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Additive and non-additive effects of day and night temperatures on thermally plastic traits in a model for adaptive seasonal plasticity

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Developmental plasticity can match organismal phenotypes to ecological conditions, helping populations to deal with the environmental heterogeneity of alternating seasons. In contrast to natural situations, experimental studies of plasticity often use environmental conditions that are held constant during development. To explore potential interactions between day and night temperatures, we tested effects of circadian temperature fluctuations on thermally plastic traits in a seasonally plastic butterfly, *Bicyclus anynana*. Comparing phenotypes for four treatments corresponding to a full-factorial analysis of cooler and warmer temperatures, we found evidence of significant interaction effects between day and night temperatures. We then focused on comparing phenotypes between individuals reared under two types of temperature fluctuations (warmer days with cooler nights, and cooler days with warmer nights) and individuals reared under a constant temperature of the same daily mean. We found evidence of additive-like effects (for body size), and different types of dominance-like effects, with one particular period of the light cycle (for development time) or one particular extreme temperature (for eyespot size) having a larger impact on phenotype. Differences between thermally plastic traits, which together underlie alternative seasonal strategies for survival and reproduction, revealed their independent responses to temperature. This study underscores the value of studying how organisms integrate complex environmental information toward a complete understanding of natural phenotypic variation and of the impact of environmental change thereon.

KEY WORDS: **Environment-by-environment interactions, circadian temperature fluctuations, adaptive developmental plasticity, *Bicyclus anynana*, seasonal polyphenism, environmental "dominance".**

Phenotypic diversity results from complex interactions between organisms and their environments, which happen at different time scales. External environmental conditions contribute to selecting phenotypic variants across generations, but also to generating variation through effects on organismal development and phenotype expression. The phenomenon by which environmental conditions affect developmental rates and/or trajectories, leading to the production of distinct phenotypes from the same genotype, is called developmental plasticity (reviewed in West-Eberhard

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2003; Beldade et al. 2011). This plasticity is both a property that can evolve and one that is thought to impact adaptive evolution (reviewed in Nettle and Bateson 2015; Lafuente and Beldade 2019), including how organisms deal with environmental perturbation (Sgrò et al. 2016; Snell-Rood et al. 2018; Rodrigues and Beldade 2020). Developmental plasticity is adaptive when the phenotypes generated in response to the conditions experienced during development are better adjusted to the environment organisms will experience as adults than an unvarying phenotype would be (Ghalambor et al. 2007). In this manner, plasticity offers a means for organisms to cope with environmental heterogeneity, such as that characteristic of alternating yearly seasons. Seasonal polyphenism refers specifically to distinct phenotypes being produced in response to seasonally variable environmental factors, such as temperature and photoperiod (Brakefield 1996; Nijhout 2003; Simpson et al. 2011; Yang and Pospisilik 2019). Compelling examples in insects include wing development in aphids (Braendle et al. 2006), wing pigmentation in butterflies (van der Burg and Reed 2021), and diapause in a variety of species (Nylin 2013).

Effects of external environmental factors on phenotype have been amply documented for various traits and species, as have genetic-by-environment (GxE) interactions (e.g., Lazzaro et al. 2008; Ingleby et al. 2010; Lafuente et al. 2018). Unlike what happens for the genetic effects (G) underlying phenotypic variation, environmental effects (E) were traditionally not partitioned into different components whose impact on phenotype expression and evolution might be distinct. Partitioning genetic variance into additive and interaction components (Falconer and Mackay 1996) takes into account that there are multiple genes and multiple alleles whose individual effects can depend on genetic context (GxG interactions, including epistasis and dominance). In contrast, much less attention has been given to potential environment-by-environment (ExE) interactions, especially in studies of developmental plasticity in animals. Experimental studies of developmental plasticity in animals often focused on the effects of single environmental factors that are held constant during the time it takes organisms to complete development. This is in stark contrast with the complexity of natural situations, where multiple and highly dynamic environmental variables appear in different combinations (Jackson et al. 2021), which could have trait- and genotype-specific effects (e.g., Verspagen et al. 2020). Considering the environment as an irreducible unit does not reflect the plethora of possible natural scenarios, including novel combinations of cues and novel cue dynamics, which organisms might experience when colonizing new environments or as a consequence of environmental perturbation.

We still know little about how organisms perceive and integrate complex environmental information into expression of coherent phenotypes. Toward a more complete account of phenotypic variation, and in particular about effects of environmental perturbation, recent studies have started to address phenotypic effects of combinations of different environmental variables, including combinations of temperature and other factors (examples in Rodrigues and Beldade 2020). When in combination, environmental factors might act redundantly, or have effects that are additive or synergistic in some manner (Piggott et al. 2015; Westneat et al. 2019). Non-additive effects of distinct environmental variables can be thought of as akin to "environmental epistasis" (Samir et al. 2015), and a number of studies have explored such ExE interactions (e.g., Ciannelli et al. 2004; Stoehr and Wojan 2016), including a growing body of work on so-called multiple stressor effects (Piggott et al. 2015; Jackson et al. 2021). Less attention has been given to environmental factors that change during the time it takes organisms to complete development. However, variables such as temperature, which impact many aspects of biology, especially in ectotherms, are typically highly dynamic, varying more or less predictably and across time scales (e.g., within a day, between days, between months) (reviewed in Colinet et al. 2015). We can ask about whether periods of exposure to distinct temperatures affect phenotype expression in a manner that is additive or one that reflects some type of "environmental dominance", with particular periods or particular temperatures affecting phenotype more strongly. This is what we explore here, specifically in relation to circadian temperature fluctuations (see also Zhao et al. 2014; Vangansbeke et al. 2015; Liefting et al. 2017). Despite the prevalence and importance of circadian fluctuations in ambient temperature, we know too little about combined effects of day and night temperatures on thermally plastic traits in animals, such as those making up the seasonal syndrome of the butterfly *Bicyclus anynana*.

B. anynana has become a valuable experimental model of adaptive developmental plasticity, where we can integrate information about the evolution and ecological significance of plasticity with knowledge about its physiological and genetic underpinnings (Brakefield et al. 2009). In its natural habitat in sub-Saharan Africa, these butterflies typically have two seasonal forms that differ in various traits associated with alternative seasonal strategies for survival and reproduction (see box 1 in Rodrigues and Beldade 2020 for a recent overview). Relative to the wet-season form, the dry-season form is larger and delays reproduction until host plants become available to feed a new generation of larvae (Brakefield and Larsen 1984; Halali et al. 2020). Dry-season individuals also have less conspicuous wing patterns and their overall brown coloration is thought to provide camouflage against the background of dry leaves, thereby helping resting butterflies escape predators' attention (Windig et al. 1994; van Bergen and Beldade 2019). Wet-season butterflies, on the contrary, presumably minimize predator attack by deflecting the attention of predators away from the body, towards their

A

Treatment	12hr light	12hr dark
19	19°C	19°C
23	23°C	23°C
27	27°C	27°C
27-19	27°C	19°C
19-27	19°C	27°C

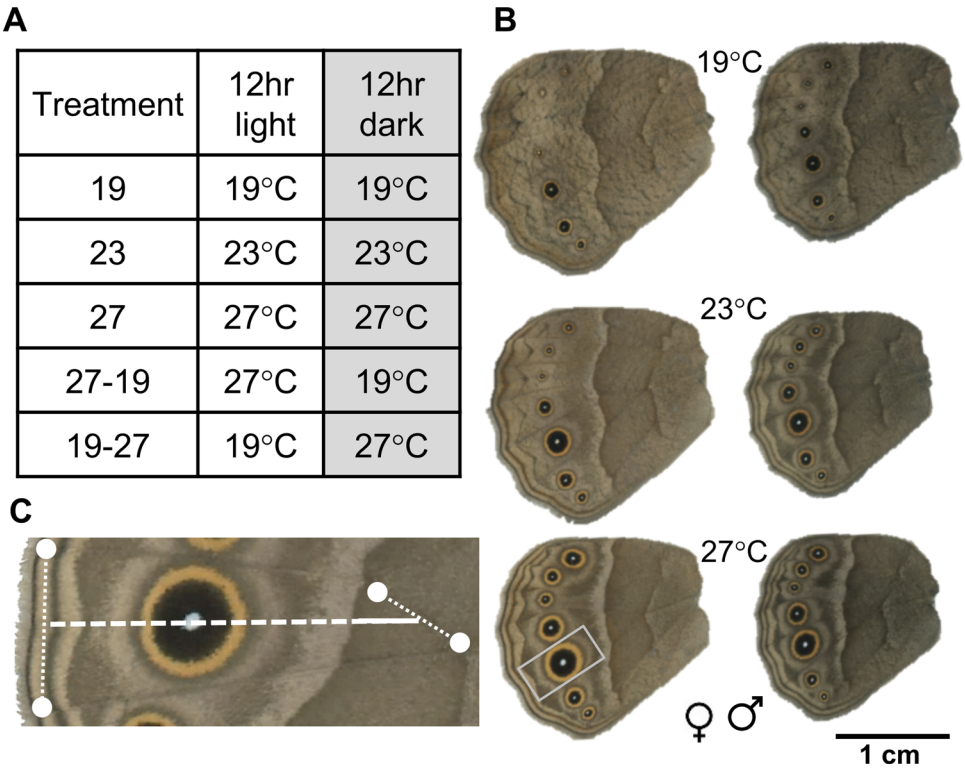

Figure 1. **Treatments and wing pigmentation phenotypes. (A) Thermal regimes with constant and fluctuating temperatures in association to the light-dark circadian cycle. (B) Examples of hindwings (ventral surface) from female and male adults from the different constant temperature treatments. (C) Section of a female hindwing (region corresponding to rectangle in panel (B) where landmarks (white circles) defined two contiguous transects (white dashed line) passing through the center of the fifth eyespot. The proximal portion of the transect (solid line) includes the approximate region used to phenotype the brightness of background.**

wing margins decorated with conspicuous wing pattern elements called eyespots (Lyytinen et al. 2004; Prudic et al. 2015). The temperature experienced during the final stages of development (from last larval instar until pupae) is the main environmental cue determining which seasonal morph is produced (Kooi and Brakefield 1999). Developmental temperature affects the dynamics of ecdysone titers, which, in turn, regulate the response of a suite of plastic traits (e.g., Oostra et al. 2014; Mateus et al. 2014; Monteiro et al. 2015). With few exceptions (Brakefield and Mazzotta 1995; Brakefield and Kesbeke 1997), laboratory studies of *B. anynana* plasticity used temperatures held constant during the light and dark hours of the day.

