Temperature effects on genetic and physiological regulation of adaptive plasticity

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CHAPTER 5. THERMAL PIGMENTATION PLASTICITY: PRELIMINARY RESULTS AND FUTURE DIRECTIONS ON THE SHAPE OF REACTION NORMS AND COLOR ANALYSIS

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ABSTRACT

Developmental plasticity, the ability of a single genotype to express different phenotypes in different environments, may evolve as an adaptive response to seasonality and is typically characterized by reaction norms. Temperature, one of the most important and common environmental factors regulating development is of extreme importance in regulating seasonal plasticity of insect´s pigmentation patterns, namely in butterflies. Here, we would like to explore the genotype (G), temperature (T), and GxT effects on *Bicyclus anynana* pigmentation patterns. *B. anynana* butterflies exhibit developmental plasticity for pigmentation patterns as an adaptive response to the alternating wet and dry seasons in their natural environment. In addition, this system also shows developmental plasticity for life-history traits. In order to explore GxT effects on *B. anynana* pigmentation patterns we derived artificial selected lines expressing extreme wet season-like or dry-season-like phenotypes at intermediate temperatures and characterized thermal reaction norms for several traits for a wide range of temperatures. Finally, for the first time in this species, we performed qualitative analysis of color and color patterns across temperature. Our preliminary results show that, for both sexes, there is a significant GxT interaction which confirms mean differences between the unselected stock and artificial selected lines responses in shape and height of reaction norms across temperature. Future directions include developing a detailed formal mathematical treatment of the influence of external

environment on development to characterize shape of thermal reaction norms. Curiously by selecting on extreme pigmentation patterns we were able to change other traits such as survivorship and pupal development time. These correlated responses to selection likely reflect genetic pleiotropy. However, we should be cautious about interpreting correlated responses between wing pattern (target of selection) and life-history traits, as we have no replication of the selection lines (see Material and Methods). We also show, for wing background color, that for low temperatures there are three groups of pigments and for high temperatures four well distinct groups. Our preliminary results also revealed a possible new color appearing at the most extreme low temperatures. We do not know what causes these differences, but we suggested that the orange color might correspond to a pigment from a different type or to a modification of a product of the melanin biosynthesis pathway. Our future work includes developing a general method to quantify color patterns possible to apply to most of the organisms.

INTRODUCTION

Coping with fluctuating external conditions is an important challenge for many organisms, such as those living in seasonal environments. Developmental plasticity, the ability of a single genotype to produce distinct phenotypes depending on the conditions experienced during development, can be a solution to cope with environmental fluctuations. The alternative phenotypes resulting from developmental plasticity include changes in behaviour, physiology, morphology, growth, and life-history traits which can result in a better match between the adult form and the conditions the organism will live in (e.g. Schlichting & Pigliucci 1998, Pigliucci 2005, West-Eberhard 2003, Beldade *et al.* 2011). Plasticity can be represented graphically by reaction norms describing phenotypic variation as a function of the environment. These provide an important tool for studying developmental sensitivity to the environment (e.g. Debat & David 2001, Lewontin 2006, Sultan 2007). The shape and height of reaction norms differ between traits and genotypes, and heritable variation for these properties of reaction norms provide the raw material for natural selection to shape the evolution of plasticity.

Plastic traits do not need to vary continuously along a gradient of the environmental cue responsible for the plasticity. In fact, reaction norms can be nonlinear, as in the case of threshold polyphenisms (e.g. Nijhout 2003, Beldade *et al.* 2011), or can have complex shapes, as in the case of pigmentation variation in adult mesothorax and abdomen segments of *Drosophila melanogaster* in response to

temperature (e. g. Gibert *et al.* 2000). Such shapes have been observed especially for environmental values outside the range organisms have adapted to (Neyfakh & Hartl 1993, reviewed in Pigliucci 2001).

One of the environmental cues most often associated to developmental plasticity is temperature, a key environmental factor in eco-evo-devo studies. In this context, thermal sensitivity of ectotherm performance has been extensively studied (e.g. Van der Have & de Jong 1996, Sinclair *et al.* 2003, Hoffmann *et al.* 2003, Fischer *et al.* 2010) being well known that temperature has a large impact on insects, from direct effects on enzymatic reactions to physiological effects that affect development (Lee Jr. 1991). In many cases, the temperature experienced during development is predictive of the environment where the adult forms will live, and of a number of important ecological parameters that can impact fitness.

Thermal plasticity in insect pigmentation is common in nature (e.g. Beldade *et al.* 2011, Gibert *et al.* 2007) and of extreme importance in visual communication (e.g. mate choice, camouflage), thermoregulation or photo-protection (reviewed in True 2003, Wittkopp & Beldade 2009). Additionally, insect pigmentation has been the target of many evo-devo studies that have attempted to characterize the regulatory genes and enzymes responsible for pigmentation development and its evolution (e.g. Jeong *et al.* 2006, Gibert *et al.* 2004, 2007, Wittkopp & Beldade 2009). Still, the sophistication and extent of the genetic analysis has not been matched by detail in quantitative methods for characterizing pigmentation phenotypes, in term of colors and color patterns. In regarding to that, here we are putting a large effort into the analysis of wing color and color pattern beyond measuring eyespots or band widths.

