1 **Title:**

2 Complex effects of day and night temperature fluctuations on thermally plastic traits in a
3 seasonal plasticity model.

4

5 **Authors:**

6 Yara Katia Rodrigues ¹, Erik van Bergen ¹, Filipa Alves ¹, David Duneau ^{2*}, Patrícia Beldade
7 ^{1,2*}

8

9 **Affiliations:**

10 ¹ Instituto Gulbenkian de Ciência, Oeiras, Portugal

11 ² UMR 5174 - CNRS, Evolution et Diversité Biologique, University Paul Sabatier, Toulouse,
12 France

13 * equal contribution

14 Corresponding author: pbeldade@igc.gulbenkian.pt

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16

17 **Abstract:**

18 **Background:** Changes in development in response to seasonally variable environments can
 19 produce phenotypes adjusted to fluctuating seasonal conditions and help organisms cope with
 20 temporal heterogeneity. In contrast to what happens in natural situations, experimental studies
 21 of developmental plasticity typically use environmental factors held constant during
 22 development, precluding assessment of potential environment-by-environment interaction
 23 effects.

24 **Results:** We tested effects of circadian fluctuations in temperature on a series of thermally
 25 plastic traits in a model of adaptive seasonal plasticity, the butterfly *Bicyclus anynana*.
 26 Comparing phenotypes from individuals reared under two types of fluctuations (warmer days
 27 with cooler nights, and cooler days with warmer nights) and those reared under a constant
 28 temperature of the same daily average allowed us to identify complex patterns of response to
 29 day and night temperatures. We found evidence of additive-like effects (for body size), but also
 30 different types of “dominance”-type effects where one particular period of the light cycle (for
 31 development time) or one particular extreme temperature (for eyespot size) had a relatively
 32 larger contribution to phenotype expression. We also gathered evidence against the hypothesis
 33 that thermal plasticity in development time drives thermal plasticity in other traits.

34 **Conclusions:** Combined effects of fluctuating day and night temperatures include additive-like
 35 effects as well as different types of environmental-dominance interaction effects. Differences
 36 between plastic traits reveal independent responses to temperature, and possible independent
 37 assessment of temperature conditions. Our study underscores the importance of understanding
 38 how organisms integrate complex environmental information towards a complete understanding
 39 of natural phenotypic variation and of the potential impact of environmental change thereon.

40

41 **Keywords:**

42 Environment-by-environment interactions; Circadian temperature fluctuations; Adaptive
 43 developmental plasticity; *Bicyclus anynana*; Seasonal polyphenism; environmental
 44 “dominance”

45

46 **Background**

47 Phenotypic diversity results from complex interactions between organisms and their
 48 environments, which happen at different time scales. External environmental conditions
 49 contribute to selecting phenotypic variants across generations, but also to generating variation
 50 by affecting organismal development. The phenomenon by which environmental conditions
 51 affect developmental rates and/or trajectories, leading to the production of distinct phenotypes
 52 from the same genotype, is called developmental plasticity [1]. This plasticity is adaptive if the
 53 phenotypes generated in response to the environmental conditions experienced during
 54 development are better adjusted to the environmental conditions the organisms will experience
 55 during adulthood [1,2]. In this manner, plasticity offers a means for organisms to cope with
 56 environment heterogeneity, such as that characteristic of alternating seasons. Seasonal
 57 polyphenism refers to distinct phenotypes being produced in response to seasonally variable
 58 environmental factors, such as temperature and photoperiod [3,4]. Compelling examples include
 59 wing development in aphids [4,5], wing pigmentation in butterflies [3,6–8], and diapause in a
 60 variety of animals [9,10].

