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RESEARCH

Habitat effects on local adaptation and plasticity of thermal tolerance across life stages in tropical *Bicyclus* butterflies

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Abstract
Climate change impacts on ectotherms will be a consequence of an interplay between species-specific evolutionary effects, population-level local adaptation, and developmental or plastic effects in individuals. While variation in thermal tolerance resulting from species physiological differences and local adaptation are well researched, how variation in plasticity across habitats might impact vulnerability to climate change remains poorly understood. We studied microhabitat (understory vs. open) distributions and the plasticity in thermal tolerance of four *Bicyclus* butterfly species across forest and ecotone (savanna-forest transition zone) habitats in Cameroon. For each species, we performed common garden experiments at two stable temperature regimes (20 and 30 °C) and quantified larval and adult thermal tolerance. We found clear differences in distributions across species such that two species were more associated with open microhabitats (*B. dorothea* and *B. vulgaris*) while two others were more understory associated (*B. sanaos* and *B. sandace*), with variation across seasons and habitats (forest vs. ecotone). Three species exhibited higher plasticity in critical thermal maximum (CT_{max}) in the ecotone relative to the forest indicating the importance of the interaction between habitat and developmental temperatures in influencing thermal tolerance. Microhabitat distributions were also consistent with trends in thermal tolerance; the most understory-associated species had both the lowest average CT_{max} and lowest plasticity in CT_{max} in the ecotone. Our findings suggest that microclimate and thermal adaptation shape plastic responses to thermal tolerance, and these factors will likely result in heterogenous responses to climatic change for tropical insects.

Keywords Climate change · Developmental plasticity · Habitat preference · Ecotone · Forest · Microhabitat

Introduction

The accelerating rate of global warming presents a serious challenge to most organisms, especially ectotherms whose physiological and biochemical processes are strongly affected by environmental temperature (Urban 2015; Pacifici et al. 2015). To cope with the rapidly

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changing climate, organisms can: adapt through evolutionary changes (Hoffman and Sgro, 2011; Schou et al. 2014; Diamond 2017), shift their geographical distribution (Parmesan 1996; Thomas 2010), or acclimate to novel environments through phenotypic plasticity, including behavioural thermoregulation (Kearny et al., 2009; Rodrigues and Beldade 2020). While studies have increasingly characterized the genetics underlying thermal adaptation (Ware-Gilmore et al. 2023; Milucki et al., 2024) and plasticity in thermal tolerance (Gunderson and Stillman 2015; Lockwood et al. 2018; Rodrigues and Beldade 2020), knowledge gaps remain, especially in the tropics (Merilä 2012; Sheldon 2019).

Phenotypic plasticity allows organisms to cope with rapidly changing environments (Ghalambor et al. 2007; Bonamour et al. 2019). At the physiological level, plasticity in thermal tolerance traits can be the result of developmental conditions (e.g., temperature) experienced by immature stages (Stillman 2003; Sgro et al., 2016; Kellerman et al., 2017) that may provide a means to increase their fitness and those of adults (though not always), and thereby contribute to the future survival of populations or species facing climate variation (Rodrigues and Beldade 2020; Buckley 2022). In fact, adult fitness of arthropods is determined by the growth and development rates of immature stages that are themselves controlled by temperature (Forster et al. 2011; Soltani Orang et al. 2014) through the well-known temperature-size rule (Atkinson 1994) and the metabolic theory of ecology (Brown et al. 2004). These two theories broadly highlight that, in ectothermic organisms, high developmental temperatures should lead to small-sized individuals and high metabolic rates in resulting adults that can determine responses to elevated temperature.

Habitats (e.g., forest vs. grassland) and microhabitats (e.g., canopy vs. understory, or shaded forest vs. open forest) can also exert strong influence on thermal tolerance of species (Moiroux et al. 2013). For example, Montejo-Kovacevich et al. (2020) demonstrated the role of habitat as an important predictor in determining thermal sensitivity of ten *Heliconius* butterfly species in the Ecuadorian Andes – highland species had significantly lower heat tolerance compared to populations found in the lowland of the same area. Similarly, in Ghana, Woon et al. (2022) found that termites from forest (covered by canopy) have considerably lower heat tolerance compared to those from savanna open environments exposed to more elevated temperatures. Microhabitats provide shelters and refugia which can provide an escape from extreme conditions and are also important considerations for quantifying exposure that can influence thermal adaptation. In army ants, microhabitat is a primary predictor of thermal tolerance with below-ground species being particularly sensitive to temperature change as a consequence of adaptation to lower temperatures (Baudier et al. 2015). Habitat at multiple scales (e.g. elevation, land cover, and vertical) can therefore shape thermal tolerance of insects and determine their distributions and vulnerability to climate change (Alruiz et al. 2022).

Variability in the thermal sensitivity of life stages represents an additional complicating factor underlying the prediction of insect responses to climate change (Kingsolver et al. 2011). In the fall army worm (*Spodoptera frugiperda*), for example, adults exhibited lower maximum thermal tolerance (CT_{max}) than larvae (Phungula et al. 2023). In the butterfly *Bicyclus anynana*, Klockmann et al. (2017) found that heat tolerance was highest for pupae and lowest in eggs and hatchlings. For butterflies generally, relative to adults, larvae may also have a lower ability to thermoregulate, with limited capacity to move long distances towards optimal thermal conditions or away from extremes (Ashe-Jepson et al. 2023). Climate change responses in ectotherms are then expected to be a complex interplay between

the thermal sensitivity of immatures stages, which generally have a reduced mobility (e.g., larvae of butterflies) and that of adults as their mobility can help them escape from deleterious effects of warming through behavioural thermoregulation (Stevenson 1985; Abram et al. 2017) and contribute to the evolution of thermal response of the species (Lafuente and Beldade 2019).

