	0.351	-0.093	-0.329	-0.139
Diameter fourth eyespot hind wing	0.346	-0.093	-0.335	-0.121
Diameter fifth eyespot hind wing	0.356	-0.064	-0.339	-0.105
Diameter sixth eyespot hind wing	0.338	-0.047	-0.314	-0.044
Diameter seventh eyespot hind wing	0.181	0.078	-0.243	-0.038
Eigenvalue	55.56%	18.85%	54.17%	21.36%

Eigenvalues are expressed as percentage of total variance accounted for by the first principal component and second principal component.

diameter of wing spots loaded heavily on PC1 while the length and the width of the fore and hindwings had the highest loadings on PC2 for both males and females (Table 2). PC1 and PC2 varied across seasons and habitat (Fig. 4). The diameter of the eyespots (PC1) are the main wing pattern characters varying across season and habitat while PC2 showed less variation, suggesting that the length and the width of the wings are not seasonally plastic and are less affected by habitat (Fig. 5). Within each habitat, PC1 fluctuated seasonally and simultaneously, but the pattern of variation was not identical across habitats. The diameter of ocelli in the ecotone population varied drastically from one season to another while in forest populations, a slight variation of this trait

Table 3. Statistical results of the nested ANOVA models for the relationships between the principal component 1 and 2 and environmental factors

Response trait	Factors	df	Mean sq.	F value	P value
PC1	Sex	1	0	0.003	<0.001
	Seasons	3	847.0	214.1	<0.001
	Habitat	1	786.6	198.9	<0.001
	Seasons*habitat	3	86.0	21.7	<0.001
	Seasons*habitat/sites	8	102.4	25.9	<0.001
	PC2	Sex	1	0.5	0.2
PC2	Seasons	3	11.8	6.2	<0.001
	Habitat	1	5.9	3.1	0.080
	Seasons*habitat	3	8.9	4.7	0.002
	Seasons*habitat/sites	8	8.3	4.4	<0.001