61

62 Effects of external environmental factors on development have been amply documented for
 63 various traits and species [11,12], as have gene-by-environment (GxE) interactions [13,14].
 64 Efforts to partition genetic effects into additive and interaction components take into account
 65 that there are multiple genes and alleles whose individual effects can depend on the genetic
 66 context (GxG effects). In contrast, much less attention has been given to potential environment-
 67 by-environment (ExE) interactions [15–17]. Traditionally, experimental studies of
 68 developmental plasticity have focused on the effects of single environmental factors held
 69 constant during the time it takes to complete development. This is in stark contrast with the
 70 complexity of natural situations, where multiple and highly dynamic environmental factors can
 71 have distinct effects on different genotypes and plastic traits. Towards a more complete account
 72 of phenotypic variation, recent studies have started to address phenotypic effects of
 73 combinations of different types of cues [18–20]. Less attention has been given to changes in

74 particular environmental factors during development [21,22]. Environmental factors such as
 75 temperature fluctuate regularly not only with the yearly seasons, but also with the daily light-
 76 dark cycle. Despite the prevalence and importance of circadian fluctuations in ambient
 77 temperature, we still lack a clear understanding of the combined effects of day and night
 78 temperatures on thermally plastic traits, such as those described for the seasonally plastic
 79 butterfly *Bicyclus anynana*.

80

81 *B. anynana* has become a valuable experimental model of adaptive developmental plasticity,
 82 where we can integrate information about the evolution and ecological significance of plasticity
 83 with knowledge about its physiological underpinnings [7,23–25]. In its natural habitat in sub-
 84 Saharan Africa, these butterflies typically have two seasonal forms that differ in various traits in
 85 association with alternative strategies for avoiding predation and for reproduction. Relative to
 86 wet-season form butterflies, dry-season form individuals are larger and delay reproduction until
 87 host plants become available for a new generation of larvae [8,26,27]. Dry-season individuals
 88 also have less conspicuous wing patterns and their dull brown coloration is thought to provide
 89 camouflage against the background of dry leaves, thereby helping resting butterflies escape
 90 predators' attention [8,28,29]. Wet-season butterflies, on the contrary, minimize predator attack
 91 by deflecting the attention of predators away from the fragile body, towards their wing margins
 92 decorated with conspicuous wing pattern elements called eyespots [30,31]. The main
 93 environmental cue determining which form will be produced is the temperature experienced
 94 during development [8,32]. Developmental temperature affects the dynamics of ecdysone titres,
 95 which, in turn, regulates the response of a suite of plastic traits [24,33]. With only two
 96 exceptions [34,35], laboratory studies of *B. anynana* plasticity used temperatures held constant
 97 during light and dark hours of the day.

98

99 Here, we compared a series of thermally plastic traits between individuals reared under three
 100 constant temperatures or under circadian temperature fluctuations with the same daily average
 101 as the intermediate constant temperature (Fig. 1a). To probe 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. We tested the null hypothesis of no interaction between day and night temperatures by comparing the effect of temperature fluctuations with those of the constant temperature of the same daily average. We also tested the null hypothesis of no association between temperature and light phase by comparing the two types of fluctuations. We found differences between target traits in relation to the combined effects of day and night temperature, including additive and non-additive effects of different kinds. Finally, our data also provide evidence against a previous suggestion that the effect of circadian temperature fluctuations on different thermally plastic traits is a consequence of their direct effects on development time.

Methods

Butterflies and temperature treatments

We used a captive outbred population of the tropical butterfly *B. anynana* [23] 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 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 cages with 22 L1 each that were split into five temperature treatments. Three treatments had constant temperatures: 19°C and 27°C extremes (simulating typical average temperatures 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 fluctuations with the light-dark cycle between the two extreme temperatures (Fig. 1a). For each of these five thermal regimes, we had four replicate cohorts in four independent cages. 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 -

130 20°C. Wings were dissected and stored at 4°C until phenotypic analysis.