Tropical insects suffer from a range of knowledge gaps and lack of research which challenges predictions of their responses to environment change (Slade and Ong 2023). Modelling studies have highlighted a possible high vulnerability to future warming in tropical insects given that environmental temperatures are already close to their upper thermal limits (Deutsch et al. 2008; Kingsolver et al. 2013). However, microclimates and thermoregulation may provide relief from thermal extremes for tropical insects (Bonebrake et al. 2014). How these different factors that shape thermal tolerance – plasticity, habitat, microhabitat, and life stage – then interact to influence thermal sensitivity should be a priority for understanding climate change impacts of insects, especially in the tropics.

Butterflies of the genus *Bicyclus* (Kirby, 1871) are endemic to Africa where they occupy many habitat types (Aduse-Poku et al. 2017). *Bicyclus* are also well known for often exhibiting seasonal polyphenism in morphological features (Brakefield and Larsen 1984, Dongmo et al. 2018) and local adaptation to habitat types in thermal tolerance, e.g. *B. dorothea* (Dongmo et al. 2021). During the past four decades, several species from the genus have been extensively used to explore many aspects of phenotypic plasticity such as wing morphology and behaviours under laboratory and natural conditions (e.g., Windig et al. 1994; van Bergen et al., 2017, Halali et al. 2021a, b).

In this study, we make use of the diversity of macro and microhabitats present in Cameroon to examine their roles in driving plasticity in thermal tolerance. We studied populations of four *Bicyclus* species (*B. dorothea*, *B. sanaos*, *B. sandace*, and *B. vulgaris*) originating from two contrasting habitats: tropical rainforest characterized by relatively small variation in temperatures and a forest-savanna transition ecotone known to be more variable thermally (Tsalefack et al., 2003). First, we conducted field surveys in each habitat (forest and ecotone) to assess the seasonal microhabitat (open vs. understory) associations of the target species. We hypothesized that the seasonal microhabitat association and distribution of the four *Bicyclus* species would be consistent with patterns of thermal tolerance quantified under laboratory conditions. We also hypothesized that forest populations of the four *Bicyclus* species experiencing low temperature variation would be less thermally plastic than their ecotone counterparts that are exposed to a greater thermal variation in the wild. To test this, we subjected populations of these butterfly species to one of two constant temperatures (approximate minimum and maximum temperatures for populations in shaded/understory ecotone and forest habitats) during their development in a common garden environment. Specifically, we predicted that more open associated butterflies (possibly *B. vulgaris* and *B. dorothea*) would have higher thermal tolerance relative to more understory species (possibly *B. sandace* and *B. sanaos*).

Methods

We sampled four species of *Bicyclus* butterflies across two habitats in Cameroon, ecotone and forest. We conducted ecological surveys to assess the association of the species with two microhabitats, either understory or open, in both habitats (see *Microhabitat associations* below). From each of these habitats we established lab colonies to test thermal tolerance under distinct thermal conditions (see *Plasticity in thermal tolerance* below).

Habitats and study species

Cameroon has a complex topography and its vegetation and climate show dramatic variation across the country's extensive latitudinal gradient (~2–14°N) (Servant and Servant 2000). Vegetation types across the country range from tropical dense forest in the south, isolated montane forests in the north-west, to large savannas and Sahelian landscape in the north (Djoufack 2011). We sampled in two localities of Cameroon - ~450 km apart - Ndikiniméki (N 4.76986, E 12.7332) and Somalomo (N 3.37405, E 12.7332) (Fig. S1). The average altitude in Ndikiniméki is ~800 m at sea level (a.s.l) and the area receives rainfall seasonally twice a year amounting to 1,700 mm/year with a monthly mean temperature ranging between 22 and 25 °C (Tsalefac et al. 2003; Dongmo et al. 2018). The vegetation is described as ecotone, largely composed of a mosaic of gallery forests and savannas (Smith et al. 1997). Somalomo is a small village situated in the southern part of Cameroon dominated by dense tropical rainforest and is in the vicinity of one of the country's largest protected areas, the Dja Faunal Reserve. The average altitude of Samolomo is ~600 m a.s.l. The location has an equatorial climate receiving rainfall seasonally twice a year amounting to ~2,000 mm/year and monthly mean temperature varies between 22 and 24 °C (Dongmo et al. 2018).

Four *Bicyclus* species were selected for thermal tolerance assessment based on their presence in both forest and ecotone habitats and their microhabitat preference (especially shaded vs. open habitats) as described by previous studies (Larsen 2005; Vande Weghe 2010). *Bicyclus dorothea* is known to prefer open forest areas such as roads or forest fringes, but is mostly absent from primary forest (Vande Weghe 2010; Dongmo et al. 2017). *Bicyclus vulgaris* is found in similar habitats but is also common in pre-forest, dense savannah and agricultural lands (Larsen 2005). *Bicyclus sandace* has been recorded from a range of habitats and its habitat preferences appears to vary across regions. For example, Vande Weghe (2010) found that *B. sandace* is abundant in Gabonese forest, but absent in open savannas, while Larsen (2005) noted that it was common in forest habitat, agriculture lands and also in dense savannah. From our observation in Cameroon, *B. sandace* occupies openings in the forest and forest galleries but we also found some individuals in the savannah and the species appears to be typically more tied to the vicinity of forests than *B. vulgaris*. Finally, *B. sanaos* is mostly found in rainforests and in ecotones where it prefers understories and is generally not found in open savannah (Larsen 2005; Vande Weghe 2010).