The tropical Nymphalid *B. anynana* has been established as a laboratory model for research on developmental plasticity (e.g. Beldade & Brakefield 2002, Brakefield *et al.* 2009, Beldade *et al.* 2011). This African butterfly exhibits phenotypic plasticity in pigmentation in response to natural wet–dry seasonality which is externally cued principally by temperature in the final larval and early pupal stages (Brakefield & Reitsma 1991, Brakefield *et al.* 1996, Kooi & Brakefield 1999). Resulting changes in adult wing patterns are associated to seasonal changes in the resting background color and different strategies to minimize predation. The wet season form has conspicuous wing patterns with large eyespots and lighter wing background color, whereas the dry season form has very reduced eyespots and a more cryptic appearance with darker wing

color resembling the brown background of dry leafs (Brakefield 1997, Beldade *et al.* 2011, Mateus *et al.* 2014). In addition to wing pattern, *B. anynana* adults of the wet and dry seasons also differ in life-history strategies (e.g. Brakefield & Reitsma 1991, Brakefield & Frankino 2009, Oostra *et al.* 2011, 2014). In the field, adults spend the harsh dry season being relatively inactive and delay reproduction until the beginning of the wet season. Conversely, the relatively short lived adults of the wet season form are more active and reproduce rapidly.

In the laboratory, individuals that develop at warmer temperatures show wet season-like phenotype with eyespots of large size, while individuals that develop at cooler temperatures show a dry season-like form with eyespots of reduced size (Brakefield *et al.* 1996). At intermediate temperatures, lab reared butterflies show intermediate phenotypes (e.g. Brakefield *et al.* 1998). Standing genetic variation for *B. anynana* wing patterns enabled researcher to derive artificial selected lines that, at intermediate temperature, are similar to one or the other of the natural dry and wet season forms (Brakefield *et al.* 1996). This strategy achieved gradual response to artificial selection on the height, but not the shape, of thermal reaction norms for *B. anynana* wing patterns (Brakefield *et al.* 1996, Wijngaarden & Brakefield 2001) and, unfortunately, lines were lost before the full characterization of the basis of phenotypic differences. Here, in order to better explore genotype (G), environmental (E), and GxE effects on *B. anynana* pigmentation development, we have invested in 1) re-deriving lost artificial selected lines expression extreme wet season-like or dry-season-like phenotypes at intermediary temperatures, and 2) characterizing thermal reaction norms for a wider range of temperatures that is usually explored in this species (including more intermediate temperatures to better assess reaction norms shape, and also more extreme temperatures). As only the outer ends of the reaction norms are thought to be exposed to selection in the field, by using intermediate and extreme values we expect to be able to explore how "hidden" parts of the response curves are affected by this thermal gradient.

The results presented here are still preliminary. We hope can fuel future work on: 1) changes in wing color beyond eyespot or band size, 2) shape of reaction norms of artificially selected lines, 3) characterization of correlated response to selection on wing pattern for other seasonally-varying traits.

MATERIAL AND METHODS

Artificial selection lines for wing pattern wet and dry-season phenotypes

We used a large outbred laboratory stock (WT) of *B. anynana* butterflies established from about 80 gravid females captured in Malawi (Brakefield *et al.* 2009). The lab population has been maintained at an adult population size of about 500 individuals/generation under controlled conditions. Larvae were reared on young maize plants sprayed with anti-fungic solution and adults fed on mashed banana.

We re-derived artificial DRY and WET selection lines by selecting individuals that were most similar to either the dry-season form or the wet-season form, respectively. These artificial lines were selected from a single large population of about 2000 individuals (G0), reared from the stock at 23°C (cf. Wijngaarden & Brakefield 2001). Initially, we were using two replicate selection lines in each direction. However, a microsporidia infection in our laboratory populations resulted in the loss of one line per direction resulting in no replication for the artificial selection.

For the first three generations, butterflies from both sexes were selected on the basis of the total diameter of the large fifth eyespot on the ventral hindwing relative to the distance between the second and fifth eyespot white centers (measurement highly correlated with overall wing size, e.g. Zijlstra *et al.* 2003), (Figure 5.1A) and on color patterns characteristic of the natural dry- and wet-season forms, respectively. Smallest eyespot butterflies were used as DRY parents, and largest eyespot butterflies were used as WET parents respectively. Measurements were made in Image-J software (Abramoff *et al.* 2004) using a digitizing tablet and a micrometer eyepiece in a binocular microscope at 10x magnification. To determine measurement error, repeatability was calculated by measuring 50 individuals (25 females and 25 males) three times randomly. Repeatability was calculated from Analysis of Variance (ANOVA) with the formula $r = S^2A/(S^2 + S^2A)$, where S^2A is the between group variance and S^2 is the within group variance (c.f. Falconer & Mackay 1996). Repeatability which ranges from 0 to 1 was of 0.96 *i.e.*, the measurement error is negligible. From a total of 600 G0 females, that survived from the initial population of 2000 individuals, the 222 that showed the wet-like most (large ventral eyespots, lighter background) and those with dry-like most (smaller eyespots and darker background) were measured, and from a total of 985 G0 males 424 were measured (each individual three times, final

measurement corresponds to the average). The 40 most extreme individuals of each sex and phenotype were selected to produce the next generation.