131

132 **Quantification of phenotypic traits**

133 We quantified the response to thermal regimes for various thermally-plastic life-history and
 134 wing pigmentation traits. We monitored development time by recording the number of days
 135 from L1 larvae to pre-pupae, from pre-pupae to pupae, and from pupae to adult, and we
 136 calculated total development time by adding those. We measured two proxies of body size:
 137 pupal mass and adult wing area. For pupal mass, one-day-old pupae were weighed to the nearest
 138 0.001g (KERN ABS 80-4N scale). For wing area, we scanned the ventral surface of adult
 139 hindwings using a colour-calibrated digital scanner (Epson V600) and analysed the resulting
 140 images with a set of custom-made interactive Mathematica notebooks (Wolfram Research, Inc.,
 141 Mathematica, Version 10.2, Champaign, IL, 2015) to measure hindwing area and a series of
 142 wing pigmentation traits. For the colour pattern measurements, we first drew two contiguous
 143 transects defined by the centre of the fifth eyespot, which is often used to document wing
 144 pattern plasticity in this and other species [8,36], and four wing landmarks (on the wing margin
 145 and intersection between veins; Fig. 1b-c) in that eyespot's wing compartment. We marked the
 146 limits of each of the colour rings along the transect (central white focus, middle black disc, and
 147 external golden ring) to determine ring diameters and calculate the approximate eyespot area
 148 (considering it as a circle). The colour of eyespot rings and wing background were quantified
 149 using the mean RGB values of the pixels in 3-pixel high rectangles centred on the transect. For
 150 the wing background colour, we used the most proximal 50 pixels of the transect, corresponding
 151 to a wing region without any defined colour pattern element (Fig. 1c). RGB values were
 152 converted to HSB (Hue, Saturation, and Brightness) using the *rgb2hsv* function in R.
 153 Background colour was characterized by the brightness value in the HSB colour space; high
 154 brightness values corresponding to lighter colours.

155

156 **Statistical analyses**

157 We compared phenotypes between temperature treatments, each of which included four

158 replicate cages with up to 22 individuals per cage. All statistical tests were done with R [37],
 159 separately for males and females. When appropriate, Normal distribution and homoscedasticity
 160 of the residuals were tested with Shapiro-Wilk normality tests and Brush-Pagan tests,
 161 respectively. We used a general linear hypotheses test (glht) to test for differences between
 162 thermal regimes, followed by Tukey post Hoc pairwise comparisons ($\alpha=0.05$) to ascertain
 163 differences between pairs of treatments (package *multcomp* in R).

164
 165 First, to test for differences in development time, we used a Cox proportional hazards model to
 166 determine whether “treatment” influenced the proportions of adult eclosions over time (package
 167 *Survival* in R). For the different developmental stages and each sex, we tested the model *Coxph*
 168 *survival (time, eclosion) ~ replicate + treatment*. Second, to test for differences in body size
 169 (pupal weight and wing area) and wing pigmentation (eyespot size and wing background
 170 colour), we applied a linear model and tested the model: *trait ~ replicate + treatment*. The trait
 171 “relative eyespot size” corresponded to the ratio between eyespot area and wing area, for which
 172 the assumption of a normal distribution of the residuals was confirmed by a Shapiro test.
 173 Finally, to test for the correlation between developmental time and relative eyespot area, we
 174 used a correlation test with a Spearman method, with the *p* values corrected and adjusted by the
 175 False Discovery Rate (FDR) [38]. The same type of analysis was used to investigate the
 176 correlation between developmental time and relative eyespot size using a dataset combining
 177 previously published data on development time [39] and on eyespot size [36] in *B. anynana*.

178

179 **Results**

180 We tested the effect of circadian temperature fluctuations on different thermally plastic traits:
 181 development time (Fig. 2), body size (Fig. 3), and wing pigmentation (Fig. 4). We first
 182 compared phenotypes between the three treatments with constant temperatures to assess the
 183 direction and strength of thermal plasticity in our *B. anynana* population and experimental
 184 conditions. We then compared phenotypes between the three treatments of the same daily
 185 average temperature to assess the contribution of day and night temperatures to the phenotype.