Here, we compared a series of thermally plastic traits between individuals reared under three constant temperatures or under circadian temperature fluctuations with the same daily average as the intermediate constant temperature (Fig. 1A). To test the effects of the association between temperature and light, we included two regimes with temperature fluctuations: warmer days and cooler nights, as well as the reverse situation. This design allowed us to test the hypothesis of dominance-like interactions between day and night temperatures on plastic trait expression. We found differences between traits in relation to the combined effects of day and night temperature, including additive and dominance-like effects of different kinds. Our data also provide evidence that the effect of temperature fluctuations on different thermally plastic traits cannot solely be a secondary consequence of direct temperature effects on development time.

Methods

BUTTERFLIES AND TEMPERATURE TREATMENTS

We used a captive outbred population of the tropical butterfly *B. anynana* (Brakefield et al. 2009) kept in climate-controlled conditions with 65% humidity and 12-12 h light-dark cycles (Sanyo MLR-351H or Aralab FITOCLIMA 1000 EH incubators). Caterpillars were fed with young organic maize plants and adults with sliced banana on wet cotton. To set our experiment, we collected eggs from a large cohort of adults housed at 27°C and allowed them to hatch at the same temperature. Each day for a period of 4 days, we collected first instar larvae (L1) and randomly assigned them to cages with 22 L1 each that were split into five temperature treatments. Three treatments had constant temperatures: 19°C and 27°C extremes (typical temperatures used to induce development of the dry and wet seasons, respectively), and an

intermediate of 23°C. Two additional treatments had a daily average temperature of 23°C, but cyclical light-dark fluctuations between the two extreme temperatures (Fig. 1A). For each of these five thermal regimes, we had four replicate cohorts in four independent sleeve cages (*ca*. 22 cm length × 12.5 cm width × 100 cm height). The position of the cohorts within each incubator was changed regularly, and food availability was monitored daily. We checked larval cages daily and transferred pre-pupae into individual cups where they were monitored for pupation and adult eclosion. Adults were allowed to fully stretch their wings before being frozen at −20°C. Wings were cut and stored at 4°C until phenotypic analysis.

QUANTIFICATION OF PHENOTYPIC TRAITS

We quantified the response to thermal regimes for various thermally plastic life-history and wing pigmentation traits. We monitored development time by recording the number of days from L1 larvae to pre-pupae, from pre-pupae to pupae, and from pupae to adult, and we calculated total development time by adding those together. We measured two proxies of body size: pupal mass and adult wing area. For pupal mass, one-day-old pupae were weighed to the nearest 0.001 g (KERN ABS 80–4N scale). For adult wings, we used a flatbed photographic scanner (Epson V600) to image the ventral surface of hindwings. The scanner was color-calibrated using an IT 8.7/2 reflective calibration target and the appropriate color profiling software, in accordance with the ISO 12641–2 standard. The resulting images were analyzed with a set of custom-made interactive Mathematica notebooks (Wolfram Research, Inc., Mathematica, Version 10.2, Champaign, IL, 2015) to measure hindwing area and a series of wing pigmentation traits. For the color pattern measurements, we focused on the fifth eyespot, which is often used to document wing pattern plasticity in this and related species (e.g., Windig et al. 1994; van Bergen et al. 2017). We first drew two contiguous transect lines defined by the eyespot center and four-wing landmarks (on the wing margin and intersection between veins) in that wing compartment (Fig. 1C). We marked the limits of each of the color rings (central white focus, middle black ring, and external golden ring) along the transect to determine ring radii and calculate the approximate eyespot diameter and area (considering the eyespot as a circle). The colors of eyespot rings and wing background were quantified using the mean RGB values of the pixels in 3-pixel high rectangles centered on the transect (see also van Bergen and Beldade 2019). For the wing background color, we used the most proximal 50 pixels of the transect, corresponding to a wing region without any defined color pattern element (Fig. 1C). RGB values were converted to HSB (hue, saturation, and brightness) using the *rgb2hsv* function in R. Background color was characterized by the brightness value in the HSB color space; low brightness values corresponding to darker colors.

STATISTICAL ANALYSES

We compared phenotypes between temperature treatments, each of which included four replicate cages with up to 21 eclosed adults per cage (data in Supporting Information S1). Where appropriate, the R syntax used for the different tests is shown (in italics) and explained. All statistical tests were done with R (R Core Team 2016), separately for males and females, as we wanted to focus on testing for additive versus non-additive effects of day and night temperature, rather than re-evaluating previously documented sex differences in trait values and/or sex-specific responses to temperature (e.g., Oostra et al. 2011). However, we provide information about sex-by-treatment interactions as obtained from likelihood ratio tests comparing the goodness-of-fit of competing models (with versus without interaction). Normal distribution and homoscedasticity of the residuals were tested with Shapiro-Wilk normality tests and Brush-Pagan tests, respectively.

We first conducted a set of analyses to test whether the interaction between day and night temperatures was statistically significant for our target traits. In this case, the "23" treatment was disregarded and the comparisons were done between the remaining four treatments (Fig. 1A). This corresponds to a full factorial analysis of 19°C and 27°C as day and night temperatures (dT and nT, respectively), which were considered as explanatory categorical variables. For eyespot area, we tested the model *eyespot area* $\sim$ *wing area* $+ dT * nT +$ *(1|replicate)*, where *wing area* is a covariate and the term *(1|replicate)* corresponds to accounting for *replicate* as a random factor. For each of the other target traits, we tested the model *trait* $\sim dT * nT +$ *(1|replicate)*.

Next, we compared phenotypes between the three treatments with constant temperatures (19, 23, 27) to assess the direction and strength of thermal plasticity in our *B. anynana* population and experimental conditions, and between the three treatments of the same daily average temperature (19-27, 27-19, 23) to explicitly test for potential dominance-like effects. In this case, temperature treatments were considered as categorical explanatory variables.

To test for differences in adult eclosion success, we used a mixed generalized linear model with a binomial distribution of the error. We coded the eclosion variable as 1 (success) and 0 (failure) and considered replicate experiments as a random effect.

To test for differences in development time among individuals that eclosed successfully, we used the framework of a survival analysis. We fitted a parametric survival regression model (function *survreg* in the R package *Survival)* to determine whether treatment (i.e., thermal regime, considered as a fixed factor) influenced the proportion of eclosions over time. This model assumes a lognormal distribution (choice based on maximum like-

lihood comparison with the other commonly used distributions) and contained a Gaussian random effect to account for the four replicate cages (Thomas and Reyes 2014). This analysis was done for total development time from L1 to adult, as well as the duration of the larval and pupal stages. For pre-pupae, the short duration of the stage did not allow the use of a parametric model and we, therefore, used a Cox proportional hazards model (function *coxme* in the R package *Coxme*; Therneau and Grambsch 2000) with the same structure as the parametric survival regression model. For each sex, we tested the model *survival* (*time, eclosion*) $\sim$ *treatment* + *(1|replicate)*, where the term *(1|replicate)* corresponds to accounting for *replicate* as a random factor.

To test for differences in body size (pupal weight and wing area) and wing pigmentation (relative eyespot size and wing background brightness) including the four replicate cages as a random effect, we used a generalized linear mixed model with the function *lmer* from the R package *lme4* (Bates et al. 2015). This method allows for *p*-values to be obtained from likelihood ratio tests comparing the goodness of fit of competing models (with versus without variables). We tested the model *trait* $\sim$ *treatment* + (*1|replicate*), a syntax that corresponds to testing for the effect of treatment (i.e., thermal regime, considered as a fixed factor) and accounting for differences between replicates (random factor). For eyespot size, and to account for thermal plasticity in wing size, we considered "eyespot area" as the response variable and included "wing area" as a covariate: *eyespot area* $\sim$ *treatment* * *wing area* + (*1|replicate*).

To ascertain differences between pairs of thermal regimes, we used a *general linear hypotheses test* (glht) using Tukey post hoc pairwise comparisons (i.e., fitting an adequate model followed by a glht (with alpha = 0.05) from the package *multcomp* in R; Hothorn et al. 2008). This method allows to contrast several factors adjusting the *p*-value for multiple testing and can be applied to generalized linear models and Cox models alike.

Finally, to test for the correlation between developmental time and relative eyespot area, we used the Pearson method, both on our dataset and on an independent dataset combining previously published data on *B. anynana* development time (Oostra et al. 2011) and eyespot size (van Bergen et al. 2017).

