This is the peer reviewed version of the following article: Rodrigues, Y. K., van Bergen, E., Alves, F., Duneau, D., & Beldade, P. (2021). Additive and non-additive effects of day and night temperatures on thermally plastic traits in a model for adaptive seasonal plasticity. Evolution, 75(7), 1805–1819, which has been published in final form at https://doi.org/10.1111/evo.14271.

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions

**TITLE**

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

**Short Title:**

Effects of day-night temperature fluctuations.

**Authors & affiliations:**

Yara Katia Rodrigues$^{1,2}$, Erik van Bergen$^{1,3}$, Filipa Alves$^1$, David Duneau$^{1,4,*}$, Patricia Beldade$^{1,4,5,*}$

* equal contribution

1 Instituto Gulbenkian de Ciência, Oeiras, Portugal
2 Current address: Atlantic Technical University (UTA), Mindelo, São Vicente island, Cabo Verde
3 Current address: Department of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland
4 UMR 5174 - CNRS, Evolution et Diversité Biologique, University Paul Sabatier, Toulouse, France
5 CE3C: Centre for Ecology, Evolution, and Environmental Changes, Faculty of Sciences, University of Lisbon, Portugal

**Corresponding author:**

Patrícia Beldade pbeldade@fc.ul.pt

**Authors’ 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.

Funding

Instituto Gulbenkian de Ciência's Ph.D. program, Programa de Pós-Graduação Ciência para o
Desenvolvimento (PGCD) (support to Y.K.R.)
Portuguese science funding agency, Fundação para a Ciência e Tecnologia, FCT: PhD 410
fellowship to Y.K.R. (SFRH/BD/114404/2016), and research grant to P.B. (PTDC/BIA411
EVF/0017/2014).
French research funding agency, Agence Nationale de la Recherche, ANR: Laboratory of
Excellence TULIP, ANR-10-LABX-41 (support to D.D.).
French research centre, Centre National de la Recherche Scientifique, CNRS: International
Associated Laboratory, LIA BEEG-B (support to D.D. and P.B.).
The People Program (Marie Curie Actions) of the European Union's Seventh Framework
Program (FP7/2007-2013) under REA grant agreement PCOFUND GA-2013-609102, through
the PRESTIGE program coordinated by Campus France (support to D.D.).

Acknowledgements

We thank Elvira Lafuente for help with data analyses, Vicencio Oostra and Maaike de Jong for
access to their published data, and Carolina Peralta and Pedro Castanheira for help with animal
husbandry.

Data accessibility

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

Competing interests

The authors declare no competing financial interests

ABSTRACT

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 towards a complete understanding of natural phenotypic variation and of the impact of environmental change thereon.

**KEYWORDS**

environment-by-environment interactions; circadian temperature fluctuations; adaptive developmental plasticity; *Bicyclus anynana*; seasonal polyphenism; environmental “dominance”
INTRODUCTION

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 et al. 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. Towards 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 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 titres, 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 hrs 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 four days, we collected first instar larvae (L1) and randomly assigned them to different temperature treatments.
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. 22cm length x 12.5cm width x 100cm 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 0.001g (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,
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 supplementary file 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 ~ 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 ~ 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 likelihood 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 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) ~ 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 ~ 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 ~ 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 (additional file 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 (additional file 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, pre-pupal, 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 pre-pupal 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 towards 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 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 (additional file 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-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 \( df=2, \chi^2=21.1, p=2.6e-5 \)), 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 together: \( df=2, \chi^2=23.1, p=9.8e-6 \)). This is in line with previously described thermal plasticity for \textit{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: 
\[ df=2, \chi^2=0.23, p=0.89; \]  
\[ \text{eyespot size: } df=2, \chi^2=1.35, p=0.51 \]: 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 Fig. 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-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).

**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

![Figure 5. Correlation between relative eyespot size and development time.](image-url)
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.

### Combined effects of day and night temperatures on thermally plastic traits

If day and night temperatures contributed equally to phenotype expression, i.e. 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 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, 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 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 of 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 to 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 (e.g. Verspagen et al. 2020), 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, i.e. 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.