After G0, smaller populations of about 500 larvae per generation were reared at 23°C. From the resulting G1 individuals, 171 extreme females and 229 males were measured, and in G2 207 females and 224 males were measured. For each line, the 40 most extreme females and 40 most extreme males were allowed to mate to produce the next generation. After generation G3, upon obvious reduction of phenotypic variation within line, we started selecting by eye, targeting eyespot size and also background color, the 40 most extreme individuals of each sex.

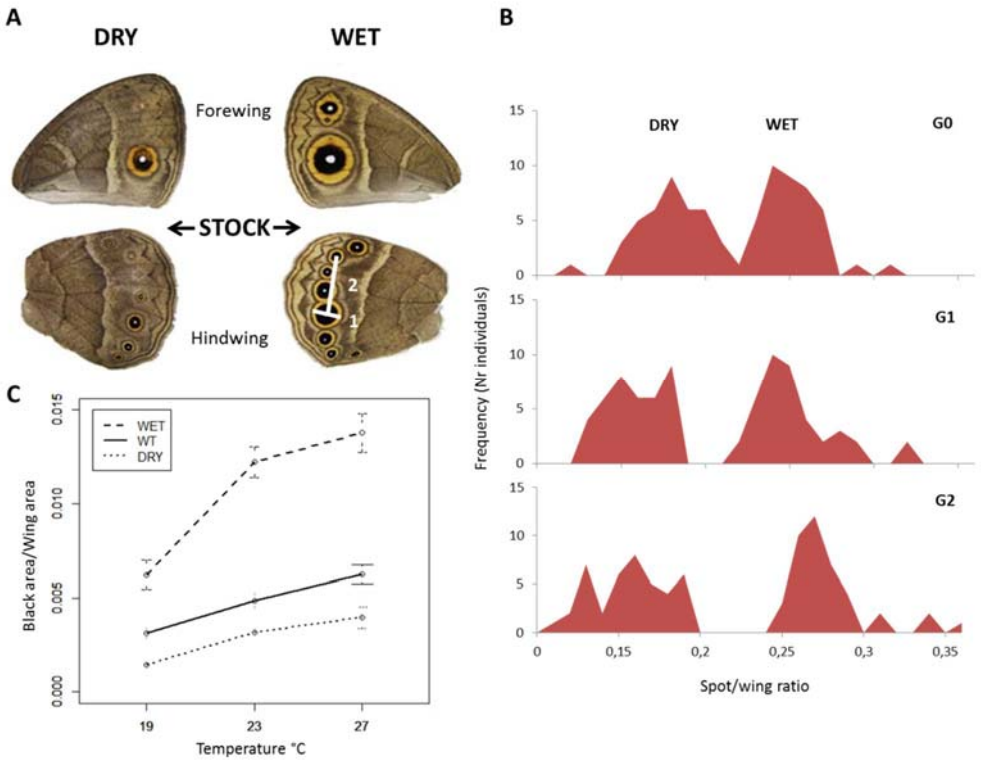


Figure 5.1 - Selection of DRY and WET lines. A) The photos correspond to representative female wings after 10 generations of selection (reared at 23˚C). **B)** Frequency distributions of the size of the fifth eyespot on the hindwing relative to the wing size for 40 selected female butterflies of the DRY and WET lines for the three first generations of artificial selection. After that we applied selection by eye targeting eyespot size and also background color. **C)** Reaction norms for the black area (corrected for total wing area) across developmental temperatures after 10 generations of artificial selection for eyespot size of female *B. anynana* (individuals reared at the same conditions as individuals of CHAPTER 4, however we did not measure the white center size not being possible to represent total eyespot size). This shows that after 10 generations (individuals reared at the same time as those from different pigmentation lines analyzed in CHAPTER 4) it was already possible to distinguish completely different phenotypes

across temperature. Error bars represent 95% of Confidence Interval (CI) for the mean and the sample sizes are 29-30 for WET line, 30-38 individuals for WT (unselected controls), and 29-30 for DRY line.