Given these species descriptions in the literature (and based on our experience) we then predicted that *B. sanaos* would be the most forest-restricted butterfly of the four, present in lower abundances in ecotone and most restricted to understory microhabitats for both forest and ecotone habitats. We also predicted that both *B. vulgaris* and *B. dorothea* would be most abundant in both ecotone habitats and in open area microhabitats, while *B. sandace* would

be intermediate in preference for particular microhabitats. Phylogenetically, *B. dorothea*, *B. sandace*, and *B. vulgaris* all belong to the "*dorothea*-group" and are closely related to one another while *B. sanaos* belongs to the "*martius*-group" (Aduse-Poku et al. 2017).

Laboratory colonies of each species were established from 438 individuals collected from populations in both ecotone and forest habitats. *Bicyclus* butterflies in more open habitats generally enter reproductive diapause in the dry season (Brakefield et al., 1991; Halali et al. 2020) so we collected butterflies in the wet season in two sampling bouts in each habitat: *B. dorothea* and *B. vulgaris* were collected in October 2018 and *B. sandace* and *B. sanaos* in September 2019.

Microhabitat associations

We conducted a 20-day survey of two microhabitat types: closed understories and open areas. These surveys were conducted in both of the investigated habitats/sites (ecotone and forest) during both the wet and dry seasons. The dry season in both localities (Somalomo and Ndikiniméki) usually lasts from mid-November to mid-March and the wet season from mid-March to mid-November. For the dry season, we conducted sampling in January 2019 for Somalomo (forest) and January 2020 for Ndikiniméki (ecotone), while wet season sampling was conducted in August 2019 for Ndikiniméki and September 2019 for Somalomo.

In each habitat, we chose six stations for carrying out fruit-baited trapping with a minimum distance of at least 1 km between stations. The exact location of the trap station depended on the availability of the two microhabitats, "open area" and "closed understory". At each station, we set two traps: one in an open area and the other in the closed understory at least 70 to 100 m apart. Standard conical pop-up butterfly bait traps (Bioquip 1422 cone trap) were used for sampling. Each trap was placed such that the base hung at about 20 cm above ground. For each trap, we added overripe banana mixed with locally sourced palm wine that had been fermented for at least one day. We applied tangle-foot on the hanging ropes of the trap as well as on the branches of the trees where the trap was set to prevent ants from accessing the traps. To avoid recaptures of the same individual butterflies, the position of the traps in each station was changed (at least 100 m from the previous position) every two days within each microhabitat and trapped individuals were marked on their wings using small scratches on the wings to enable us to identify any recaptured individuals.

To characterize habitat associations, the traps were inspected, re-baited (if necessary) daily and butterflies collected between 9 AM to 4 PM. Trapped specimens were identified in the field using available identification literature (Larsen 2005; Vande Weghe 2010). Specimens that were difficult to identify accurately in the field were brought back to the laboratory for further identification. The ambient temperature of each microhabitat was recorded using Thermochron iButton data loggers (model: DS1922, ±0.5 °C accuracy) suspended at 2 m from the ground and protected from direct sunlight. The position of each data logger was changed every two days within the microhabitat. Data loggers were programmed to record the temperature at 30-minute intervals throughout the 20-day survey.

Plasticity in thermal tolerance

For our common garden experiment, all collected butterflies were transported to the International Institute of Tropical Agriculture (IITA) entomological laboratory based in Yaoundé,

Cameroon. Overall, we obtained 438 wild caught individuals distributed as follow: Somalomo: (*B. dorothea* $N=68$; *B. sandace* $N=18$; *B. sanaos* $N=27$), Ndikiniméki: (*B. dorothea* $N=102$; *B. sandace* $N=40$; *B. sanaos* $N=32$; *B. vulgaris* $N=62$). For each species from the two habitats, we placed five females and five males into cages ($24\times24\times24$ cm) made of white polyester screen (to facilitate air circulation), containing mashed banana as adult food and distilled water-soaked cotton (Brakefield et al. 2009). These set-ups were kept in a shade house with mean temperature of ~26 °C, mean relative humidity of ~78% and a photoperiod of L12:D12. Pot-grown millet (*Pennisetum glaucum*) was chosen for oviposition due to its of cultivation under laboratory conditions. Eggs were collected and transferred to moist black filter paper in Petri dishes, and hatchlings were then transferred onto fresh potted-lawn grass, *Axonopus compressus*, a natural host plant of these species readily available at the vicinity of the laboratory. These first-generation larvae were grown in large cages ($30\times30\times30$ cm) until the adult stage. However, despite multiple trials, we were unable to rear and maintain forest populations of *B. sanaos* under these laboratory conditions. For this reason, we were unable to quantify the thermal tolerance proxies (CT_{min} and CT_{max}) for the forest population of *B. sanaos.*

Adults of the first fully lab-reared (F1) generation were kept in the same conditions as the founding individuals. Following eggs laid by the F1 females, first instar larvae (i.e., second generation larvae F2) were placed on *A. compressus* and randomly allocated to climate cabinets (I-36 VL Percival Scientific Inc., Perry, IA, USA) set at 20–30 °C, at 75% RH and a photoperiod of L12:D12. Fifth instar larvae (F2) and one-day-old adults (F2) of ecotone and forest populations of all four species reared at 20 °C and 30 °C were used in the thermal tolerance assays. These temperatures are roughly equivalent to expected minimum (~20 °C) and maximum (~30 °C) temperatures experienced by ecotone and forest *Bicyclus* species in shaded habitats (Dongmo et al. 2021) – which should then approximate the thermal conditions of the understory microhabitat. The large difference in temperature across the two experiments also maximized the ability to detect differences in thermal tolerance across the developmental temperatures and estimate plasticity (see *Data analysis* below). The number of larvae and adults assessed for thermal tolerance (CT_{min} and CT_{max}) for each temperature treatment are illustrated in Table S1.