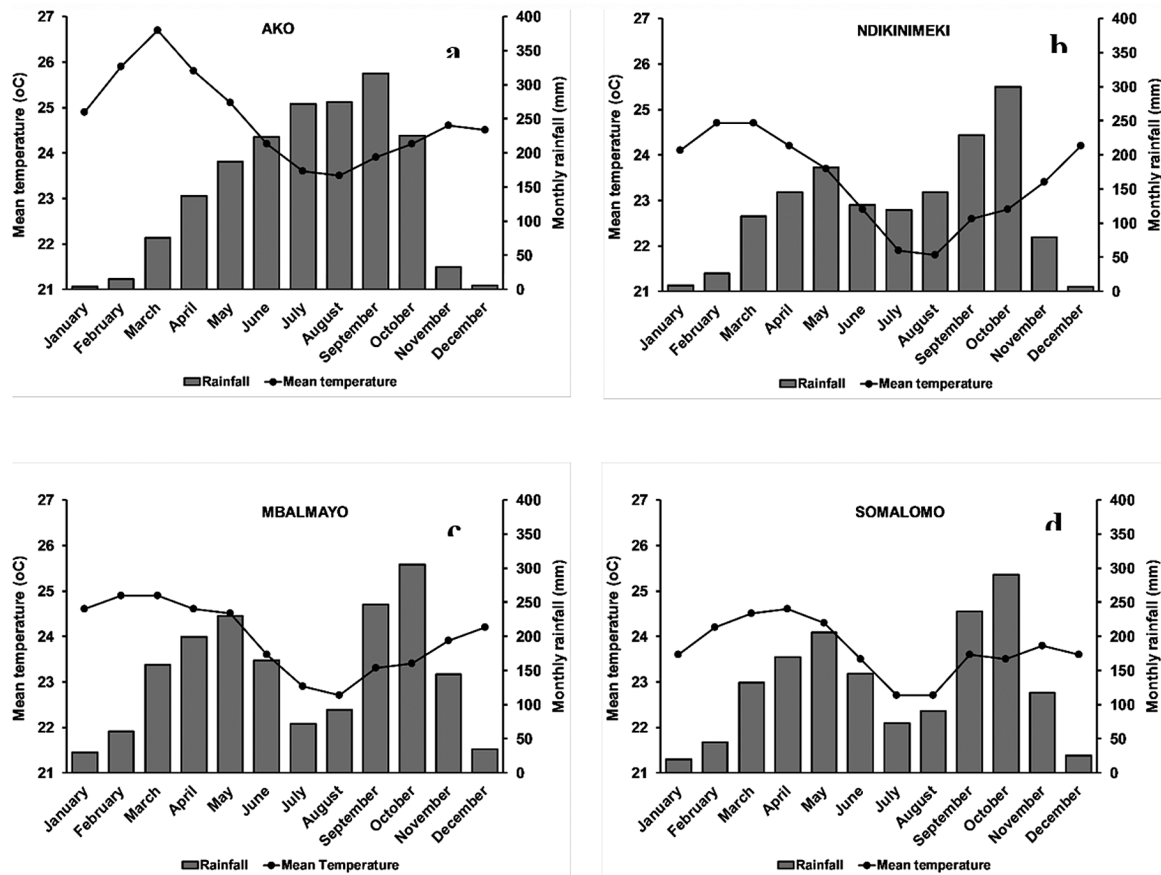


Fig. 3. Temperature and rainfall profiles of each study site: (a) Ako; (b) Ndikiniéki; (c) Mbalmayo; and (d) Somalomo.

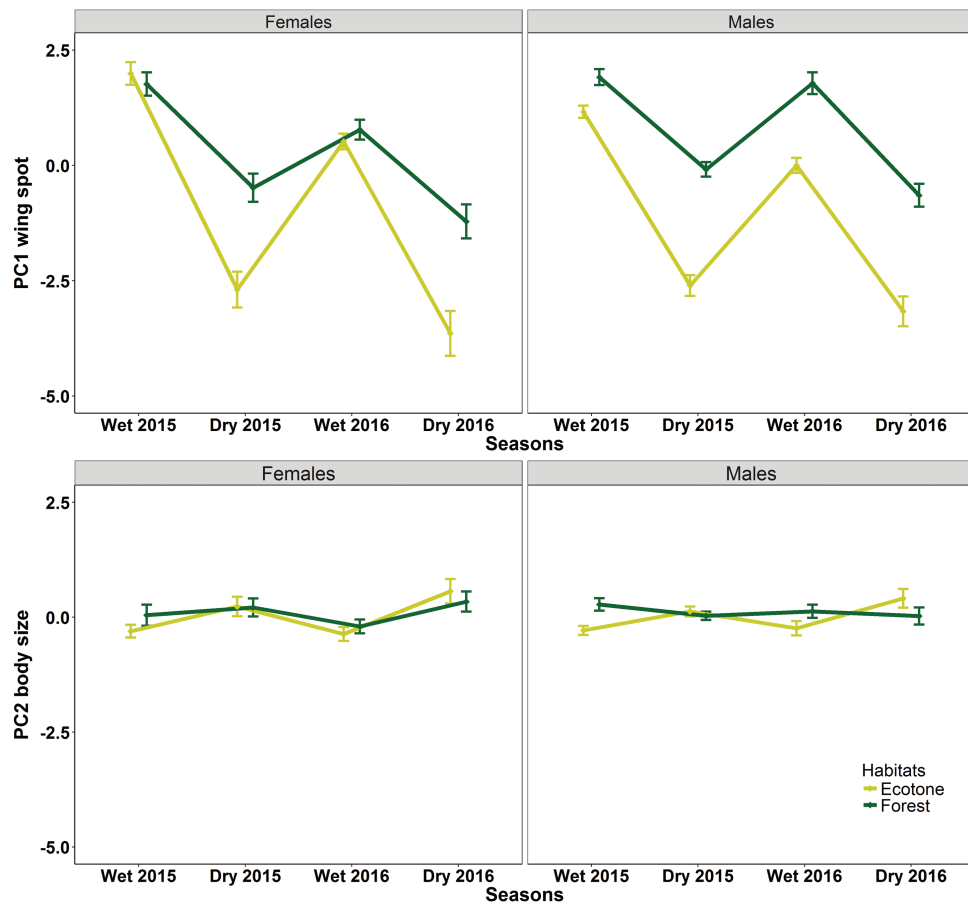


Fig. 4. Seasonal changes in the first two principal components of *Bicyclus dorothea* wing patterns in different populations in Cameroon inhabiting forest and ecotone habitats over the transition from a wet to dry season.

was observed. Nested ANOVA performed using PC1 and PC2 showed a high response for these traits to season, habitat and their interaction though PC2 showed no effect of habitat (Table 3).