Thermal reaction norms

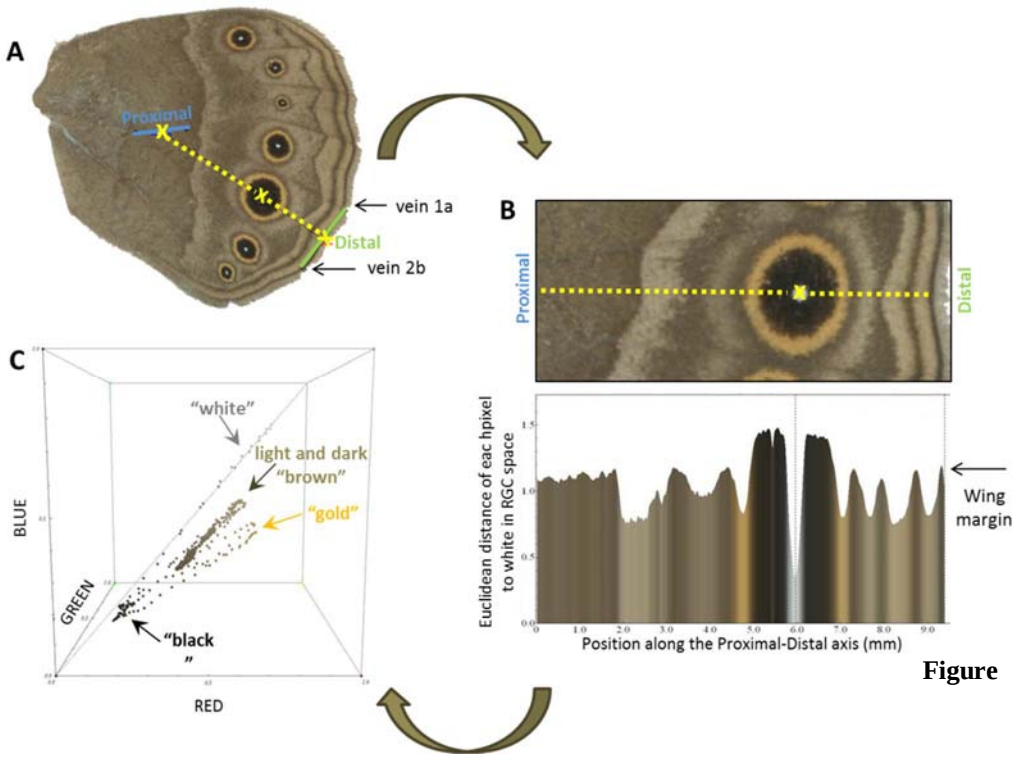
To characterize thermal reaction norms, we reared 120 first-instar larvae from the WT, DRY and WET lines at nine different temperatures and measured wing pattern in the resulting adults. For the artificially selected lines, we collected eggs after 19 generations of selection intensity at 23°C. First-instar larvae from each stock were transferred in batches of 40 individuals onto separate net sleeves, with two maize plants each. Sleeves with larvae were placed in climate-controlled chambers set at 15°C, 17°C, 19°C, 21°C, 23°C, 25°C, 27°C, 29°C or 31°C ± 0.5°C, with 65-70% relative humidity and 12:12hr light/dark cycle. The lowest and the highest temperatures are below and above, respectively, the temperatures typically used in the lab to induce the formation of dry-season (19°C) and wet-season (27°C) phenotypes and which are believed to natural conditions (Brakefield & Reitsma 1991). Sleeves were monitored each two days, plants were watered or replaced when necessary, and pre-pupae were collected and transferred to individual pots until adult eclosion. Pupation and eclosion days were recorded each two days. Adults were frozen 24h after eclosion and their wings were cut and stored in the freezer until analysis.

Phenotypic measurements

We developed a new method to obtain and process high quality images of wing pattern in a standard and semi-automated manner (F Alves and P Beldade, *manuscript in preparation*). With this method color- and light-calibrated image acquisition of flat adult wings are taken by using a high-resolution photographic scanner (Epson Perfection v600 Photo scanner), (pictures available in http://wingpatterns.igc.gulbenkian.pt). VueScan 9x32 9.3.18 software (Steinhoff 2011) was used for setting color-calibration (white point for Red=0.5, Blue=0.5, and Green=0.52; black point for Red, Blue, and Green=0; curve low=0.25, and high=0.75; brightness of 1; and TIF in 24RGB). Images are then processed and analyzed using custom-code in *Mathematica* software (Wolfram 1996).

The total size (diameter) of the fifth eyespot on the ventral surface of the right hindwing as well as the background color of each individual image are "sampled" by

drawing a transect through three visible landmarks: the eyespot center, intersections of veins 1a and 1b with the margin (Distal), and the cross-vein between 1a and 1b (Proximal), (Figure 5.2). We then extract images up to 11 pixels-high centered on the transect and obtained average color values (RGB scale) for each pixel along the transect. These color values are plotted on three-dimensional RGB color space to visualize different color "qualities" and to quantify pixels corresponding to different colors (density of points within defined RGB limits). To assess color pattern, we plotted the Euclidean distances between each of the transect´s pixels RGB to the white reference (1, 1, 1) in the RGB space (hereafter "distance to white"). Total transect size was used as a measurement to correct for overall wing size.