We estimated the upper and lower thermal limits using a dynamic method (i.e., temperatures were raised or decreased until thermal stress observed) which is an ecologically relevant measure of thermal tolerance consisting of exposing an organism to a gradual heating or cooling at a fixed rate within an environmental chamber (Terblanche et al. 2011). Using this approach, we took two different measurements: (1) the critical thermal minimum (CT_{min}), defined as the temperature at which an experimental individual is no longer able to move any appendages following a gradual cooling of the environment at a given rate; (2) the critical thermal maximum (CT_{max}), defined as the temperature point before an experimental individual begins to make uncoordinated movements, due to excess heat following a gradual increase of the temperature at a given rate (Lutterschmidt and Hutchison 1997).

Typically, larvae in *Bicyclus* butterflies develop into five instars (Condamine, 1973). Before assessment of thermal tolerance of the fifth instar larvae, we weighed each individual using a high precision Jewellery GEM50 scale at the nearest 0.001 mg. The larval weight was assessed to determine if it can have a potential implication in thermal response as larvae were reared under two different temperature regimes. We then placed larvae individually in 47 mm Petri dishes with parts of their covers cut out and replaced by a fabric wire

mesh to allow air circulation. The Petri dishes were placed in a climate cabinet set to the same temperature (20–30 °C) that was used during the rearing of each group. Adults were not weighed and kept individually in small plastic 250-mL bottles with about 25 holes of ~5 mm diameter. The temperature inside the climate cabinet was then lowered (for CT_{min}) or raised (for CT_{max}) at a constant rate of 0.25 °C/min, following Terblanche et al. (2011). CT_{max} for larvae was determined when the larva started twitching its body or regurgitating a greenish liquid. In contrast, the CT_{min} for larvae was the lowest temperature at which each individual was not able to make any movement, and unable to hold onto the wall of the Petri dishes during the experiment.

In adults, the CT_{min} was determined by noting the temperature at which an individual was no longer able to move its appendages (antennae, legs, wings) while CT_{max} was the temperature at which individuals started making uncoordinated movements. We used each experimental individual only in a single trial (CT_{max} or CT_{min}) to avoid effects from previous exposure influencing the results. During assessments, we observed the behaviour of (individual) larvae and adults through a glass window in the main door of the climate cabinet to determine its CT_{min} or CT_{max}. Through the same window, we monitored the real-time temperature variation within the cabinet using a water-proof thermometer probe (DE:30 W, DER EE, New Taipei City, Taiwan). Overall, we reared 1498 forest individuals (all species, see details in Table S1) at two temperature treatments (i.e., 786 at 20 °C and 712 at 30 °C), and 3015 ecotone individuals (1616 at 20 °C and 1399 at 30 °C). We then performed CT_{min} and CT_{max} experiments on a total of 2866 s generation fifth instar larvae, and 1647 s generation (F2) adult butterflies (all species, habitats and rearing temperatures combined (see Table S1).

Data analysis

We used R version 4.4.1 (R Core Team, 2024) for all statistical analyses. We investigated whether thermal tolerance traits i.e., CT_{min} and CT_{max} for larvae and adults were affected by rearing temperature and habitat in each species. In order to do so, we used linear mixed models (*lmer* function in R) with rearing temperature, habitat (forest or ecotone), and their interactions as fixed effects, larval weight as a covariate and species as a random intercept. Pairwise comparisons with Tukey's posthoc test was conducted using the package *emmeans* (Lenth et al., 2022) for mean separation between groups. The degrees of significance in thermal tolerance traits was estimated by calculating the effect size (Cohen's d) using the mean and standard deviation of the data at 20 °C and 30 °C for each species using the package *Durga* (Khan and McLean 2024).

Furthermore, following Stillman (2003), we investigated the developmental plasticity of upper and lower thermal limits of larvae and adults of each species in each habitat by calculating the absolute difference between the mean CT_{max} of the two rearing temperatures (i.e., $\Delta CT_{max} = CT_{max}$ 30 °C - CT_{max} 20 °C) of all individuals; the same formula was applied for CT_{min} (i.e., $\Delta CT_{min} = CT_{min}$ 30 °C - CT_{min} 20 °C). A bootstrap was applied to the estimations of ΔCT_{max} and ΔCT_{min} with 10,000 replications using the function "boot" of the package boot ver. 1.3–31 (Canty and Ripley 2024). Positive (or negative) values indicate the ability of species to increase (or decrease) its upper (or lower) thermal limits following rearing at low or high temperatures (Stillman 2003).

To test for the variation in abundance of the four *Bicyclus* species in each microhabitat (open or understory), we fitted separate generalized linear models (with a negative binomial error or zero inflated negative binomial distribution to avoid overdispersion) for each species using the *glm.nb* or *zeroinfl* functions built in the package "*pscl*" v1.5.9 (Zeileis et al. 2008). In each model, we used habitat (forest or ecotone), microhabitat (open or understory), and seasons (wet or dry) as predictors along with their interactions. To check for the overdispersion of the models, we visually inspected the normality of residuals versus fitted values using the Q-Q plots. We also calculated the indicator value index (IndVal) of each butterfly species to verify their affinity/preference to microhabitat types. The indicator value index quantifies the fidelity and the specificity of a given species in relation to a particular habitat or sites. Higher IndVal number correspond to strong fidelity/association to a particular habitat or site (Dufrêne and Legendre, 1997). The IndVal calculations were performed using the "*multipatt*" function of the package "*indicspecies*" v. 1.7.12 (Caceres and Legendre, 2009).