Results

We tested the effect of circadian temperature fluctuations on various thermally plastic traits: development time (Fig. 2), body size (Fig. 3), and wing pigmentation (Fig. 4). Except for a lower eclosion success for individuals from 19°C relative to 27°C (glht, $z = 3.04$, $p = 0.02$), there was no difference between all other pairs of thermal regimes in the chance of larvae reaching adulthood (Supporting Information S2). By focusing on the four thermal regimes corresponding to a full-factorial analysis of the two extreme temperatures (19°C and 27°C) in the two light periods (day and night), we established that there was a statistically significant interaction between day and night temperatures for most target traits (Supporting Information S3; see below). We then considered all five thermal regimes (Fig. 1A, Tables 1 and 2) to quantify thermal plasticity and to explicitly test for possible dominance-like effects between alternating temperatures. First, we compared phenotypes between the three treatments with constant temperatures to quantify the direction and strength of thermal plasticity in our *B. anynana* population and experimental conditions. Then, we compared phenotypes between the three treatments of the same daily average temperature to assess the contribution of day and night temperatures to phenotype. Finally, to test the hypothesis that temperature-induced changes in wing pattern are mediated by direct temperature effects on development time, we tested the correlation between development time and eyespot size, using our and another independent dataset (Fig. 5).

DIFFERENT CONTRIBUTIONS OF DAY AND NIGHT TEMPERATURES TO DEVELOPMENT TIME

We confirmed thermal plasticity in *B. anynana* development time in our study population (Table 1): individuals reared at lower temperatures took longer to reach adulthood than individuals reared at higher temperatures (Fig. 2A). For both males and females, temperature affected the duration of all developmental stages monitored; warmer temperatures resulted in shorter larval, prepupal, and pupal stages (Fig. 2B).

We also found differences in development time between the three treatments with a daily average temperature of 23°C (Fig. 2C, Table 1). For both males and females, development was faster for individuals that spent the day at 27°C and the night at 19°C (27-19 treatment), compared to individuals that spent the day at 19°C and the night at 27°C (19-27 treatment). The duration of the pupal stage differed between those two thermal regimes, but the duration of the larval and prepupal stages did not (Fig. 2D). The difference between our two treatments with fluctuating temperatures revealed that the temperature experienced during the light phase had a larger, dominance-like, impact on total development time. Individuals reared with a day temperature of 27°C demonstrated a shift in development time towards that of individuals reared at constant 27°C, while the development time of individuals reared with a day temperature of 19°C shifted toward those reared at a constant temperature of 19°C. The response of individuals reared at a constant intermediate temperature of 23°C (23 treatment) relative to the two fluctuations of the same daily mean (27-19 and 19–27) appeared different for males and females (Fig. 2C-D). While for females, the 23 treatment was different from 19–27 but not from 27-19, the reverse was true for males. However, we did not detect a significant sex-by-treatment

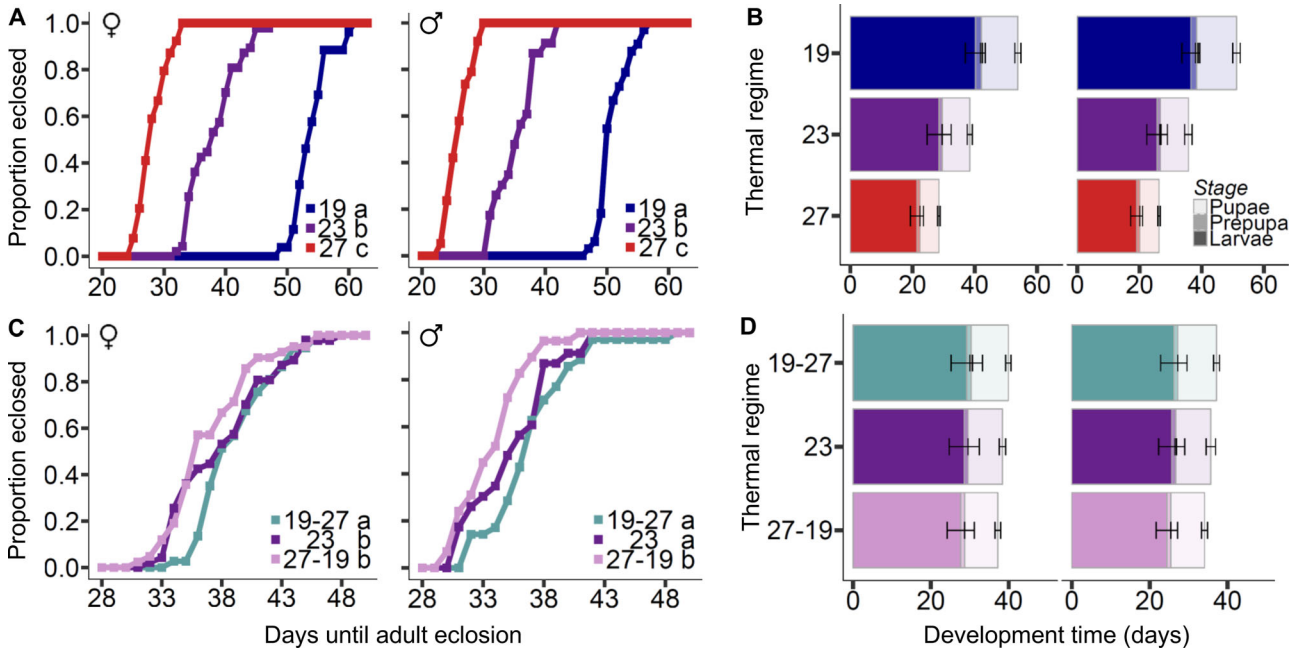

Figure 2. **Effects of constant and fluctuating temperatures on development time. Total development time (L1 to adult) and duration of different developmental stages (larvae, pre-pupae, pupae) for females and males developing under constant (A-B) or fluctuating (C-D) temperatures. Panels (A) and (C) represent the proportion of adult eclosion since the start of the experiment. While each line corresponds to the individuals of all four replicates for each treatment, the "replicate cage" effect was explicitly included in the statistical analysis (see Methods). Information on the statistical tests is available in Table 1. There were significant differences between constant temperature treatments in (A), and between the three types of treatments of same daily mean in (C) ($p < 0.001$ in all cases). Letters next to treatment legend illustrate whether pairs of treatments are significantly different (different letters) or not (same letter), *cf.* glth post hoc test. Panels (B) and (D) correspond to the duration of different developmental stages. Constant temperature treatments in (B) differed in duration of all developmental stages ($p < 0.001$ in all cases). Fluctuating temperature treatments in (D) differed significantly for the duration of specific developmental stages ($p < 0.001$ for pupae in both sexes and $p < 0.05$ for male larvae), but did not differ for other stages (Table 1).**

interaction when the sexes were analyzed together ($df = 2$, $\chi^2 = 2.6$, $p = 0.27$ for constant temperature treatments; $df = 2$, $\chi^2 = 1.4$, $p = 0.49$ for treatments with same daily mean temperature).

NO DIFFERENCE BETWEEN FLUCTUATIONS AND CONSTANT DAILY TEMPERATURE FOR BODY SIZE

For both proxies of body size quantified, pupal mass (Fig. 3A) and adult wing area (Fig. 3B), we confirmed known patterns of thermal plasticity (Table 2), with lower temperatures yielding larger individuals. Individuals reared at 19°C were significantly larger than individuals reared at 27°C, and those from 23°C were not different from 27°C in females and not different from either extreme in males. However, lack of a significant sex-by-treatment interaction suggests similar effects for males and females (pupal mass: $df = 2$, $\chi^2 = 0.9$, $p = 0.64$; wing area: $df = 2$, $\chi^2 = 1.63$, $p = 0.44$).

The full-factorial analysis (Supporting Information S3) revealed no statistically significant interaction between day and night temperatures for male pupal mass, and the comparison between the three thermal regimes with the average daily temperature of 23°C detected no significant differences for either proxy of body size (except female pupal mass; Fig. 3C and D). When the sexes were analyzed together, we confirmed a significant sex-by-treatment interaction for pupal mass ($df = 2$, $\chi^2 = 7.5$, $p = 0.02$) but not for wing area ($df = 2$, $\chi^2 = 3.18$, $p = 0.2$). Detection of no clear difference in the contribution of day and night temperatures to body size reflects seemingly largely additive rather than any dominance-like effects of day and night temperatures on this trait.