Figure

5.2 - Wing color analysis. A) Transect (yellow line), drawn through the fifth eyespot of a scanned image of a hindwing from a female reared at 27°C, defined by three wing landmarks (marked x), with the Proximal wing cell reference represented in blue and the Distal in green, respectively. **B)** Detail of the wing region including the transect defined in panel A (on the top) with the respective plot of the Euclidean distance between the color value of each of the transect's pixels to RGB-scale white (on the bottom). This type of graphical representation can be used to quantify different aspects of the wing pattern phenotypes. **C)** RGB values (average of 11 pixels from the transect's middle line) were visualized in the 3D RGB space allowing

distinguishing between color "groups": "white", "black", "gold" and "brown". Pixels plotted in respective color.

Statistical analyses

All data analyses were performed with R (R Development Core Team 2012). Eyespot size and pupal development time analyses were done separately for females and males because of sexual dimorphism in *B. anynana* wing size and life history traits (e.g. Zwaan *et al.* 2008, de Jong *et al.* 2010, Oostra *et al.* 2011). In all statistical models, we use genotype to refer to the different genetic backgrounds (DRY, WET and WT).

We analyzed eyespot total size, pupal development time and survival changes with temperature for each of the three genotypes. Before that, for eyespot size and pupal development time, parametric assumptions were considered by checking normality (Shapiro-Wilk test, alpha=0.05) and homoscedasticity (Fligner-Killeen test, alpha=0.05) of residuals, and transforming data when appropriate. When significant differences were found for the different factors in the overall models (ANOVA, alpha=0.05), we performed post-hoc comparisons between factor levels using Tukey´s honest significant differences (HSD) tests (alpha=0.01).

For eyespot size, we tested the model *eyespot size ~ transect size + genotype * temperature*, with transect size as covariate. Pupal development time was log transformed and we tested the general linear model *development time ~ genotype * temperature*. For both cases we used general linear models assuming a Gaussian distribution of the error, and with temperature (nine levels: 15°C, 17°C, 19°C, 21°C, 23°C, 25°C, 27°C, 29°C and 31°C) and genotype (three levels: DRY, WET and WT) as fixed effects. Samples sizes are not equal for both traits in general samples sizes are higher for wing pattern analysis because even when we missed pupation and/or eclosion days the individuals were still used for eyespot size measurement (see detailed sample sizes in Annex 5.1).

Survival differences were compared using the model *survival proportion ~ genotype * temperature * sex*. We used a general linear model assuming a weighted (total N) Binomial distribution of the error, and with temperature (nine levels), genotype (three levels), and sex (two levels) as fixed effects. Here, we used sex as fixed effect because we did not know from previous works (as in the case of eyespot size and development time) if there is sexual dimorphism for this life history trait.

PRELIMINARY RESULTS AND DISCUSSION

We collected phenotypic data from females and males of three different genetic lines (Figure 5.1) reared at nine temperatures. In our preliminary analyses, we show the thermal reaction norms for the total size of the fifth eyespot on the hindwing (Figure 5.3). We also show reaction norms for pupal development time (Figure 5.4) and survival rate (Figure 5.5). This analysis allowed us to determine effects of temperature (T), genetic background (G), and sex (for the survival analysis) and the interaction between them. Finally, we also illustrate differences in wing background color along the gradient of temperatures (Figure 5.6 and 5.7). We found significant effects of T, G and G x T interaction on eyespot width and pupal development time for both sexes. For survival rate, genotype appears as the main factor explaining the high rates of mortality, especially for extreme temperatures. Finally, we show wing background color changes across temperature and for extreme low temperature a different pigment color appears.

Artificial selection lines differ in height and shape (GxT) of thermal reaction norms for eyespot size

Our artificial selection at intermediate temperature produced differences in wing pattern across all temperatures leading, with the lines having well separated reaction norms (at G10 Figure 5.1C and G19 Figure 5.3). The WET line shows the highest and DRY line the lowest phenotype for all temperatures, respectively (reflected in reaction norms of different height) in agreement with what was expected after the artificial selection procedure on both phenotypic directions (Brakefield *et al.* 1996).

Reaction norms are an important tool to quantify the degree of phenotypic variance and magnitude of plasticity of morphometric and life-history traits (DeWitt *et al.* 1998, Karan *et al.* 1998, Pertoldi *et al.* 2014). By measuring thermal reaction norms of *B. anynana* unselected and selected DRY and WET lines across a range of temperatures we were able to assess the G, T and GxT effects on eyespot size variation. We could also assess possible correlated responses, to the artificial selection on wing pattern; notably, for pupal development time and survivorship. However, as we did not have replicate lines for each selection direction, the interpretation of these correlated responses should be taken as indicative rather than definitive. Significant G effect means that genetic backgrounds differ, significant T effect means that trait responses are thermally plastic, and significant GxT effects reflects differences between genetic stocks in thermal reaction norms.

Figure 5.3 and 5.4 underlies seasonal polyphenism in WT and selected lines showing the total size of the fifth eyespot on the hindwing across nine different temperatures, including intermediate and extreme values. We quantified these differences and we found that total eyespot size was significantly affected by temperature (females: F=238.49, df=8, P < 0.001; males: F=225.0730, df=8, P < 0.001), by genotype (females: F=898.43, df=2, P < 0.001; males: F=743.1025, df=2, P < 0.001), and by genotype x temperature (females: F=8.49, df=16, P < 0.001; males: F=8.2658, df=16, P < 0.001) for both sexes (see Annex 5.1).