Results

Microhabitat associations in bicyclus

Microhabitat temperature data revealed consistently higher mid-day temperatures (by about 4–5 °C) in the open ecotone sites vs. understory sites. For forest sites, this was the case in the wet season (higher by about 4–5 °C) but much less so in the dry season (only higher by about 1–2 °C) (Fig. S2). Results from the generalised linear model revealed that habitat, microhabitat, and season were significant predictors of the abundance of *B. dorothea*, *B. sandace*, *B. vulgaris*, and *B. sanaos* during the survey (Fig. 1; Table S2). Interactions between microhabitat and season were also significant across species (Table S2). *Bicyclus dorothea*, *B. sandace* and *B. vulgaris* were more abundant in ecotone where they were associated with open microhabitat and exhibited the highest numbers during the wet season. The indicator values obtained were consistent with results from the generalized linear model for *B. dorothea* (IndVal: 0.445; $p=0.002$) and *B. vulgaris* (IndVal: 0.360; $p=0.020$) as to their preference for open microhabitats in both forest and ecotone. In contrast, *B. sanaos* was more abundant in the understory microhabitat (IndVal: 0.499; $p<0.001$), and most were recorded in the ecotone during the wet season (Fig. 1; Table S2). Temperature patterns were also consistent with expectations as ecotone open sites had highly elevated temperatures relative to the habitat and microhabitat combinations (Fig. S2).

Plasticity in thermal tolerance

For adults, rearing temperature was a significant predictor for CT_{min} in all species and exhibited a greater difference for ecotone populations of *B. dorothea* (Cohen's d=2.088, 95% CI [1.730, 2.408], *B. sandace* (Cohen's d=2.681, 95% CI [2.083, 3.105] and the forest population of *B. vulgaris* (Cohen's d=1.197, 95% CI [0.905, 1.498]) (Table 1; Fig. 2). The effect size estimation revealed that adults reared at higher temperature had the higher CT_{max} in both habitats for *B. dorothea* (forest: Cohen's d=1.228, 95% CI [0.714, 2.098]; ecotone: Cohen's d=1.380, 95% CI [0.897, 1.982]) and only in ecotone habitat for *B. vul-*

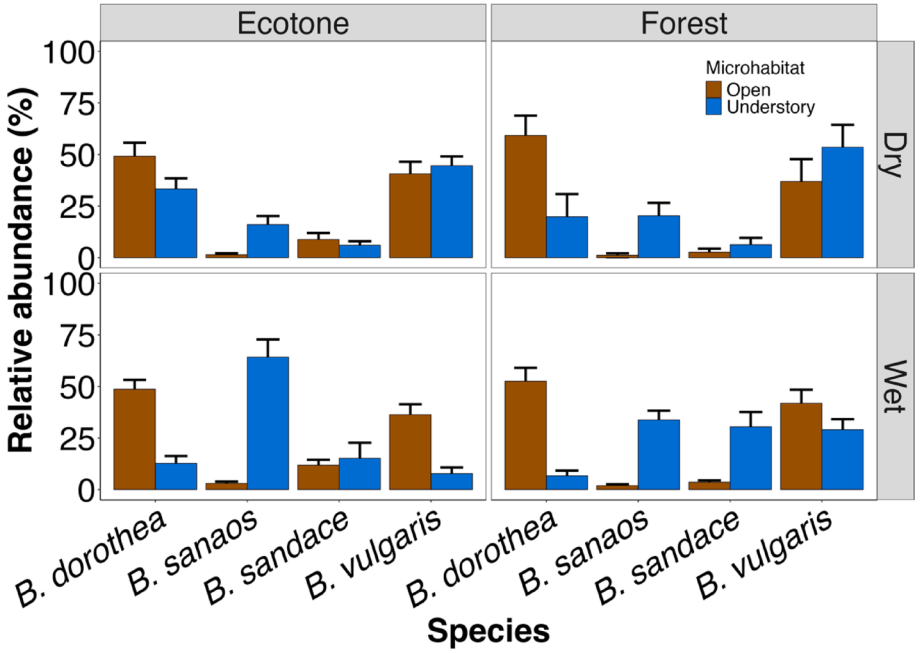

Fig. 1 Relative abundance of each species across microhabitats in the forest and ecotone sites during 20-day surveys in the wet and dry seasons

garis (Cohen's d = 1.694, 95% CI [1.281, 2.071]) and *B. sandace* (Cohen's d = 0.912, 95% CI [0.441, 1.832]).

For larvae, there was a strong effect of rearing temperature characterized by an increase CT_{min} values with increasing temperature in most species and habitat combinations (Fig. 3), except in *B. sandace* (Cohen's d = 0.377, 95% CI [0.100, 0.687]). For example, for *B. dorothea*, effect size revealed significant differences in CT_{min} between the two rearing temperatures in ecotone (Cohen's d = 1.407, 95% CI [1.173, 1.648]) and in forest (Cohen's d = 0.711, 95% CI [0.336, 1.105]) populations. For *B. vulgaris*, rearing temperature only affected CT_{min} in forest (Cohen's d = 1.320, 95% CI [1.043, 1.574]) but not in the ecotone (Cohen's d = 0.251, 95% CI [−0.034, 0.520]) populations. Larval weight did not have a significant effect for any species (estimate: 0.002 ± 0.001; $p = 0.065$). Similar to CT_{min}, rearing temperature was a strong predictor of CT_{max} variation for larvae in all species, except forest population of *B. sandace* (Cohen's d = −0.325, 95% CI [−0.653, 0.335) (Table 1; Fig. 3). The CT_{max} of individuals reared at 30 °C was 1 to 2 °C higher compared with those reared at 20 °C for almost all species.