DIFFERENT CONTRIBUTIONS OF COOL AND WARM TEMPERATURES TO EYESPOT SIZE

We investigated two aspects of wing pigmentation (Fig. 4, Table 2): relative eyespot size, which is a trait well known to be thermally plastic and vary between seasonal morphs, and wing background brightness. We showed significant effects of developmental temperature on wing background brightness, but only for males (Fig. 4A; significant sex-by-treatment interaction with $df = 2$, $\chi^2 = 21.1$, $p = 2.6 \times 10^{-5}$), and confirmed effects on eyespot size for both sexes (Fig. 4B), with seemingly stronger thermal plasticity for females (significant sex-by-treatment interaction when the sexes were analyzed

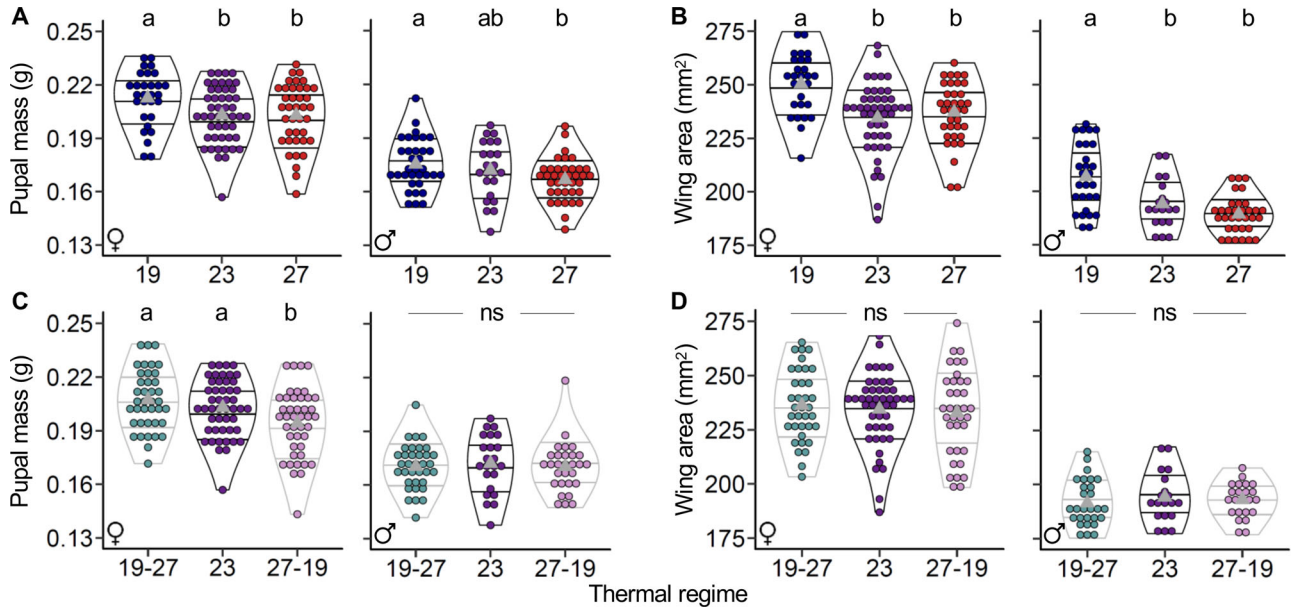

Figure 3. **Effects of constant and fluctuating temperatures on body size. Pupal mass and wing area of adult butterflies for females and males developed under constant (A and B) and fluctuating (C and D) temperatures. Each dot corresponds to one individual (all replicates plotted together but "replicate" effect included in statistical model) and the red triangles are median values. Further information on the statistical tests is available in Table 2. We found significant differences ($p < 0.05$ in all cases) in pupal mass between constant temperature treatments (A) and between treatments of same daily mean temperature (C) for females (but not males). We found significant differences in adult wing area between constant temperature treatments (B) ($p < 0.001$ for both sexes), but not between treatments of same daily mean temperature (D). When there was a significant difference between treatments, letters above each treatment illustrate whether pairs of treatments are significantly different between them (different letters) or not (same letter), *cf*. glth post-hoc pairwise-comparison test. *ns* refers to non-significant differences between treatments.**

together: $df = 2$, $\chi^2 = 23.1$, $p = 9.8 \times 10^{-6}$). This is in line with previously described thermal plasticity for *B. anynana* wing pigmentation (van Bergen and Beldade 2019), with larger and brighter eyespots in animals reared at warmer temperatures (Fig. 4E).

Regarding the comparison between constant and fluctuating temperatures of the same daily average, we found similar results for males and females (confirmed by lack of significant sex-by-treatment interaction when sexes were tested together; brightness: $df = 2$, $\chi^2 = 0.23$, $p = 0.89$; eyespot size: $df = 2$, $\chi^2 = 1.35$, $p = 0.51$): no differences for wing brightness, and clear differences for eyespot size (Fig. 4C-E). Individuals reared at either of the two fluctuating temperature regimes had larger eyespots than those reared at the constant temperature of 23°C, and were not significantly different from each other. The exposure to 27°C for half of each day, regardless of whether lights were on or off, resulted in larger eyespots, suggesting that the higher temperature had a stronger, dominant-like, effect on this trait. Size and color of individual eyespot rings (central white focus, middle black ring, and external golden ring) are illustrated in Figure 4E, and have been shown before to differ between temperatures and between sexes (van Bergen and Beldade 2019).

CORRELATION BETWEEN EYESPOT SIZE AND DEVELOPMENT TIME BETWEEN BUT NOT WITHIN TEMPERATURE TREATMENTS

It had been previously suggested that thermal plasticity in traits such as eyespot size, rather than a direct response to temperature, is a correlated response to temperature-induced changes in development time (Brakefield and Kesbeke 1997; Zijlstra et al. 2004). This hypothesis is not consistent with our results, which show that individuals reared at 19–27 developed more slowly than those from 27-19 (Fig. 2) but had similar eyespot size (Fig. 4). We, thus, went on to investigate the correlation between development time and relative eyespot size, both across and within temperature treatments (Fig. 5).

Across constant temperature treatments with largely non-overlapping development times, we found an overall strong negative correlation between development time and relative eyespot area, for both females and males (Fig. 5A and B). However, within temperature treatments, no correlations between development time and relative eyespot size were statistically significantly different from zero. This result was confirmed using an additional independent dataset put together from previously published work (Oostra et al. 2011; van Bergen et al. 2017) that included two extra intermediate constant temperature treatments (Fig. 5C).

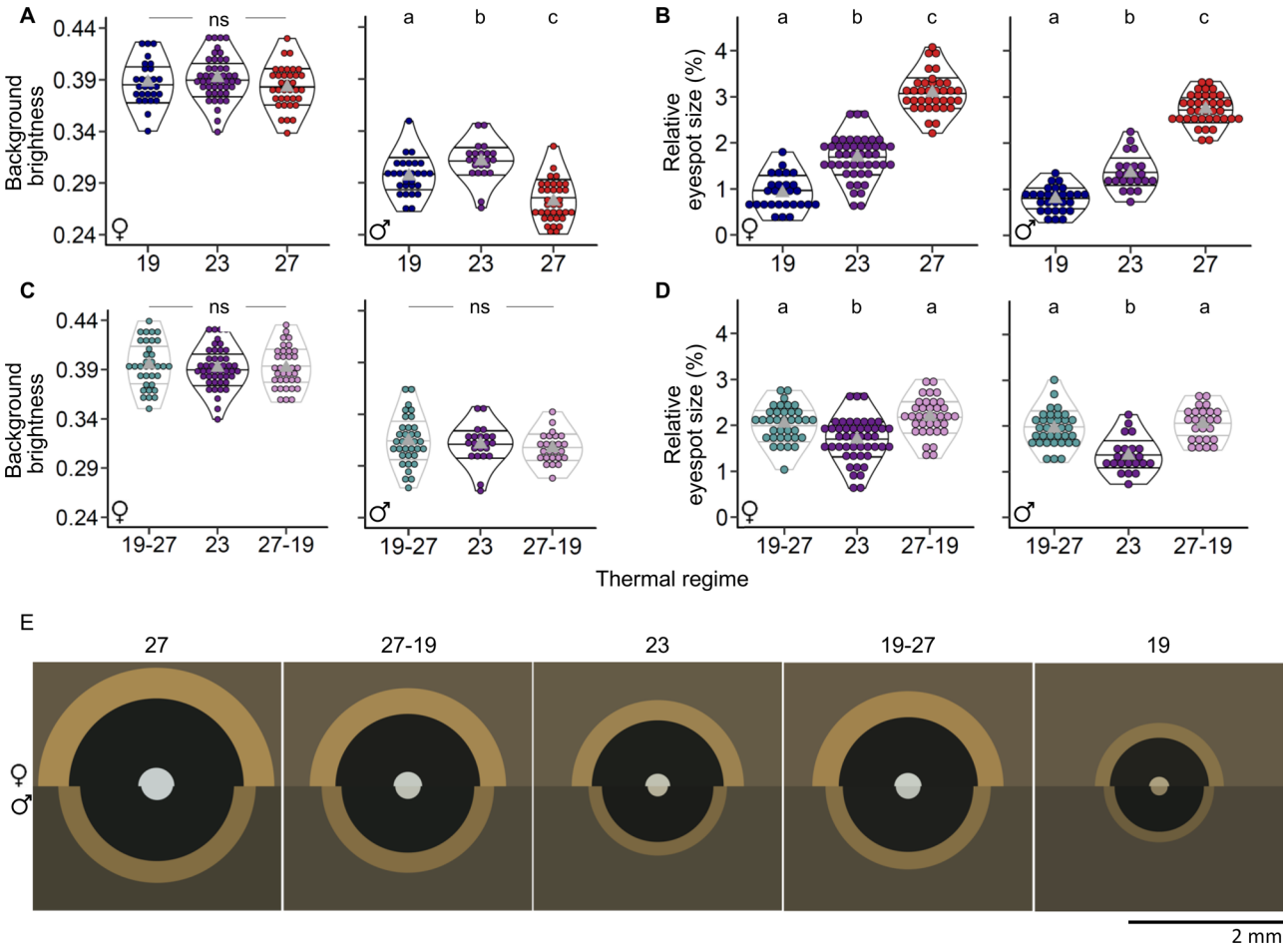

Figure 4. **Effects of constant and fluctuating temperatures on wing pigmentation. The background color and relative eyespot size from females and males developed under constant (A and B) and fluctuating temperatures (C and D). Each dot corresponds to one individual (all replicates plotted together but "replicate" effect included in the statistical model) and the red triangles are median values. Further information on the statistical tests is available in Table 2. We found differences in brightness of wing background color between constant temperature treatments (A) for males ($p < 0.001$) but not females, and no significant differences between treatments of same daily mean temperature (C) for either sex. We found significant differences in eyespot size (using wing area as covariate) for both sexes ($p < 0.001$ in all cases) between constant temperature treatments (B), and also between treatments with the same daily mean (D). When there was a significant difference between treatments, letters above each treatment illustrate whether pairs of treatments are significantly different between them (different letters) or not (same letter), *cf.* glth post hoc pairwise-comparison test. *ns* refers to non-significant differences between treatments. (E) Representation of mean RGB color for the pixels of the wing background, as well as relative area and colors of eyespot rings from different thermal regimes.**