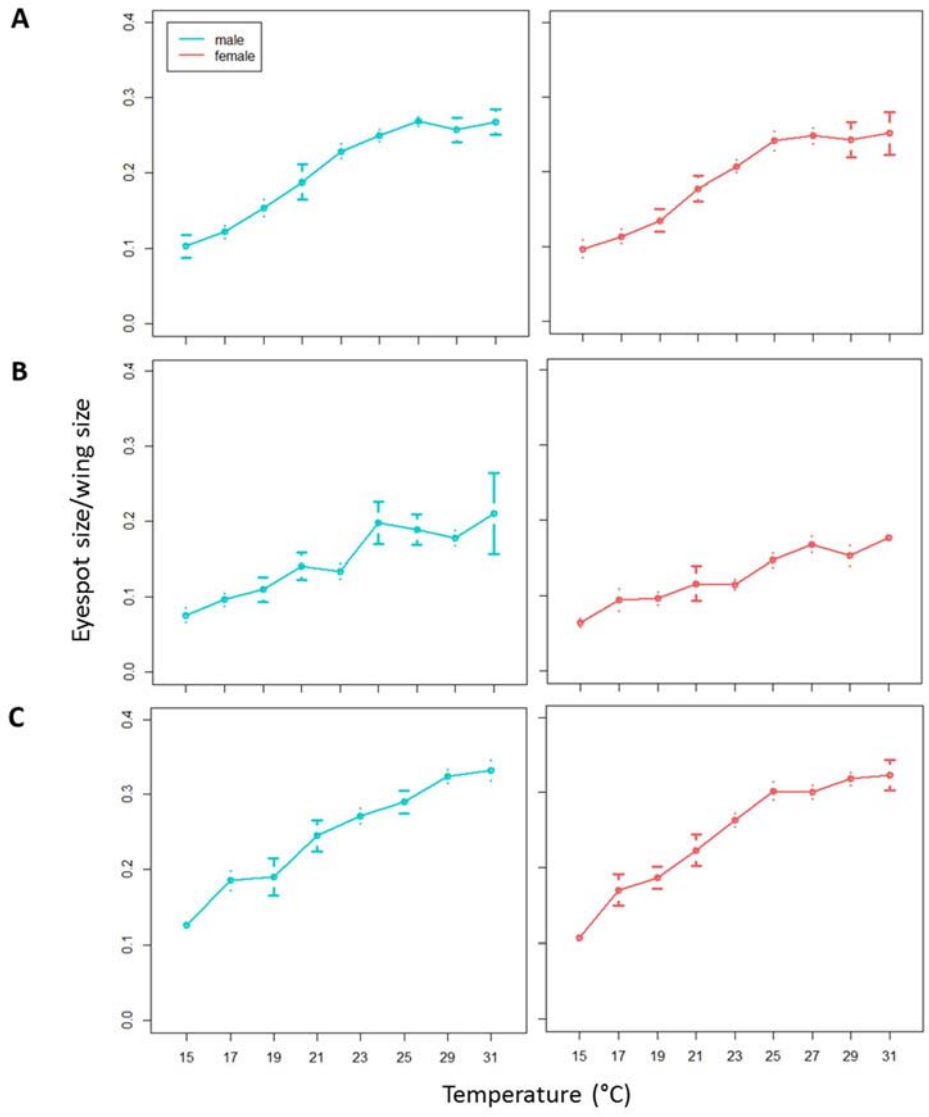


Figure 5.3 - Thermal reaction norms for eyespot size for unselected (WT-A) and artificial selected lines (DRY-B and WET-C). For the total width of the fifth eyespot on the hindwing (relative to corresponding wing size) of three genotypes (A ,B,C), we plotted the mean value as a function of temperature and use bars to represent the standard deviation. Females (right, pink)

and males (left, blue) are represented separately because of the already known sexual dimorphism in *B. anynana* wing size and patterns. We tested for the effect of temperature and genotype using the model *eyespot size ~ transect size + genotype * temperature* (see Material and Methods and Annex 5.1). When we found significant effects of temperature or/and genotype on trait value $P < 0.05$, we compared across temperatures (Tukey HSD, $P < 0.01$, see Annex 5.1 for sample sizes and statistical details). There is a significantly GxT interaction which confirms differences between lines in thermal reaction norms.