Discussion and Conclusions

The results demonstrate that habitat and season have strong effects on wing pattern elements in *B. dorothea*. Among all wing characters studied, the diameter of eyespots was highly sensitive to the effect of habitat, sex, seasons and the interaction between habitat and seasons. All populations examined in this study show a certain degree of plasticity with respect to the diameter of their eyespots. This variation is strongly seasonal and more pronounced in ecotone populations than in their forest counterparts. Generally, this study revealed two important patterns in seasonal polyphenism in *B. dorothea*: 1) a general observation indicating that ecotone populations tend to be more variable than forest populations and 2) change in eyespot size occurs seasonally suggesting an adaptation of these populations to alternating changes in climate annually (Brakefield and Larsen 1984, Brakefield and Reitsma 1991, Brakefield et al. 2007).

Previous studies on seasonal polyphenism in *Bicyclus* butterflies in a single locality in Malawi showed that seasonal forms of these butterflies differ drastically in color and wing pattern elements over the wet and dry seasons (Windig et al. 1994). A similar example was also found in Australia where butterflies belonging to the genus *Mycaliesis* Hübner, 1818 exhibit the same pattern of plasticity of their wing elements over seasons (Braby 1994). This plastic response

in *Bicyclus* butterflies to wet and dry seasons was shown to be directly related to changes in environmental components (Brakefield and Reitsma 1991, Windig et al. 1994, Brakefield and Zwaan 2011). The role of temperature was highlighted as the main factor inducing these different morphs (Roskam and Brakefield 1996, Brakefield et al. 2007) though biotic factors like food plant quality can also influence the development of wing patterns and hence, can drive plastic responses in the field (Kooi et al. 1996). One of the key climatic characteristics of ecotones is the greater annual variation in environmental variables (Longman and Jenik 1992). In these regions, while rainfall patterns are generally unimodal with large differences between day and night temperatures (Hirota et al. 2010, Ibanez et al. 2013), tropical rainforests are often characterized by a bimodal rainfall pattern, with relatively small differences between day and night temperatures.

Brakefield and Reitsma (1991) highlighted that temperature decreases during the dry season in a unique study area in Malawi was the principal cue leading to the dry season form (with reduced wing spots) of five *Bicyclus* species in the wild. The strong plasticity observed at ecotones during the dry season can be explained by the heterogeneous nature of the environment. Ecotones are generally mosaics of forest fragments surrounded by tree-less savanna. Together, vegetation patterns and temperature fluctuations increase environmental heterogeneity at the microhabitat level. In regions with a unimodal rainfall pattern, dry seasons tend to be longer and drier than those of regions with bimodal rainfall patterns (though this also depends on altitude), leading to drier environments in