For the three species for which we have data, ΔCT was always higher for ecotone populations relative to forest for both larvae and adults (Fig. 4). We found a similar pattern for CT_{min} except that *B. vulgaris* had higher plasticity in forest relative to ecotone (Fig. 4).

Table 1 Summary of statistical results of the linear mixed model for the relationship between thermal tolerance traits (CT_{min} and CT_{max}) and environmental predictors for second-generation larvae and adult individuals in four *Bicyclus* species

Stage	Traits	Model parameters	Estimates	Lower CI	Upper CI	t-value	*P*-value
Larva	CT_{min}	**Fixed effects**					
		Intercept	5.132	4.662	5.597	21.117	<0.001
		Habitat	0.435	0.238	0.629	4.359	<0.001
		Rearing temperature	0.913	0.679	1.146	7.641	<0.001
		Larval weight	0.002	−0.000	0.003	1.853	0.065
		Habitat*Rearing temperature	−0.180	−0.458	0.098	−1.263	0.207
		Random effects					
			logLik	**AIC**	**LRT**	**df**	***P*-value**
		1 | Species	−2202.1	4416.3	61.001	1	<0.001
	CT_{max}	**Fixed effects**					
		Intercept	45.271	44.792	45.720	192.893	<0.001
		Habitat	0.320	0.172	0.461	4.352	<0.001
		Rearing temperature	0.818	0.629	1.002	8.605	<0.001
		Larval weight	−0.001	−0.002	0.000	−1.009	0.313
		Habitat*Rearing temperature	0.523	0.303	0.742	4.667	<0.001
		Random effects					
			logLik	**AIC**	**LRT**	**df**	***P*-value**
		1 | Species	−2143.2	4299.2	30.178	1	<0.001
Adult	CT_{min}	**Fixed effects**					
		Intercept	4.739	4.223	5.257	19.946	0.004
		Habitat	0.977	0.758	1.191	8.858	<0.001
		Rearing temperature	1.789	1.609	1.970	19.403	<0.001
		Habitat*Rearing temperature	−1.033	−1.327	−0.739	−6.887	<0.001
		Random effects					
			logLik	**AIC**	**LRT**	**df**	***P*-value**
		1 | Species	−1302.6	2615.3	54.535	1	<0.001
		Fixed effects					
	CT_{max}	Intercept	42.177	41.170	43.180	13.195	<0.001
		Habitat	2.124	0.940	3.315	784.109	<0.001
		Rearing temperature	0.129	0.100	0.160	784.363	<0.001
		Habitat*Rearing temperature	−0.087	−0.132	−0.040	784.173	<0.001
		Random effects					
			logLik	**AIC**	**LRT**	**df**	***P*-value**
		1 | Species	−1546.6	3103.2	61.089	1	<0.001

Discussion

Plasticity in thermal limits will be a key mechanism through which species can effectively manage some of the impacts of rapid climate change. We found strong habitat-specific (forest vs. ecotone) plasticity across populations in addition to clear microhabitat affinities (open vs. understory) for all species. For both larvae and adults, ΔCT_{min} and ΔCT_{max} were higher in the ecotone for all three species (except for ΔCT_{min} in *B. vulgaris*). We also found that microhabitat associations match thermal tolerance patterns – the most open-habitat associated species (*B. dorothea* and *B. vulgaris*) had the highest thermal tolerance while *B. sanaos* was the most-understory associated species and had the narrowest thermal tolerance.

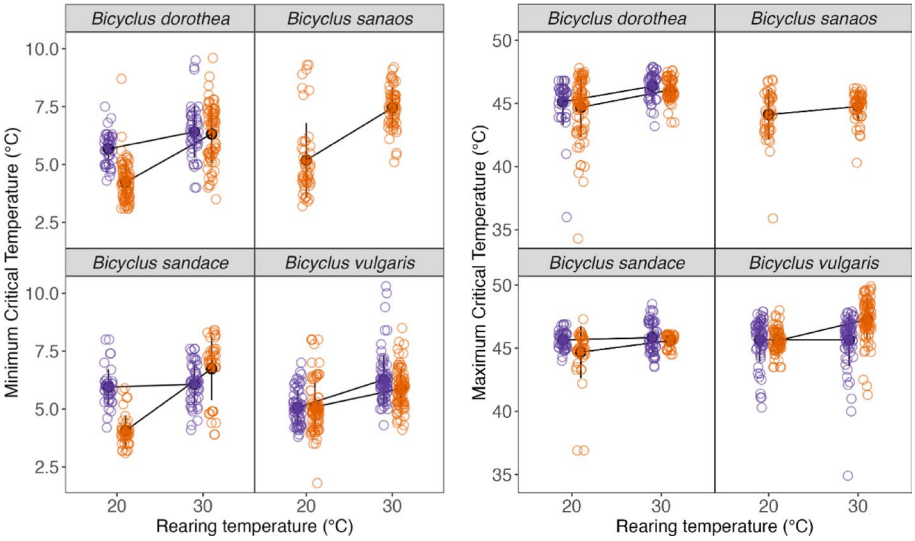

Fig. 2 Plasticity in critical thermal minimum (CT_{min}) and maximum (CT_{max}) of second-generation (F2) adults of four *Bicyclus* species (*B. dorothea*, *B. vulgaris*, *B. sandace* and *B. sanaos*) initially reared at two constant temperatures (20 and 30 °C) and originating from two habitats (forest [in purple] vs. ecotone [in orange]) in Cameroon. No data is available for the forest population of *Bicyclus sanaos* because all our trials failed while carrying out the common garden experiment. Error bars represent the standard error of the mean