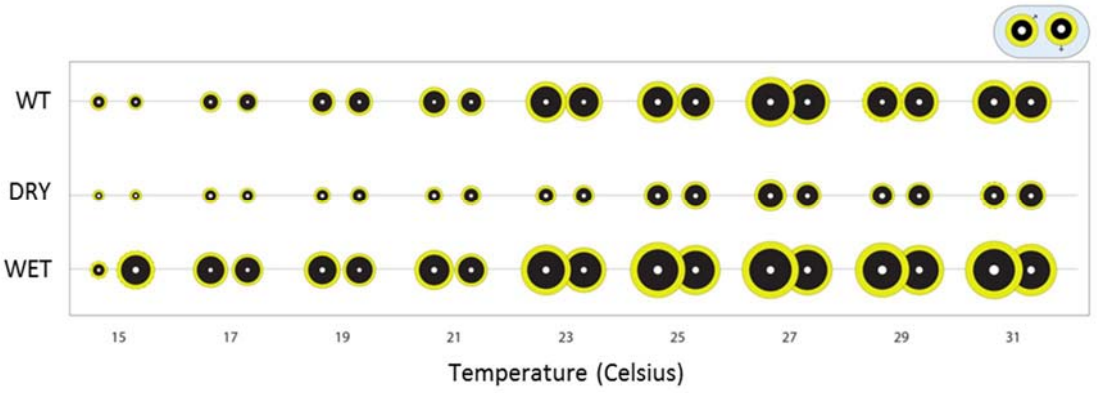


Figure 5.4 - Developmental plasticity for eyespot different ring sizes across temperature, for females and males, of the WT, DRY and WET genotypes. The figure depicts the central tendencies computed as the median of the (unimodal) distributions for the size of the different rings of the fifth eyespot on the hindwing: white, black and gold. For each temperature (15°C to 31°C), males are represented always by the left eyespot and females by the right eyespot (see legend on the right top corner of the figure). Eyespots represented by dashed lines correspond to small sample sizes (N<5 individuals). In general this quantitative approach shows that eyespot size increases with increasing developmental temperature, while DRY and WET lines have smaller and larger eyespots across temperature.

For both sexes, there was a significant interaction GxT which suggests variation for phenotypic plasticity between lines (Figure 5.3 and 5.4). In general, WET and WT lines seem to show higher levels of plasticity with most pronounced eyespot size differences between low and high temperatures relative to the DRY line (see difference between means in Tukey HSD results in Annex 5.1). These results are in agreement with previous works where lines selected for wet-like phenotype at intermediate temperature showed higher sensitivity to temperature in comparison with the line selected for dry-like phenotype (Brakefield *et al.* 1996). A previous study had described an artificial selected line of *B. anynana* that only produced wet season-like large eyespots across all temperatures (from 17°C to 27°C) but still did have larger eyespots at higher

temperatures, which means that phenotypic plasticity is still retained (Brakefield *et al.* 1996).

The existence of phenotypic plasticity demonstrates that the eyespot developmental pathway is under environmental control. The DRY and WET artificial selection lines show that plasticity can be changed trough selection (see also Brakefield *et al.* 1996). Wijngaarden & Brakefield 2000 demonstrated, through different combinations of crosses between genetically different selection lines, that those lines differed in 5 to 10 polymorphic genes that would have contributed to the evolution of these divergent phenotypes. The separable genetic and environmental effects on eyespot size development show that the unselected stock contains allelic variation for influencing eyespot size, and that selection could change those allele frequencies to produce genetically divergent lines. It is unclear how that allelic variation impacts hormone dynamics.

Development time showed correlated response to artificial selection on wing pattern

Because we know that pupal development time and wing pattern show strong genetic and phenotypic correlations, due to shared hormonal effects (Zijlstra *et al.* 2004, Oostra *et al.* 2011) we also explored thermal plasticity in pupal development time for our lines.

Figure 5.5 illustrates thermal plasticity in WT and selected lines for pupal development time. We found that pupal development time was significantly affected by temperature (females: $F=1282.2820$, $df=8$, $P < 2.2e-16$; males: $F=1193.2487$, $df=8$, $P < 2.2e-16$), by genotype (females: $F=227.1087$, $df=2$, $P < 2.2e-16$; males: $F=257.0595$, $df=2$, $P < 2.2e-16$), and by genotype x temperature (females: $F=2.6512$, $df=16$, $P < 0.000504$; males: $F=3.0861$, $df=16$, $P < 5.138e-05$) for both sexes (see Annex 5.1). Figure 5.5 shows that for all genotypes pupal development time decreases with increasing temperature, similarly for both sexes (as in Oostra *et al.* 2011). For total pupal development time, the DRY line shows the highest reaction norms (i.e. longer pupal development across temperature) in comparison with the WET line and the unselected stock that are similar (Figure 5.5). This means that artificial selection on ventral eyespot size at intermediate temperature lead to correlated responses in pupal development time.

Previous works demonstrated that pupal development time and wing pattern show strong genetic correlations due to shared hormonal underpinnings (Zijlstra *et al.* 2004, Oostra *et al.* 2011). However, it was also shown that there was substantial genetic

variation allowing antagonistic selection to uncouple the two traits (Zijlstra *et al.* 2003, 2004). It was suggested that response to selection on development time resulted from shifts in hormone dynamics, while response to selection on eyespot size resulted from later changes in developmental mechanisms of pattern determination (Zijlstra *et al.* 2004). It is unclear what the mechanisms are for our response to selection on eyespot size and correlated changes in development time.