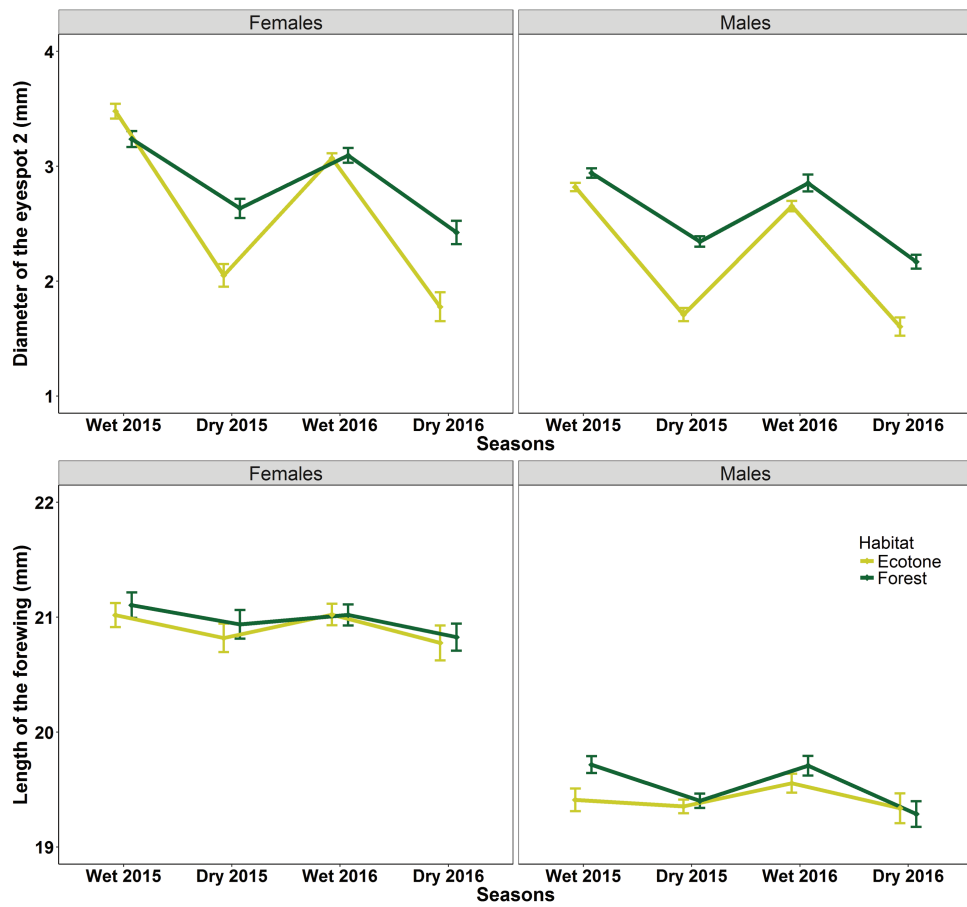


Fig. 5. Seasonal changes in the length of the forewing and the second diameter of the forewing of *B. dorothea* wings in different populations in Cameroon inhabiting forest and ecotone habitats over the transition of the wet to dry seasons.

ecotones during the dry season compared to forest environments (Tsalefac et al. 2003). Hence, while tropical rainforests have almost constant mean monthly temperatures and availability of larval host plant throughout the year, larval host plants are likely only available in the wet season in ecotone habitats where there is considerable fluctuation in mean monthly temperatures over seasons (Chan et al. 2016). Predation very likely plays an additional key role in maintaining wing spot plasticity. In *B. anynana* changes in wing patterns are known to be adaptive in that dry season forms are cryptic while conspicuous eyespots in wet season forms can contribute to deflection of and survival from predator attacks (Lyytinen et al. 2004, Prudic et al. 2015).

It is well known that behavioral responses are among the most immediate responses of animals in general and butterflies in particular to climatic variation (Walther et al. 2002, Parmesan 2006). Two strategies can be used by many satyrine species: 1) adults will cease their reproduction during unfavorable dry seasons and females will start reproducing at the onset of the rainy season (Brakefield and Larsen 1984), or 2) persisting adult females during the dry season will move into an aggregate of moist refugia in the understory where they will sometimes lay their eggs on the few remaining fresh host plants for larval growth (Braby 1995). Larvae from these eggs will then develop in an environment with favorable temperatures and will likely yield adults with reduced spots. The high plasticity observed in ecotone populations suggests adaptation to complex changes (such as lack of larval food plants and predator avoidance) occurring during the dry season—but the mechanisms behind these patterns require further study. Exploring seasonal polyphenism in life history traits

such as reproductive diapause, egg fertility, population dynamics of *Bicyclus* species across the ecotone and forest gradient will provide insights into such evolutionary aspects of phenotypic plasticity.

Our study showed that *B. dorothea* exhibits seasonal polyphenism in some of its wing pattern characters and the degree of plasticity is a function of habitat type. Populations from ecotone habitats tend to be more variable over the wet and dry seasons relative to their forest counterparts. This seasonal polyphenism is likely linked to environmental cues such as climate or vegetation, known to vary considerably in ecotone habitats, and driven by selective forces such as predation and host plant changes over time. The study here demonstrates the importance of field-based study of butterfly morphology for understanding trait and environment relationships—but this work is limited by a lack of laboratory evidence for plasticity for this particular case. Experimental approaches would well supplement these results and have the potential to address many of the open questions posed here. Such an integrated approach in the field and laboratory will help in understanding the persistence and the risk of extinction of species across diverse and species-rich ecosystems in Central Africa (Dongmo et al. 2017) and provide strategies that can be used by conservation biologists in the future under climate change (Bonebrake et al. 2018).

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