Discussion

We investigated the effects of combinations of day and night temperatures on a series of thermally plastic traits in *B. anynana* butterflies: development time, body size, and wing pigmentation. Butterflies reared under constant warmer temperatures generally had faster development, smaller bodies, and larger eyespots, matching the seasonal polyphenism described for the species, which reflects alternative seasonal strategies for survival and reproduction (Brakefield 1996; Brakefield et al. 2009; box in Rodrigues and Beldade 2020). To test for possible dominance-like effects of day and night temperatures, we focused on comparing phenotypes from individuals reared under two types of circadian temperature fluctuations and under a constant temperature of the same daily average. While butterflies from all treatments with an average intermediate temperature (constant 23, as well as fluctuating 27-19 and 19–27) had trait values that were intermediate between those from the extreme constant temperatures (19 and 27), we found striking differences between traits in the relative contribution of two alternating temperatures experienced during development to final phenotype.

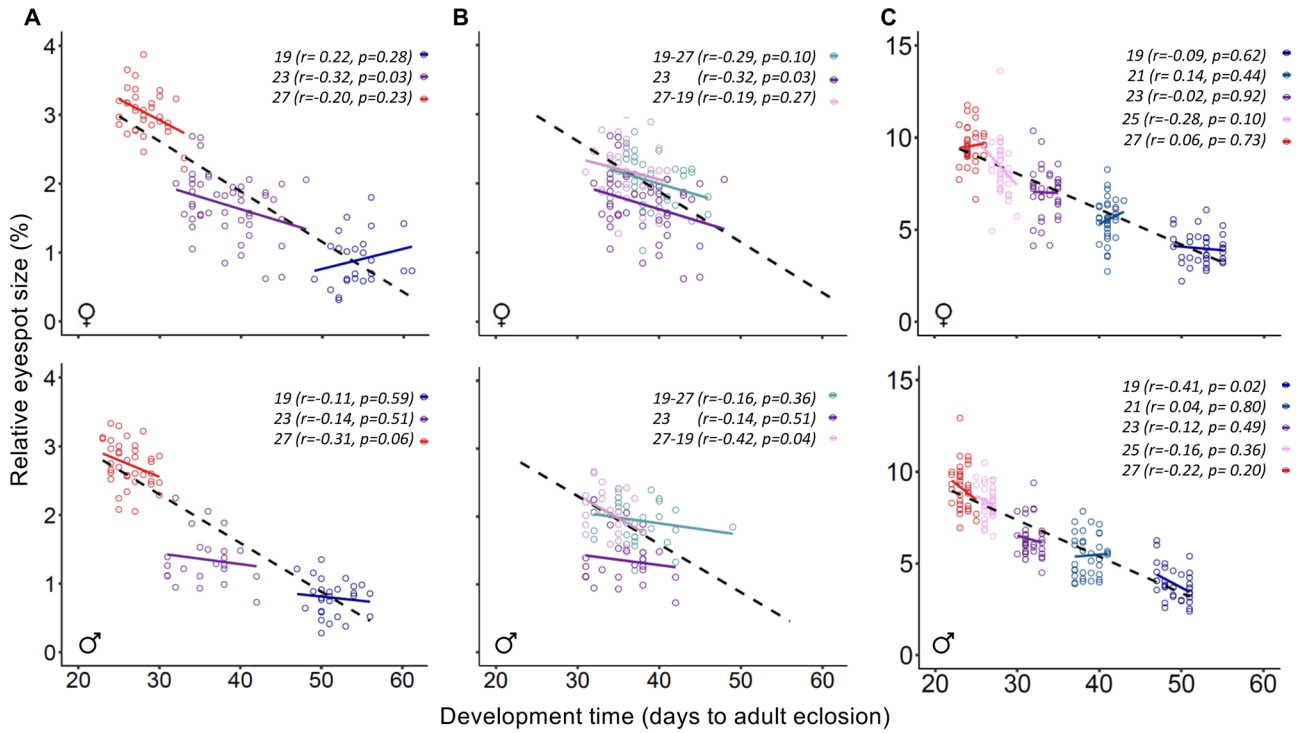

Figure 5. **Correlation between relative eyespot size and development time. Relationship between development time and relative eyespot size for females and males from our regimes with constant temperatures (A) or with daily mean temperature of 23°C (B), as well as data from published work on *B. anynana* using constant temperatures (C). Each dot corresponds to one individual and all replicates are plotted together, separately for females and males. Lines correspond to the best fit line: same color as dots for relationships within each of the different thermal regimes, and black lines for relationship across all data points. Parametric correlation test based on Pearson's correlation coefficient (*r*) showed a significant negative correlation when data points from all treatments were considered together (dashed black lines): $r = -0.79$ for females and $r = -0.82$ for males in A and B; $r = -0.84$ for females and $r = -0.86$ males in C ($p < 0.0001$ in all cases). The correlations within treatments and corresponding *p*-values are given in the figure.**

COMBINED EFFECTS OF DAY AND NIGHT TEMPERATURES ON THERMALLY PLASTIC TRAITS

If day and night temperatures contributed equally to phenotype expression, that is, if their effects were purely "additive", to borrow from the terminology used to partition genetic variance, we expected to have no difference between the two types of fluctuations (our 27-19 and 19–27 regimes), and also no difference between those and the treatment with constant temperature of the same daily average (our 23 regime). We found evidence for such additive effects (for body size; Fig. 3), but also for dominance-like effects where one particular period of the light cycle (for development time; Fig. 2) or one particular extreme temperature (for eyespot size; Fig. 4) had a relatively larger impact on phenotype. We could distinguish between different types of dominance-like effects because, relative to the more ecologically relevant scenario of warmer days with cooler nights, which had been studied before (Brakefield and Mazzotta 1995; Brakefield and Kesbeke 1997), we added a treatment with cooler days and warmer nights. We found that the temperature experienced during the day had a stronger effect on development time than the temperature experienced during the night (Fig. 2C), and that the warmer temperature experienced, during whatever period of the light-dark cycle, had a stronger effect on eyespot size than the cooler temperature (Fig. 4D). Previous studies had shown that for some, but not all, traits, animals reared under day-night temperature fluctuations differed from those reared under constant temperatures (e.g., Brakefield and Mazzotta 1995; Brakefield and Kesbeke 1997; Zhao et al. 2014; Vangansbeke et al. 2015; Liefting et al. 2017; Salachan et al. 2017; Bai et al. 2019). However, without an experimental treatment with cooler days and warmer nights, it is not possible to disentangle temperature from light phase effects, and to identify the distinct types of non-additive effects we document here ("day-dominance" for development time, and "warm-dominance" for eyespot size).

In terms of the effects of fluctuating day and night temperatures on development time, the number of hours spent at a particular temperature seems to have been "weighed" differently depending on light phase. It had been previously suggested that the acceleration of development resulting from warmer days could be related to *B. anynana* caterpillars feeding mostly

Table 1. Results of statistical analysis for variation in development time.

	Total dev time		Larvae		Pre-pupae		Pupae	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Constant temperatures								
females	290.3	2.9×10^{-61}	246.4	6.3×10^{-54}	103.7	3.0×10^{-23}	250.3	5.5×10^{-54}
males	281.2	2.2×10^{-60}	230.8	1.9×10^{-49}	60.4	7.9×10^{-14}	205.2	1.6×10^{-44}
Fluctuations & 23°C								
females	18.5	9.8×10^{-05}	8.5	0.10	5.0	0.08	28.4	1.5×10^{-06}
males	19.0	7.6×10^{-05}	7.9	0.02	0.5	0.77	25.5	4.1×10^{-06}

χ^2 LRT test statistic and corresponding *p-value* (*df* = 2) relative to the data in Figure 2, testing the effect of temperature treatment on duration of development and of different developmental stages with a parametric survival analysis (Lognormal distribution), except for the time as pre-pupae (tested with a cox regression survival analysis).

during dark hours and assimilating resources during light hours (Brakefield and Mazzotta 1995). Cooler nights could presumably sustain higher feeding rates, and warmer days allow higher assimilation efficiency. Either or both of these could result in faster development in regimes with warmer days. Studies in different insects have, indeed, documented associations between temperature and various metabolism-related variables, including food ingestion efficiency (Rall et al. 2010), depletion of energy reserves (Klepsatel et al. 2016, Klepsatel et al. 2019), lipid storage (Jang and Lee 2018), and effects of macro-nutrient diet in development (Kutz et al. 2019). And studies in other lepidopterans have documented seasonal plasticity in metabolism (Kivelä et al. 2019). Aside from association to food acquisition and processing, potential day-night differences in temperature perception could also contribute to day temperature having a higher impact on development time. The issue of how often and exactly when developing organisms "acquire information" about external conditions is largely unresolved (Frankenhuis and Panchanathan 2011). Within specific windows of environmental sensitivity during development (e.g., Snell-Rood et al. 2015; Fawcett and Frankenhuis 2015; Panchanathan and Frankenhuis 2016; Kingsolver and Buckley 2020), it remains unclear whether organisms assess external conditions continuously or at discrete time points. A "dominance" effect of the conditions experienced during the light hours could reflect assessment of temperature mainly occurring during that period of the day. However, the differences that we found between traits would imply that such an "assessment effect" would need to be trait- and/or developmental stage-specific. Understanding where (which tissues), when (which periods of development and periods of the day), and how (which mechanisms) external temperature is sensed is needed to explain interactions between day and night temperatures on thermally plastic traits, in this and other systems. Recent studies, in both animals and plants, have been providing molecular insight into the existence of distinct mechanisms for how cold versus high temperatures are sensed (Guillaume-Schöpfer et al. 2020; Nogueira Freitas and Voets 2020) and affect biological processes (Lloyd et al. 2018).