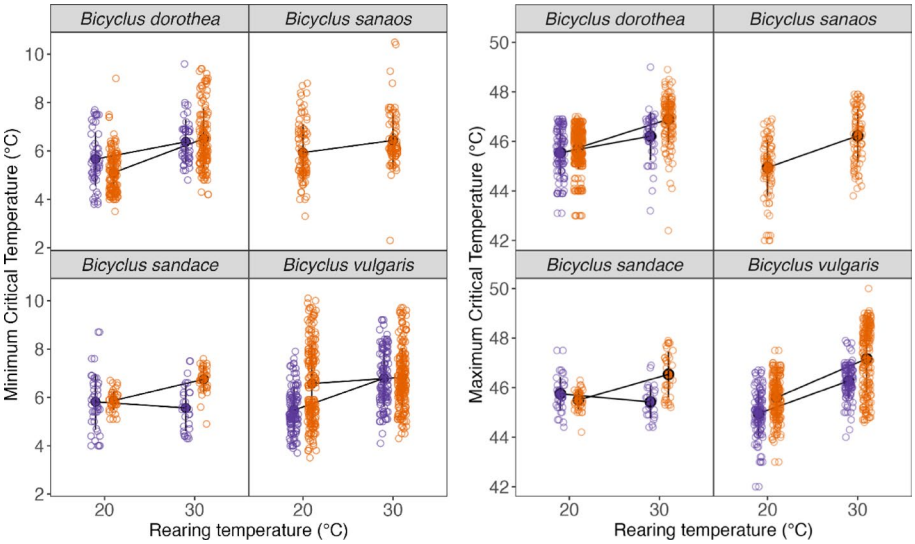

Fig. 3 Plasticity in critical thermal minimum (CT_{min}) and maximum (CT_{max}) of second-generation larvae of four *Bicyclus* species (*B. dorothea*, *B. vulgaris*, *B. sandace* and *B. sanaos*) initially reared at two constant temperatures (20 and 30 °C) and originating from two contrasted habitat (forest [in purple] vs. ecotone [in orange]) in Cameroon. *Bicyclus sanaos* is not included for forest habitat because all our trials failed during common garden experimentation. Error bars represent the standard error of the mea

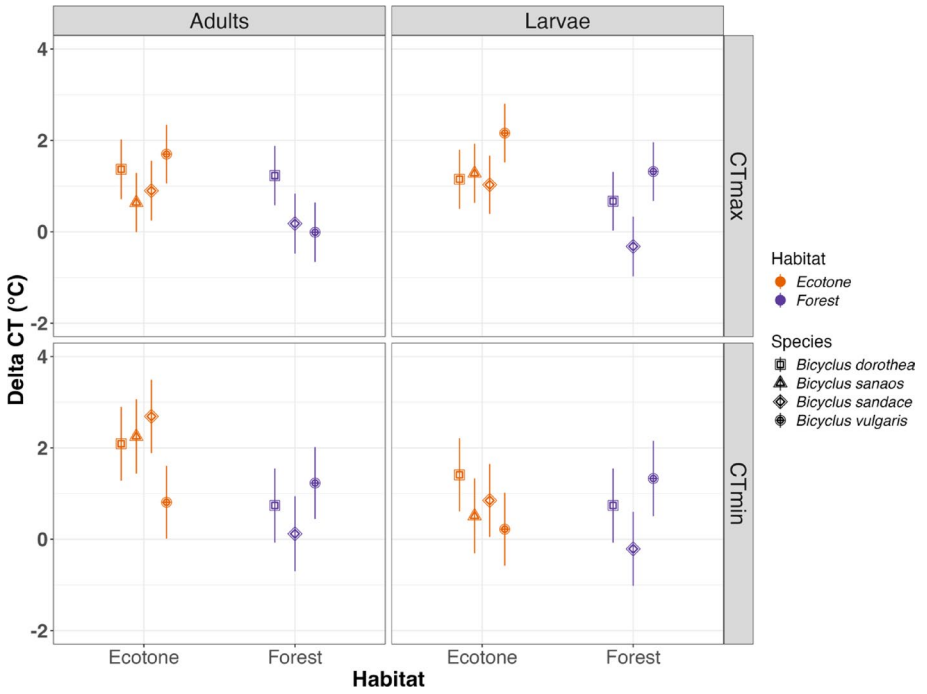

Fig. 4 Plasticity in upper and lower thermal limits in larvae and adults of four *Bicyclus* species originally from two habitats (ecotone vs. forest). Plasticity here is defined as the mean difference in thermal limits between individuals developed at 20 °C and 30 °C; values were bootstrapped 10,000 times

However, our results highlight that such predictions can be complicated by habitat-specific patterns in plasticity and microhabitat associations – the combination of thermal tolerance levels, plastic responses, and microhabitat usage will likely drive heterogeneous responses to warming for tropical species.

We found strong microhabitat associations across the four species, setting up the opportunity to examine the interactions between microhabitat associations and broader habitat/temperature developmental origins. Both *B. vulgaris* and *B. dorothea* were commonly associated with both understory and open habitats, across seasons and habitats. Fermon et al. (2000) found that both of these species were associated with open habitats as well. Conversely, *B. sanaos* was only reliably found in understory microhabitats, regardless of season or habitat – consistent with its reputation as a rainforest associated species (Oostra et al. 2014). *B. sandace* could be found in both microhabitats but was most abundant in the forest understory during the wet season. The microhabitat abundance survey therefore also revealed the importance of seasonal variation, which was not explicitly addressed in our plasticity experiments. For logistical purposes, we derived our lab colonies from wet season individuals while results for dry season individuals could exhibit differential patterns – especially when microhabitat associations vary across seasons, as they do here for Cameroon *Bicyclus*.