186 We found different responses for different traits, including additive and non-additive effects of
187 different types. We finally tested the correlation between development time and eyespot size,
188 using our and another independent dataset (Fig. 5).

189

190 **Different contributions of day and night temperatures to development time**

191 We confirmed thermal plasticity in *B. anynana* development time in our study population:
192 individuals reared at lower temperatures took longer to reach adulthood than individuals reared
193 at higher temperatures (Fig. 2a). For both males and females, temperature affected the duration
194 of all developmental stages monitored; individuals from warmer temperatures had shorter larval,
195 pre-pupal, and pupal stages (Fig. 2b).

196

197 We also found differences in development time between the three treatments with a daily
198 average temperature of 23°C (Fig. 2c). For both males and females, development was faster for
199 individuals that spent the day at 27°C and the night at 19°C (27-19 treatment) compared to
200 individuals that spent the day at 19°C and the night at 27°C (19-27 treatment). The duration of
201 the pupal stage differed between treatments, but not the duration of the larval and of the pre-
202 pupal stages (Fig. 2d). The difference between our two treatments with fluctuating temperatures
203 revealed that the effect of day and night temperature on development time is not additive and
204 that the temperature experienced during the light phase had a larger impact on total development
205 time. Individuals reared with a day temperature of 27°C demonstrated a shift in development
206 time towards individuals reared at constant 27°C, while the development time of individuals
207 reared with a day temperature of 19°C shifted towards those reared at a constant temperature of
208 19°C. The response for individuals reared at a constant temperature of 23°C relative to the two
209 fluctuations of the same daily mean (27-19 and 19-27) differed between males and females (Fig.
210 2c-d).

211

212 **No difference between fluctuations and constant daily temperature for body size**

213 For both proxies of body size we quantified, pupal mass (Fig. 3a) and adult wing area (Fig. 3b),

we confirmed known thermal plasticity patterns, with lower temperatures yielding larger individuals. For both sexes, individuals reared at 19°C were significantly larger than individuals reared at 23°C or 27°C, which did not differ significantly between each other.

Overall, we found no significant differences between individuals reared under constant versus fluctuating temperatures of the same daily average (Fig. 3c-d). This corresponds to an additive-like effect of day and night temperatures on body size. The only exception was female pupal mass, where we found a significant difference between the two fluctuating temperature treatments. Like for development time, the temperature experienced during the light phase had a stronger effect on the phenotype (Fig. 3c).

Different contributions of cool and warm temperatures to eyespot size but not wing background brightness

We investigated two aspects of wing pigmentation (Fig. 4): relative eyespot size, which is a trait well known to be thermally plastic, and wing background colour, which had not been quantified before despite suggestions of it also varying between seasonal forms. As had been noticed but not formally quantified before, females are lighter than males and wing background colour depends on rearing temperature, but only for males (Fig. 4a). Plasticity for eyespot size was much stronger, with significant differences between all three constant temperature treatments (Fig. 4b). This is in line with the well described thermal plasticity for *B. anynana* eyespot size, with larger eyespots in animals reared at warmer temperatures, for both males and females.

Regarding the comparison between constant and fluctuating temperatures of daily average of 23°C, we found similar results for males and females: no differences for wing darkness (Fig. 4c) and clear differences for eyespot size (Fig. 4d). 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. Results for overall eyespot area were consistent with those for the area of individual eyespot rings (central white

242 focus, middle black disc, and external golden ring), which also showed sex and temperature
243 differences in actual colours (Fig. 4e and Additional file 1).

244

245 **Correlation between eyespot size and development time between but not within** 246 **temperature treatments**

247 It had been previously suggested that thermal plasticity in traits such as eyespot size, rather than
248 a direct response to temperature, is a correlated response to temperature-induced changes in
249 development time [29,35,40]. This is not consistent with our results which show that individuals
250 reared at 19-27 developed slower than those from 27-19, but both had larger eyespots than
251 individuals reared at 23°C. We thus went on to investigate the correlation between development
252 time and eyespot size, both across and within temperature treatments. We used our dataset with
253 five thermal regimes, as well as an additional independent dataset put together from published
254 work that includes two extra constant temperature treatments [36,39] (Fig. 5).