INDEPENDENT EFFECTS OF TEMPERATURE ON DIFFERENT TRAITS MAKING UP A PLASTICITY SYNDROME

Typically, seasonal morphs differ in a suite of traits that respond to seasonably variable environmental conditions, and reflect seasonally variable strategies for survival and reproduction. In the case of *B. anynana*, the thermal plasticity "syndrome" includes the traits monitored here, as well as various others traits, such as starvation resistance, longevity, and reproductive investment (recent overview in Rodrigues and Beldade 2020). Supported also by laboratory data on correlated responses to artificial selection

Table 2. Results of statistical analysis for variation in body size and wing pigmentation.

	Pupal mass		Wing area		Wing background		Eyespot size*	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Constant temperatures								
Females	8.0	0.02	19.7	5.2×10^{-5}	4.3	0.12	173.3	3.0×10^{-37}
Males	7.1	0.03	32.3	9.5×10^{-8}	53.2	2.8×10^{-12}	166.8	2.1×10^{-35}
Fluctuations & 23°C								
Females	14.5	0.7e-03	1.13	0.56	1.0	0.62	29.0	4.8×10^{-07}
Males	0.21	0.90	2.2	0.32	2.0	0.37	42.6	5.6×10^{-10}

χ^2 LRT test statistic and corresponding *p-value* (*df* = 2) relative to the data in Figures 3 and 4, testing the effect of temperature treatment on body size and wing patterns with a GLMM.

*The analysis of eyespot size used wing area as covariate (cf. Methods section).

on development time (Zijlstra et al. 2004), it had been suggested that temperature affects development time directly, and it is the ensuing changes in development time that lead to changes in other thermally plastic traits (Brakefield and Kesbeke 1997; Zijlstra et al. 2004; Brakefield and Frankino 2006).

Butterflies developing at lower temperatures take longer to complete development and have smaller eyespots than those developing at warmer temperatures, and there was a clear negative correlation between development time and eyespot size when testing across temperature treatments. However, for individuals developing under the same thermal regime, development time, which can differ by several days, did not correlate with eyespot size. This situation, reminiscent of the Simpson's paradox or Yule–Simpson effect (Hernán et al. 2011), was true both for our dataset and for data from another independent study (Fig. 5). These data suggest that temperature-induced changes in development time are unlikely to account for temperature-induced changes in eyespot size, such as those we documented for fluctuating temperatures, and argue for a direct effect of temperature on different thermally plastic traits. Additional support for this comes from the different shapes of reaction norms for traits belonging to the thermal plasticity syndrome, and from the fact that manipulations of the ecdysone dynamics known to mediate this plasticity have trait-specific effects (Mateus et al. 2014; Oostra et al. 2014; Monteiro et al. 2015). Differences in the shape of reaction norms can help account for differences in the response to day-night temperature fluctuations. In particular, mathematical properties of non-linear reaction norms, such as Jensen's inequality (see Colinet et al. 2015), can partly account for the type of dominance effect of one particular period of the light cycle that we observed for eyespot size, and others have observed for other traits (Vangansbeke et al. 2015). These results underscore the value of teasing apart effects of day and night warming in studies assessing the impact of climate change on phenotypic variation; particularly since trait-specific responses can break up putatively adaptive trait correlations and, as such, affect organismal fitness.

EFFECTS OF CIRCADIAN TEMPERATURE FLUCTUATIONS ON TRAIT EXPRESSION AND TRAIT EVOLUTION

The combined effects of day and night temperatures on phenotype expression are especially well studied in plants (e.g. effects on the regulation of flowering time; Jin and Zhu 2019; Qiu et al. 2019), and have been documented also for various fitness-related traits in different animal taxa (e.g., Zhao et al. 2014; Vangansbeke et al. 2015; Liefting et al. 2017; Salachan et al. 2017; Bai et al. 2019). The close association between effects of light and temperature on biological processes is revealed by some overlap in the molecules involved in sensing the two types of cues (e.g., phytochromes in *Arabidopsis* (Jung et al. 2016; Legris et al. 2016; Qiu et al. 2019), or cryptochrome in *Drosophila* (Gentile et al. 2013; Harper et al. 2017)), and by the observation that both light and temperature can reset the circadian clock (Goda et al. 2014; Chu et al. 2016). On the other hand, beyond the documented effects on phenotype expression (notably, via developmental plasticity), day-night temperature fluctuations also affect evolution by natural selection. Studies of adaptation under different thermal regimes have documented effects of circadian temperature fluctuations on a variety of phenotypic traits, including body size (Czarnoleski et al. 2013; Adrian et al. 2016), as well on allelic frequencies (Tobler et al. 2015).

Unlike most experimental studies of thermal developmental plasticity, we addressed the effects of short-term temperature fluctuations. Circadian fluctuating temperatures are undoubtedly closer to reality than constant temperatures. This is the scenario under which organisms have evolved in natural populations, but is often not the scenario under which animals are maintained or studied in the laboratory (but see Kong et al. 2016). In fact, while exposure to radical temperature change

can be used as a form of acute stress, it is possible that thermal constancy might also constitute a type of stress and have a negative impact on organismal performance (Schulte 2014; Kingsolver et al. 2015). Whether temperature change during development is or not perceived as a stress, capable of triggering stress responses, likely depends on how abrupt and recurrent the change is (Kingsolver et al. 2016). Studies in different animals have investigated day-night temperature fluctuations, as well as fluctuations happening at variable timescales within an organism's lifetime (e.g., Brakefield and Mazzota 1995; Zhao et al. 2014; Kingsolver et al. 2015; Vangansbeke et al. 2015; Liefting et al. 2017; Salachan et al. 2017; Bai et al. 2019; Carter and Sheldon 2020). Non-constant temperatures affect trait expression in some but not all traits investigated, with the extent of the phenotypic difference between fluctuating versus constant temperatures often varying with the amplitude of the fluctuations.

It remains unclear how organisms integrate complex environmental information, such as that where multiple environmental factors change during the time it takes to complete development, and still produce coherent phenotypes (Ketola et al. 2014). What is clear is that a better understanding of the interactions of organisms with their changing environments will need to consider effects of complex environments (see Rodrigues and Beldade 2020), including multiple and highly dynamic environmental factors, on both trait expression (phenotypic plasticity) and trait evolution (resulting in adaptation) (Jackson et al. 2021). It has been argued that it is important to consider developmental plasticity in the context of studying adaptation to environmental perturbation, including that resulting from climate change (e.g., Sgrò et al. 2016, Snell-Rood et al. 2018, Rodrigues and Beldade 2020). In that it can match organismal phenotypes to ecological conditions, plasticity can help populations cope with environmental heterogeneity, as illustrated by the phenomenon of seasonal polyphenisms (Simpson et al. 2011; Yang et Pospisilik 2019). Developmental plasticity can further help (or hinder; e.g., Jensen et al. 2018; Oostra et al. 2018; Lockley and Eizaguirre 2021) not only the immediate survival but also future adaptation of populations facing environmental perturbation (Reed et al. 2011; Bonamour et al. 2019; Rodrigues and Beldade 2020) or colonizing novel environments (Ghalambor et al. 2007; Bilandžija et al. 2020).

Conclusions

We found evidence for different types of combined effects for day- and night-time temperatures on a suite of thermally plastic traits associated with distinct seasonal strategies for survival and reproduction in *B. anynana* butterflies. While day and night temperatures can have largely additive effects on phenotype expression, we also identified different types of non-additive effects. These include dominance-like effects where one particular period of the circadian cycle or one particular extreme temperature had a relatively larger contribution to end phenotype. Differences between traits revealed their independence in the response to temperature, which might relate to trait-specific windows of environmental sensitivity and/or trait-specific assessment of environmental conditions. Explaining effects of dynamic temperatures on trait expression will require a better understanding of the precise mechanisms by which animals perceive and respond to external temperature fluctuations. Our study underscores the importance of understanding how organisms integrate complex environmental information towards a complete understanding of natural phenotypic variation, and of the potential impact of environmental change thereon. Instead of considering the environment as an irreducible unit, that is, not taking into account that it is made up of many and dynamic variables, it can be valuable to consider that combinations of external conditions can have non-additive effects on trait expression, as well as on organismal fitness.