For *Bicyclus* in this study, both ΔCT_{max} and ΔCT_{min} increased in the warmer ecotone habitat while changes were not constant in the forest. Consistent with our results, Shah et

al. (2017) found that thermal acclimation ability was higher in more seasonal temperate environments in mayflies compared to less seasonal tropical environments. The relationship between absolute CT_{max} and ΔCT_{max} is predicted to be negative (Stillman 2003; Pörtner et al. 2006) reflecting a trade-off between maintaining high tolerance vs. acclimation ability. However, for the *Bicyclus* species studied here, thermal tolerance variation across species was low relative to variation across habitats and rearing temperature. We also found lower thermal tolerance in forest-associated species which has been observed previously for a range of tropical ectotherms (Simon et al. 2015; Nowakowski et al. 2018), and in comparisons of ecotone vs. forest populations in particular (Landry Yuan et al., 2018, Dongmo et al. 2021). Our results suggest that different species and populations across habitats are likely to employ variable strategies for withstanding thermal extremes using some combination of thermal tolerance dependent on rearing temperature (ΔCT) and thermal tolerance which is not (e.g., habitat determined).

Ontogeny and environmental context will further dictate the sensitivity of tropical insects to climate change. In addition to temperature, plasticity is a consequence of complex multifactorial environmental factors such as light, food, humidity and other habitat-linked variables (Rodrigues and Beldade 2020; Ling and Bonebrake 2022). Moreover, both thermal tolerance and its plasticity can vary by developmental stage in insects (Kingsolver and Buckley 2020). We found that plasticity (ΔCT) tends to be lower for ecotone larvae in *Bicyclus* but was otherwise consistent across life stages and habitats. Carter and Sheldon (2020) found that metabolism was plastic in adults but not in pupae of dung beetles. A meta-analysis by Weaving et al. (2022) found that insect juveniles generally have greater plasticity in thermal tolerance relative to adults. In another study, Lockwood et al. (2018) found a high thermal tolerance in tropical strain embryo of *Drosophila melanogaster* compared to the temperate strain, but no difference in adults from the same distinct strains. Our findings depart from this pattern as in our case, the ΔCT was similar between larval and adult individuals for the forest populations and slightly higher (by about 1 °C) in adults for the ecotone. Such findings are important as they highlight other potential unknown factors governing thermal plasticity in ectotherms. For example, Overgaard et al. (2011) show that widely distributed *Drosophila* species versus tropical restricted species in Australia were not driven by plasticity in thermal tolerance, but likely explained by innate thermal tolerance limits. Similarly, Kellermann et al. (2018) observed that desiccation tolerance in 32 *Drosophila* species was tightly linked with phylogenetic signal rather than environmental factors.

Evolutionary and genetic mechanisms underlie variation in thermal tolerance observed in this study (García-Robledo and Baer 2021). In *Bicyclus anynana*, Franke et al. (2019) found that adult temperatures caused variation in the expression of antioxidant markers and upregulated certain metabolic pathways which may affect thermal tolerance. Developmental temperatures can also affect the regulation of gene expression pathways for insects (Alston et al. 2020). Such mechanisms may likely underlie some of the patterns and variation across developmental temperatures we observe here. However, phenotypic plasticity in *Bicyclus* could potentially hinder evolutionary responses to rapid global warming if formerly reliable environmental cues become maladaptive (Oostra et al. 2018). There can also be reproductive consequences to high tolerance and high plasticity (Bogan et al. 2024). Continued investigation of the genomic and metabolic pathways that determine tolerance will allow for improved assessments of species vulnerability to climate change.

Microhabitats can play a role in shaping species thermal sensitivity and vulnerability (Sunday et al. 2014). This is particularly true for butterflies that can exploit a wide range of thermal variability within landscapes (Bonebrake et al. 2014). For *Bicyclus*, understories and open microhabitats can show marked variation in thermal fluctuations and could allow or restrict species occupancy based on their thermal tolerance. *B. sanaos* are rarely found in open microhabitats while *B. dorothea* and *B. vulgaris* regularly occur in both open and closed habitats. Corroborating with their microhabitat preference, we found that *B. sanaos* had the narrowest thermal tolerance. Interestingly, *B. sandace* exhibited low plasticity for thermal tolerance in forest where it was largely restricted to understory microhabitats but higher plasticity in ecotone where it was found in both open and understory. This suggests that microhabitat thermal conditions can drive a species' thermal tolerance and plasticity. Finally, a comparative study showed that ancestral *Bicyclus* were likely forest-linked and colonized savannah habitats around 8 − 3 million years ago and species in both these habitats show marked divergence in life-history traits including diapausing strategy, body size, growth rates and fecundity (Halali et al. 2021a, b). Such differences in life history are likely to factor into structuring thermal tolerance and shaping differences between forest and open habitat species.

Our results demonstrate that plasticity differences across habitats, and associated microhabitats, are likely to be important in driving responses to thermal variation and climate change. Complex interactions between habitat and climate at multiple scales, and across life stages, will ultimately shape ecological and evolutionary patterns relevant to projecting climate change impacts on biodiversity. For tropical insects, habitats and microhabitats will drive variation in microclimates that will determine exposure to thermal variation (Kemppinen et al. 2024) – more closed microhabitat or forest species may have lower plasticity in thermal tolerance. Because different species and populations have variable microhabitat preferences and thermal tolerance can depend on developmental temperature, heterogeneous responses to warming could be a consequence of these important interactions.

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Data availability All data and scripts are available on figshare: https://figshare.com/s/bbcac4091f4cd368374e.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Research was carried out in accordance with permission by the Ministry of Scientific Research and Innovation of Cameroon (MINRESI).

Consent to participate Not applicable.

Consent to publish Not applicable.

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