255

256 Across constant temperature treatments, with largely non-overlapping development times, we
257 found an overall strong negative correlation between development time and eyespot size, for
258 both females and males (Fig. 5a-b). However, within temperature treatments, there were no
259 correlations between development times and relative eyespot sizes that were statistically
260 significantly different from zero. This result was confirmed using an independent dataset from
261 previously published studies that included more intermediate temperature treatments (Fig. 5c).

262

263 **Discussion**

264 We investigated the effects of combinations of day and night temperatures on a series of
265 thermally plastic traits in *B. anynana* butterflies. We confirmed and quantified thermal plasticity
266 in our experimental population and conditions for development time, body size, and eyespot
267 size, and documented thermal plasticity in wing background colour. Butterflies reared under
268 warmer temperatures generally had faster development, smaller bodies and larger eyespots,
269 matching the seasonal polyphenism described for the species [6,7,24,25]. To assess potential

interaction effects of day and night temperatures, we then compared phenotypes from individuals reared under two types of circadian temperature fluctuations and under a constant temperature of the same daily average.

Combined effects of day and night temperature 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 effects, we should 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 (23°C). We found evidence of such additive effects (for body size; Fig. 3), but also of “dominance”-type 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 contribution to phenotype.

Two previous studies had addressed the effect of circadian temperature fluctuations on *B. anynana* but used only warmer days than nights [34,35]. While this is the more ecologically-relevant regime, in isolation, it did not allow identification of interactions between temperature and light phase. Like in those previous studies, we found that butterflies that developed under cooler nights, relative to those that developed under constant temperature of the same daily average (i.e. 27-19 versus 23 regimes), had faster development (Fig. 2) and larger eyespots (Fig. 4), but mostly did not differ for the other traits under study (except female pupal mass; Fig. 3). Moreover, we showed that cooler nights speeded up development largely by shortening the duration of the post-feeding pupal phase (Fig. 2b). The explanation previously proposed to account for such effects on total development time was that *B. anynana* caterpillars eat mostly during the dark hours and assimilate those resources during light hours [34]. Cooler nights can presumably sustain higher activity levels and higher feeding rates, while warmer days might allow higher assimilation efficiency. Either or both of these could result in faster development. Relationships between temperature and food ingestion efficiency [41], as well as between

thermal stress and depletion of energy reserves [42] have been reported for different arthropods. Another factor that may explain different contributions of day and night temperatures relates to the open question of how often and when developing organisms “acquire information” about external environment [43,44]. Within windows of sensitivity during development (e.g. [24,32,45–47]), it remains unclear whether organisms assess environmental conditions continuously or at discrete time points. The “dominance” effect of the conditions experienced during the light hours on development time could reflect discrete sampling of the environment mainly occurring during that period of the day.

Our comparison between the two types of fluctuations allowed us to gain new insight on the combined effects of day and night temperatures on development time and eyespot size, two iconic examples of *B. anynana* seasonal plasticity. We found distinct types of interaction effects for the two traits. For development time, we found that the temperature experienced during the day had a stronger effect than the temperature experienced during the night. In the same way that individuals develop faster at 27°C relative to 19°C (Fig. 2a), individuals from 27-19 (*i.e.* day spent at 27°C) developed faster than those from 19-27 (day at 19°C) (Fig. 2c). On the other hand, we found that any period of the light-dark cycle spent at a warmer temperature lead to increased eyespot size, as is characteristic of development at warmer temperatures (Fig. 4a). Individuals developing under both types of temperature fluctuations had larger eyespots than those from the constant temperature of the same daily average (Fig. 4b).