AUTHOR CONTRIBUTIONS

Y.K.R. and P.B. conceived and designed the study; Y.K.R. performed the experiments and collected the data; F.A. developed a set of interactive Mathematica notebooks to collect wing phenotypic data; E.v.B. provided data on the extra constant thermal regimes and helped collect wing color data; Y.K.R. and D.D. performed the statistical analyses, with contribution of E.v.B.; Y.K.R., P.B., and D.D. wrote the manuscript, with input from E.v.B. All authors gave final approval for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

The article's supporting data is available as "additional file 1".

LITERATURE CITED

Adrian, G. J., M. Czarnoleski, and M. J. Angilletta. 2016. Flies evolved small bodies and cells at high or fluctuating temperatures. Ecol. Evol 6:7991–7996.

Bai, C.-M., G. Ma, W.-Z. Cai, and C.-S. Ma. 2019. Independent and combined effects of daytime heat stress and night-time recovery determine thermal performance. Biol. Open 8:bio038141.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models using lme4. J. Statistical Software 67:1–48.

Beldade, P., A. R. A. Mateus, and R. A. Keller. 2011. Evolution and molecular mechanisms of adaptive developmental plasticity. Mol. Ecol 20:1347–1363.

Beldade, P., and C. Peralta. 2017. Developmental and evolutionary mechanisms shaping butterfly eyespots. Curr. Opin. Insect Sci 19:22–29.

Bilandžija, H., B. Hollifield, M. Steck, G. Meng, M. Ng, A. D. Koch, R. Gračan, H. Ćetković, M. L. Porter, K. J. Renner, et al. 2020. Phenotypic plasticity as a mechanism of cave colonization and adaptation. eLife 9:1–27.

Bonamour, S., L. M. Chevin, A. Charmantier, and C. Teplitsky. 2019. Phenotypic plasticity in response to climate change: The importance of cue variation. Phil. Trans. R. Soc B374:20180178.

Braendle, C., G. K. Davis, J. A. Brisson, and D. L. Stern. 2006. Wing dimorphism in aphids. Heredity 97:192–199.

Brakefield, P. M., and T. B. Larsen. 1984. The evolutionary significance of dry and wet season forms in some tropical butterflies. Biol. J. Linn. Soc. Lond. Soc 22:1–12.

Brakefield, P. M., and V. Mazzotta. 1995. Matching field and laboratory environments: effects of neglecting daily temperature variation on insect reaction norms. J. Evol. Biol 8:559–573.

Brakefield, P. M. 1996. Seasonal polyphenism in butterflies and natural selection. Trends Ecol. Evol 11:275–277.

Brakefield, P. M., and F. Kesbeke. 1997. Genotype-environment interactions for insect growth in constant and fluctuating temperature regimes. Proc. R. Soc. B 264:717–723.

Brakefield, P. M., and W. A. Frankino. 2006. Polyphenisms in Lepidoptera: multidisciplinary approaches to studies of evolution. In: T. N. Ananthakrishnan, D. W. Whitman (eds) Phenotypic plasticity in insects. Mechanisms and consequences. Science Publishers, Inc., Plymouth, UK, pp.121–152.

Brakefield, P. M., P. Beldade, and B. J. Zwaan. 2009. The African butterfly *Bicyclus anynana*: evolutionary genetics and evo-devo. In Emerging model organisms: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, pp 291–329.

Carter, A. W., and K. S. Sheldon. 2020. Life stages differ in plasticity to temperature fluctuations and uniquely contribute to adult phenotype in *Onthophagus taurus* dung beetles. J. Exp. Biol 223:jeb227884.

Chu, F., J. F. Qiu, H. Tao, X. Li, M. Y. Shu, H. J. Liu, Y. SiMa, and S. Xu. 2016. Impact of cyclical changes in temperature on circadian clock genes expression in Bombyx BmN cells. Arch. Insect Biochem. Physiol 91:175–186.

Ciannelli, L., K. S. Chan, K. M. Bailey, and N. C. Stenseth. 2004. Nonadditive effects of the environment on the survival of a large marine fish population. Ecology 85:3418–3427.

Colinet, H., B. J. Sinclair, P. Vernon, and D. Renault. 2015. Insects in fluctuating thermal environments. Annu. Rev. Entomol 60:123–140.

Czarnoleski, M., B. S. Cooper, J. Kierat, and M. J. Angilletta. 2013. Flies developed small bodies and small cells in warm and in thermally fluctuating environments. J. Exp. Biol 216:2896–2901.

Guillaume-Schöpfer, D., K. E. Jaeger, F. Geng, F. G. Doccula, A. Costa, A. A. R. Webb, and P. A. Wigge. 2020. Ribosomes act as cryosensors in plants. bioRxiv 414789.

Falconer, D. S., and T. F. C. Mackay. 1996. Introduction to Quantitative Genetics. 4th edition. Prentice Hall, Essex, p. 464.

Fawcett, T. W., and W. E. Frankenhuis. 2015. Adaptive explanations for sensitive windows in development. Front. Zool 12:S3.

Frankenhuis, W. E., and K. Panchanathan. 2011. Balancing sampling and specialization: an adaptationist model of incremental development. Proc. Royal Soc. B 278:3558–3565.

Gentile, C., H. Sehadova, A. Simoni, C. Chen, and R. Stanewsky. 2013. Cryptochrome antagonizes synchronization of Drosophila's circadian clock to temperature cycles. Curr. Biol 23:185–195.

Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct. Ecol 21:394–407.

Goda, T., B. Sharp, and H. Wijnen. 2014. Temperature-dependent resetting of the molecular circadian oscillator in Drosophila. Proc. Royal Soc. B 281:20141714.

Halali, S., P. M. Brakefield, S. C. Collins, and O. Brattström. 2020. To mate, or not to mate: The evolution of reproductive diapause facilitates insect radiation into African savannahs in the Late Miocene. J Anim Ecol 89:1230–1241.

Harper, R. E. F., M. Ogueta, P. Dayan, R. Stanewsky, and J. T. Albert. 2017. Light dominates peripheral circadian oscillations in *Drosophila melanogaster* during sensory conflict. J. Biol. Rhythms 32:1–10.

Hernán, M. A., D. Clayton, and N. Keiding. 2011. The Simpson's paradox unraveled. Int. J. Epidemiol 40:780–785.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biom. J 50:346–363.

Ingleby, F. C., J. Hunt, and D. J. Hosken. 2010. The role of genotype-by-environment interactions in sexual selection. J. Evol. Biol 23:2031–2045.

Jackson, M. C., S. Pawar, and G. Woodward. 2021. The temporal dynamics of multiple stressor effects: From individuals to ecosystems. Trends Ecol. Evol 36:402–410.

Jang, T., and K. P. Lee. 2018. Context-dependent effects of temperature on starvation resistance in *Drosophila melanogaster*: Mechanisms and ecological implications. J. Insect. Physiol 110:6–12.

Jensen, M. P., C. D. Allen, T. Eguchi,, I. P. Bell, E. L. LaCasella, and W. A. Hilton. 2018. Environmental warming and feminization of one of the largest sea turtle populations in the world. Curr. Biol 28:154–159.

Jin, H., and Z. Zhu. 2019. Dark, light, and temperature: Key players in plant morphogenesis. Plant Physiol 180:1793–1802.

Jung, J. H., M. Domijan, C. Klose, S. Biswas, D. Ezer, M. Gao, A. K. Khattak, M. S. Box, V. Charoensawan, S. Cortijo, et al. 2016. Phytochromes function as thermosensors in Arabidopsis. Science 354:886–889.

Ketola, T., V. M. Kellermann, V. Loeschcke, A. López-Sepulcre, and T. N. Kristensen. 2014. Does environmental robustness play a role in fluctuating environments? Evolution 68:587–594.

Kingsolver, J. G., H. J. MacLean, S. B. Goddin, and K. E. Augustine. 2016. Plasticity of upper thermal limits to acute and chronic temperature variation in *Manduca sexta* larvae. J. Exp. Biol 219:1290–1294.

Kingsolver, J. G., J. K. Higgins, and K. E. Augustine. 2015. Fluctuating temperatures and ectotherm growth: distinguishing non-linear and time-dependent effects. J. Exp. Biol 218:2218–2225.

Kingsolver, J. G., and L. B. Buckley. 2020. Ontogenetic variation in thermal sensitivity shapes insect ecological responses to climate change. Curr. Opin. Insect Sci 41:17–24.

Kivelä, S. M., K. Gotthard, and P. Lehmann. 2019. Developmental plasticity in metabolism but not in energy reserve accumulation in a seasonally polyphenic butterfly. J. Exp. Biol 222:jeb202150.

Klepsatel, P., M. Gáliková, Y. Xu, and R. P. Kühnlein. 2016. Thermal stress depletes energy reserves in Drosophila. Sci. Rep 6:33667.

Klepsatel, P., D. Wildridge, and M. Gáliková. 2019. Temperature induces changes in Drosophila energy stores. Sci. Rep 9:1–10.

Kong, J. D., J. K. Axford, A. A. Hoffmann, and M. R. Kearney. 2016. Novel applications of thermocyclers for phenotyping invertebrate thermal responses. Methods Ecol. Evol 7:1201–1208.

Kooi, R. E., and P. M. Brakefield. 1999. The critical period for wing pattern induction in the polyphenic tropical butterfly *Bicyclus anynana* (Satyrinae). J. Insect Physiol 45:201–212.

Kutz, T. C., C. M. Sgrò, and C. K. Mirth. 2019. Interacting with change: Diet mediates how larvae respond to their thermal environment. Funct. Ecol 33:1940–1951.

Lafuente, E., D. Duneau, and P. Beldade. 2018. Genetic basis of thermal plasticity variation in *Drosophila melanogaster* body size. PLoS Genet 14:1–24.