REFERENCES


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


resistance in *Drosophila melanogaster*: Mechanisms and ecological implications.


Kooi, R. E., and P. M. Brakefield. 1999. The critical period for wing pattern induction


**TABLES AND FIGURE LEGENDS**

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

<table>
<thead>
<tr>
<th></th>
<th>Total dev time</th>
<th>Larvae</th>
<th>Pre-pupae</th>
<th>Pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>$p$</td>
<td>$\chi^2$</td>
<td>$p$</td>
</tr>
<tr>
<td><strong>Constant temperatures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females</td>
<td>290.3</td>
<td>2.9e-61</td>
<td>246.4</td>
<td>6.3e-54</td>
</tr>
<tr>
<td>males</td>
<td>281.2</td>
<td>2.2e-60</td>
<td>230.8</td>
<td>1.9e-49</td>
</tr>
<tr>
<td><strong>Fluctuations &amp; 23°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females</td>
<td>18.5</td>
<td>9.8e-05</td>
<td>8.5</td>
<td>0.10</td>
</tr>
<tr>
<td>males</td>
<td>19.0</td>
<td>7.6e-05</td>
<td>7.9</td>
<td>0.02</td>
</tr>
</tbody>
</table>

$\chi^2$ 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).

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

<table>
<thead>
<tr>
<th></th>
<th>Pupal mass</th>
<th>Wing area</th>
<th>Wing background</th>
<th>Eyespot size*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>$p$</td>
<td>$\chi^2$</td>
<td>$p$</td>
</tr>
<tr>
<td><strong>Constant temperatures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females</td>
<td>8.0</td>
<td>0.02</td>
<td>19.7</td>
<td>5.2e-5</td>
</tr>
<tr>
<td>males</td>
<td>7.1</td>
<td>0.03</td>
<td>32.3</td>
<td>9.5e-8</td>
</tr>
<tr>
<td><strong>Fluctuations &amp; 23°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females</td>
<td>14.5</td>
<td>0.7e-03</td>
<td>1.13</td>
<td>0.56</td>
</tr>
<tr>
<td>males</td>
<td>0.21</td>
<td>0.90</td>
<td>2.2</td>
<td>0.32</td>
</tr>
</tbody>
</table>

$\chi^2$ 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).
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.

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. glht 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).

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-B) and fluctuating (C-D) temperatures. Each dot corresponds to one
individually (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 treatments illustrate whether pairs of treatments are significantly different between them (different letters) or not (same letter), cf. glt post-hoc pairwise-comparison test. ns refers to non-significant differences between treatments.

**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-B) and fluctuating temperatures (C-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 treatments illustrate whether pairs of treatments are significantly different between them (different letters) or not (same letter), cf. glt 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.

**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.

### ADDITIONAL FILES

**S1. Data table**

Development time, body size, and wing pigmentation data for all individuals eclosed from each replicate cage (R1-R4) of each of the thermal regimes.

**S2. Survival from L1 larvae to adulthood under different thermal regimes**

Each dot corresponds to the proportion of 22 L1 larvae that reached adulthood in each of the four replicate cages. Numbers above the X-axis correspond to number of adults eclosed from each of the four replicate cages (separated by | symbol). Letters under the boxplots represent whether eclosion success was (different letters) or not (same letter) significantly different across temperature treatments. Information about the statistical tests on the right (bold for p-values relative to statistically significant differences).

**S3. Testing statistical interaction between day and night temperatures.**

Data were tested for the interaction of day and night temperatures (dT and nT) considered as two independent factors and taking into account the two treatments with constant temperatures (27 and 19) and the two treatments with fluctuating temperatures (27-19 and 19-27). Note that these are the same data represented in Figures 2-4, but exclude the “23” treatment. Data are represented separately for each trait in females (left) and males (right). Each dot corresponds to one individual and individuals from all
replicate cages are shown together, but “replicate cage” effect (random factor) was explicitly taken into account in the statistical model. The tables to the right display the results of the statistical tests for effects of dT, nT and their interaction (dT×nT), as described in the Materials and Methods section. P-values for statistical significant dT×nT effects are shown in bold.