Independent effects of temperature on different traits that make up a plasticity syndrome

Typically, seasonal forms differ in a suite of traits that respond to seasonably variable environmental conditions [36,39]. In the case of *B. anynana*, this thermal plasticity “syndrome” includes the traits monitored here, as well as others such as starvation resistance, longevity, and reproductive investment [23,39,48]. Supported also by laboratory data on correlated responses to selection on development time [40], it had been suggested that temperature affects development time directly, and it is the ensuing changes in development time that lead to

326 changes in other thermally plastic traits [29,35,40].

327

328 Indeed, butterflies developing at lower temperatures take longer to complete development and
 329 have smaller eyespots than those developing at warmer temperatures. However, within a
 330 thermal regime, the variation in development time between individuals, which can be of several
 331 days, did not correlate with eyespot size. This apparent case of the Simpson's paradox or Yule–
 332 Simpson effect [49] was true for our dataset and for data from other independent studies (Fig.
 333 5). These results suggest that temperature-induced changes in development time cannot account
 334 for temperature-induced changes in all other thermally plastic traits, and certainly not for
 335 changes in eyespot size seen under fluctuating temperatures, and argue for a more direct and
 336 trait-specific effect of temperature. Additional support for this comes from the different shapes
 337 of reaction norms of traits belonging to the thermal plasticity syndrome, and from the fact that
 338 manipulations of the ecdysone titres known to mediate this plasticity have trait-specific effects
 339 [33,50]. The trait-specific responses to environmental conditions are probably related to trait-
 340 specific windows of environmental sensitivity during development [32,46,47]

341

342 **Effects of circadian temperature fluctuations on development and evolution**

343 Experimental studies on different animal and plant systems have documented effects of day-
 344 night temperature fluctuations on both development (i.e. phenotype expression) and evolution
 345 (i.e. phenotype filtering by natural selection). Examples of the former include effects of day
 346 versus night temperature on the regulation of flowering time [51,52], and effects of circadian
 347 temperature fluctuations on various fitness related traits [53–57]. The close association between
 348 effects of light and of temperature on biological processes is further revealed in the overlap in
 349 sensing mechanisms for the two cues (e.g. role of phytochromes as thermosensors in
 350 *Arabidopsis* [58,59], or cryptochrome in *Drosophila* [54]), and also the observation that
 351 temperature, and not only light, can reset the circadian clock [60]. At the scale of inter-
 352 generation effects, evolution under different thermal regimes in natural and experimental
 353 populations has documented effects of circadian temperature fluctuations on a variety of

354 phenotypic traits including body size [56,57], as well as on allele frequencies [61].

355

356 Unlike most experimental studies of thermal plasticity, we addressed the effects of development
 357 under temperatures not held constant. Specifically, we studied potential interaction effects of
 358 fluctuating day and night temperatures on a suite of thermally plastic traits. Circadian
 359 fluctuating temperatures are undoubtedly closer to reality than constant temperatures, the same
 360 way that colder nights are closer to reality than warmer nights. This is the scenario under which
 361 organisms have evolved in natural populations, but is rarely the scenario under which animals
 362 are maintained or studied in the laboratory (but see e.g. [62]). In fact, even though exposure to
 363 temperature change can be used as a form of stress (e.g. [57,63]), it is possible that thermal
 364 constancy also constitutes a type of stress [64]. Whether temperature change is or not perceived
 365 as a stress capable of triggering stress responses likely depends on how abrupt (rather than
 366 gradual), how large, and how recurrent the change is [65]. Many studies of thermal stress use
 367 rather short exposures to extreme temperatures (e.g. [11,66]). Such temperature changes can
 368 affect different aspects of the organisms' biology, including developmental robustness [67] and
 369 various fitness-related traits [42]. It is unclear if the temperature changes, which typically
 370 fluctuate with the day and night cycle, can illicit any of the same type of physiological
 371 responses. It is even less well known how organisms integrate complex environmental
 372 information, such as that where multiple environmental factors change during the time it takes
 373 to complete development, and still produce coherent phenotypes for the various plastic traits
 374 [67,68]. Especially when environmental challenge includes a mismatch in what are the usual
 375 combinations of environmental factors, environment-by-environment effects are likely to have
 376 an important impact on how organisms deal with such challenge.