Lafuente, E., and P. Beldade. 2019. Genomics of developmental plasticity in animals. Front. Genet 10:1–18.

Lazzaro, B. P., H. A. Flores, J. G. Lorigan, and C. P. Yourth. 2008. Genotype-by-environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. PLoS Pathog 4:1–9.

Legris, M., C. Klose, E. S. Burgie, C. C. R. Rojas, M. Neme, A. Hiltbrunner, P. A. Wigge, E. Schäfer, R. D. Vierstra, and J. J. Casal. 2016. Phytochrome B integrates light and temperature signals in Arabidopsis. Science 354:897–900.

Liefting, M., J. Cosijn, and J. Ellers. 2017. Synergistic effect of daily temperature fluctuations and matching light-dark cycle enhances population growth and synchronizes oviposition behavior in a soil arthropod. J. Insect Physiol 96:108–114.

Lloyd, A., C. Morgan, F. C. H. Franklin, and K. Bomblies. 2018. Plasticity of meiotic recombination rates in response to temperature in *Arabidopsis*. Genetics 208:1409–1420.

Lockley, E. C., and C. Eizaguirre. 2021. Effects of global warming on species with temperature-dependent sex determination: Bridging the gap between empirical research and management. Evol Applications (https://doi.org/10.1111/eva.13226

Lyytinen, A., P. M. Brakefield, L. Lindstrom, and J. Mappes. 2004. Does predation maintain eyespot plasticity in *Bicyclus anynana*? Proc. Roy. Soc. B 271:279–283.

Mateus, A. R. A., M. Marques-Pita, V. Oostra, E. Lafuente, P. M. Brakefield, B. J. Zwaan, and P. Beldade. 2014. Adaptive developmental plasticity: compartmentalized responses to environmental cues and to corresponding internal signals provide phenotypic flexibility. BMC Biol 12:97.

Monteiro, A., X. Tong, A. Bear, S. F. Liew, S. Bhardwaj, B. R. Wasik, A. Dinwiddie, C. Bastianelli, W. F. Cheong, M. R. Wenk, et al. 2015. Differential expression of ecdysone receptor leads to variation in phenotypic plasticity across serial homologs. PLoS Genet 11:1–20.

Nettle, D., and M. Bateson. 2015. Adaptive developmental plasticity: What is it, how can we recognize it and when can it evolve? Proc. Roy. Soc. B 282:1812.

Nijhout, H. F. 2003. Development and evolution of adaptive polyphenisms. Evol. Dev 5:9–18.

Nogueira Freitas, A. C., and T. Voets. 2020. Why the emperor penguin reigns where elephants shiver. Cell Calcium 91:102263.

Nylin, S. 2013. Induction of diapause and seasonal morphs in butterflies and other insects: Knowns, unknowns and the challenge of integration. Physiol. Entomol 38:96–104.

Oostra, V., M. A. de Jong, B. M. Invergo, F. Kesbeke, F. Wende, P. M. Brakefield, and B. J. Zwaan. 2011. Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. Proc. Roy. Soc. B 278:789–797.

Oostra, V., A. R. A. Mateus, K. R. L. van der Burg, T. Piessens, M. van Eijk, P. M. Brakefield, P. Beldade, and B. J. Zwaan. 2014. Ecdysteroid hormones link the juvenile environment to alternative adult life histories in a seasonal insect. Am. Nat 184:E79–E92.

Oostra, V., M. Saastamoinen, B. J. Zwaan, and C. W. Wheat. 2018. Strong phenotypic plasticity limits potential for evolutionary responses to climate change. Nat. Commun 9:1005.

Panchanathan, K., and W. E. Frankenhuis. 2016. The evolution of sensitive periods in a model of incremental development. Proc. Royal Soc. B 283:20152439.

Piggott, J. J., C. R. Townsend, and C. D. Matthaei. 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecol. Evol 5:1538–1547.

Prudic, K. L., A. M. Stoehr, B. R. Wasik, and A. Monteiro. 2015. Eyespots deflect predator attack increasing fitness and promoting the evolution of phenotypic plasticity. Proc. Royal Soc. B 282:20141531.

Qiu, Y., M. Li, R. J. A. Kim, C. M. Moore, and M. Chen. 2019. Daytime temperature is sensed by phytochrome B in Arabidopsis through a transcriptional activator HEMERA. Nat. Commun 10:140.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rall, B. C., O. Vucic-Pestic, R. B. Ehnes, M. Emmerson, and U. Brose. 2010. Temperature, predator-prey interaction strength and population stability. Glob. Chang. Biol 16:2145–2157.

Reed, T. E., D. E. Schindler, and R. S. Waples. 2011. Interacting effects of phenotypic plasticity and evolution on population persistence in a changing climate. Conserv. Biol 25:56–63.

Rodrigues, Y. K., and P. Beldade. 2020. Thermal plasticity in insects' response to climate change and to multifactorial environments Front. Ecol. Evol 8:271.

Salachan, P. V., and J. G. Sørensen, 2017. Critical thermal limits affected differently by developmental and adult thermal fluctuations. J. Exp. Biol 220:4471–4478.

Samir, P., J.hter. Rahul, and A. J. Link. 2015. Environmental interactions and epistasis are revealed in the proteomic responses to complex stimuli. PLoS One 10:e0134099.

Schulte, P. M. 2014. What is environmental stress? Insights from fish living in a variable environment. J. Exp. Biol 217:23–34.

Simpson, S. J., G. A. Sword, and N. Lo. 2011. Polyphenism in insects. Curr. Biol 21:738–749.

Sgrò, C. M., J. S. Terblanche, and A. A. Hoffmann. 2016. What can plasticity contribute to insect responses to climate change? Annu. Rev. Entomol 61:433–451.

Stoehr, A. M., and E. M. Wojan. 2016. Multiple cues influence multiple traits in the phenotypically plastic melanization of the cabbage white butterfly. Oecologia 182:691–701.

Snell-Rood, E. C., E. M. Swanson, and R. L. Young. 2015. Life history as a constraint on plasticity: developmental timing is correlated with phenotypic variation in birds. Heredity 115:379–388.

Snell-Rood, E. C., M. E. Kobiela, K. L. Sikkink, and A. Shephard. 2018. Mechanisms of plastic rescue in novel environments. Annu. Rev. Ecol. Evol. System 49:331–354.

Therneau, T., and P. Grambsch. 2000. The Cox model : Diagnostics. P. in N. Y. Springer, ed. Modeling Survival Data: Extending the Cox Model. Springer, New York. pp. 39–77.

Thomas, L., and E. M. Reyes. 2014. Tutorial: Survival Estimation for Cox Regression and R. J. Stat. Softw 61:1–23.

Tobler, R., J. Hermisson, and C. Schlötterer. 2015. Parallel trait adaptation across opposing thermal environments in experimental *Drosophila melanogaster* populations. Evolution 69:1745–1759.

van Bergen, E., D. Osbaldeston, U. Kodandaramaiah, O. Brattström, K. Aduse-Poku, and P. M. Brakefield. 2017. Conserved patterns of integrated developmental plasticity in a group of polyphenic tropical butterflies. BMC Evol. Biol 17:59.

van Bergen, E., and P. Beldade. 2019. Seasonal plasticity in anti-predatory strategies: Matching of color and color preference for effective crypsis. Evol. Lett 3:313–320.

van der Burg, K. R. L., and R. D. Reed. 2021. Seasonal plasticity: how do butterfly wing pattern traits evolve environmental responsiveness? Curr. Op. Genet. Dev 69:82–87.

Vangansbeke, D., J. Audenaert, D. T. Nguyen, R. Verhoeven, B. Gobin, L. Tirry, and P. De Clercq. 2015. Diurnal temperature variations affect development of a herbivorous arthropod pest and its predators. PLoS One 10:1–19.

Verspagen, N., S. Ikonen, M. Saastamoinen, and E. van Bergen. 2020. Multidimensional plasticity in the Glanville fritillary butterfly: larval performance is temperature, host and family specific. Proc. R. Soc. B 287:20202577.

Windig, J. J., P. M. Brakefield, N. Reitsma, and J. G. M. Wilson. 1994. Seasonal polyphenism in the wild: survey of wing patterns in five species of Bicyclus butterflies in Malawi. Ecol. Entomol 19:285–298.

West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford University Press, New York, p. 794.

Westneat, D. F., L. J. Potts, K. L. Sasser, and J. D. Shaffer. 2019. Causes and consequences of phenotypic plasticity in complex environments. Trends Ecol. Evol 34:555–568.

Yang, C. H., and J. A. Pospisilik. 2019. Polyphenism – A window into gene-environment interactions and phenotypic plasticity. Front. Genet 10:132.

Zhao, F., W. Zhang, A. A. Hoffmann, and C.en. Ma. 2014. Night warming on hot days produces novel impacts on development, survival and reproduction in a small arthropod. J. Anim. Ecol 83:769–778.

Zijlstra, W. G., M. J. Steigenga, P. B. Koch, B. J. Zwaan, and P. M. Brakefield. 2004. Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. Am. Nat 163:E76–E87.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data table S1: Development time, body size, and wing pigmentation data for all individuals eclosed from each replicate cage (R1-R4) of each of the thermal regimes.
Additional file S2: eclosion success (i.e. survival from L1 to adulthood).
Additional file S3: statistical interaction between day and night temperatures.
Figure S2. Testing statistical interaction between day and night temperatures.