377

378 **Conclusions**

379 We found evidence for different types of combined effects for day- and night-time temperatures
 380 on a suite of thermally plastic traits associated with distinct seasonal strategies for survival and
 381 reproduction in *B. anynana* butterflies. While, for some traits, day and night temperatures seem

382 to have largely additive effects on phenotype expression, we also identified different types of
 383 non-additive effects. These included environmental dominance-like effects where one particular
 384 period of the circadian cycle or one particular extreme temperature had a relatively larger
 385 contribution to phenotype. Differences between traits reveal their independence in the response
 386 to temperature, which might relate to trait-specific windows of environmental sensitivity and/or
 387 trait-specific assessment of environmental conditions. Our study underscores the importance of
 388 understanding how organisms integrate complex environmental information towards a complete
 389 understanding of natural phenotypic variation and of the potential impact of environmental
 390 change thereon

391

392

393 **List of abbreviations**

394 GxE: gene-by-environment interactions

395 GxG: gene-by-gene interactions

396 ExE: environment-by-environment interactions

397 L1: first instar larvae

398

399 **Declarations**

400 *Ethics approval and consent to participate*

401 Not applicable.

402 *Consent for publication*

403 Not applicable.

404 *Availability of data and material*

405 Raw data are available in Additional file 2.

406 *Competing interests*

407 The authors declare that they have no competing interests.

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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 colour data; Y.K.R., E.v.B., and D.D. performed the statistical analyses; Y.K.R. and P.B. drafted the manuscript, with input from E.v.B. and D.D. All authors gave final approval for publication.

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Additional files

Additional file 1: Effects of constant and fluctuating temperatures on eyespot colour rings.
Additional file 2: Dataset used in this study.

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601 **Figure legends**

602

603 **Fig. 1. Treatments and wing pigmentation phenotypes.**

604 (a) Thermal regimes with constant and fluctuating temperatures in association to the light-dark
605 circadian cycle. (b) Examples of hindwings (ventral surface) from female and male adults from
606 the different constant temperature treatments. (c) Section of a female hindwing (region
607 corresponding to rectangle in panel (b) where landmarks (white circles) defined two contiguous
608 transects (white dashed line) passing through the centre of the fifth eyespot. The proximal
609 portion of the transect (solid line) indicates the approximate region used to phenotype the
610 brightness of background.

611

612 **Fig. 2. Effects of constant and fluctuating temperatures on development time.**

613 Total development time (L1 to adult) and duration of different developmental stages (larvae,
614 pre-pupae, pupae) for females and males developing under constant (a, b) or fluctuating (c, d)
615 temperatures. Panels (a) and (c) represent the proportion of adult eclosions since the start of the
616 experiment. Each line corresponds to the individuals of all four replicates for each treatment.
617 There were significant differences (Coxph with $df=2$ and $p<0.005$ in all cases) between
618 constant temperature treatments in (a) ($\chi^2=245.3$ for females, $\chi^2=202.0$ for males), and between
619 the three types of treatments of same daily mean in (c) ($\chi^2=10.6$ for females, $\chi^2=16.8$ for males).
620 Letters next to treatment legend illustrate whether pairs of treatments are significantly different
621 (different letters) or not (same letter), cf. glth post-hoc test. Panels (b) and (d) correspond to the
622 duration of different developmental stages. Constant temperature treatments in (b) differed in
623 duration of all developmental stages in males (larvae: $\chi^2=171.2$; pre-pupae: $\chi^2=61.1$;
624 pupae: $\chi^2=170.0$) and females (larvae: $\chi^2=195.9$; pre-pupae: $\chi^2=104.2$; pupae: $\chi^2=203.6$; Coxph
625 with $df=2$ and $p<0.001$ for all cases). Fluctuating temperature treatments in (d) differed
626 significantly for the duration of the pupal stage for females (pupae: $\chi^2=31.0$, $df=2$, $p<0.001$)
627 and males (larvae: $\chi^2=6.7$, $p<0.03$; pupae: $\chi^2=38.8$, $p<0.001$; Coxph with $df=2$) but none of the
628 other stages (females larvae: $\chi^2=4.3$; pre-pupae: $\chi^2=4.2$ and males pre-pupae: $\chi^2=0.5$, $df=2$,
629 $p>0.05$).

630

631 **Fig. 3. Effects of constant and fluctuating temperatures on body size.**

632 Pupal mass and wing area of adult butterflies for females and males developed under constant
633 (a-b) and fluctuating (c-d) temperatures. Each dot corresponds to one individual (all replicates
634 plotted together) and the red triangles are median values. We found significant differences in
635 pupal mass between constant temperature treatments (a) for both females ($F=4.0$, $df=2$, $p=0.02$)
636 and males ($F=3.1$, $df=2$, $p=0.04$), and between treatments of same daily mean temperature (c)

for females ($F=7.5$, $df=2$, $p<0.001$) but not males ($F=0.1$, $df=2$, $p=0.91$). We found significant differences in adult wing area between constant temperature treatments (**b**) for females ($F=16.1$, $df=2$, $p<0.001$) and males ($F=17.7$, $df=2$, $p<0.001$), but not between treatments of same daily mean temperature (**d**) for females ($F=0.6$, $df=2$, $p=0.56$) or males ($F=0.9$, $df=2$, $p=0.40$). *ns* refers to non-significant differences between treatments. When there was a significant difference between treatments, letters above treatments illustrate whether they are significantly different (different letters) or not (same letter), *cf.* glth post-hoc test.

Fig. 4. Effects of constant and fluctuating temperatures on wing pigmentation.

The background colour and relative eyespot size from females and males developed under constant (**a,c**) and fluctuating temperatures (**b,d**). Each dot corresponds to one individual (all replicates plotted together) and the red triangles are median values. We found differences in brightness of wing background colour between constant temperature treatments (**a**) for males ($F=33.8$, $df=2$, $p<0.001$) but not females ($F=2.1$, $df=2$, $p=0.12$), and no significant differences between treatments of same daily mean temperature (**c**) for either sex ($F=0.9$, $df=2$, $p=0.30$ for males and females $F=0.6$, $df=2$, $p=0.40$). We found significant differences (ANOVA, $df=2$, $p<0.001$ in all cases) in relative eyespot size between constant temperature treatments (**b**) for females ($F=223.5$) and males ($F=315.3$), and also between treatments with the same daily mean (**d**) for females ($F=14.6$) and males ($F=25.6$). *ns* refers to non-significant differences between treatments. When there was a significant difference between treatments, Letters above treatments illustrate whether they are significantly different (different letters) or not (same letter), *cf.* glth post-hoc test. (**e**) Representation of mean RGB colour for the pixels of the wing background, as well as relative area and colours of eyespot rings from different thermal regimes (see also Additional file 1).

Fig. 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 colour as dots for relationships for data points of the different thermal regime, and black for relationship across all data points. Spearman's rank correlation coefficient (Spearman rho) test showed a significant negative correlation when data points from all treatments were considered together (black line): (**a**) $\rho=-0.85$ for females and $\rho=-0.88$ for males ($p<0.001$ for both), (**b**) $\rho=-0.19$ ($p=0.08$) for males and $\rho=-0.28$ for females ($p=0.001$), (**c**) $\rho=-0.87$ for males and $\rho=-0.85$ females ($p<0.001$ for both). For the correlations within treatments, *rho* and corresponding *p*-values are given in the figure.

(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

(b)

(c)

(a)

(b)

(c)

Developmental time