Survivorship differs between lines at extreme temperatures

In Figure 5.6 we show survival rate for different genotypes at different temperatures. We found that survivorship was not significantly affected by temperature and sex (see results in Annex 5.1).

Figure 5.6 shows that at cooler temperatures (<19°C) the DRY line shows higher survival rate in comparison with the WET line, while at warmer temperatures (>27°C) the WET line shows higher survivorship in comparison to the DRY line (Figure 5.6, Annex 5.1). This is especially visible for extreme low and extreme warm temperatures. At 15°C, the DRY shows a noticeably higher proportion of survival in comparison with the WET line, and at 29°C the opposite can be seen. At 31°C mortality is very high for all genotypes (Figure 5.6). For the unselected stock mortality is lower in comparison with the artificial selected lines. However, above 27°C there is an accentuated mortality. *B. anynana* occurs across sub-Saharan Africa where different populations live in very different environments (Roskam & Brakefield 1999). The lab stock, derived from a population in Malawi and adapted to the lab for many generations, represents only some of the species ability to survive an extended temperature range. Also by obtaining different results between DRY and WET lines for survivorship for different temperatures means that we were successfully once more in getting indirect correlated responses for different traits to our artificial selection on wing pattern. The fact that DRY line shows lower mortality at lower temperatures means that by artificial selection on wing pattern we probably also affected genes related with development, specifically in this case with the sensitivity to temperature.

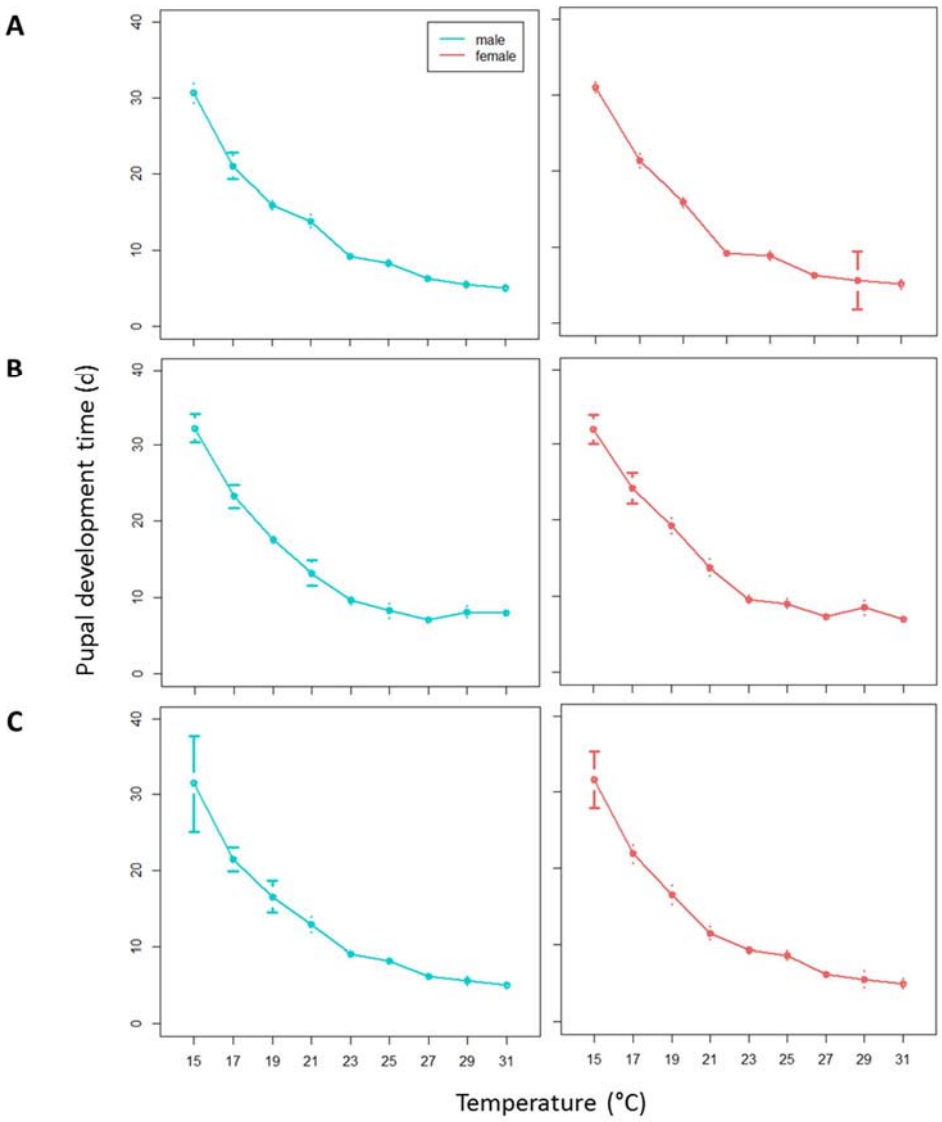


Figure 5.5 - Thermal reaction norms for pupal development time for unselected stock (WT-A) and artificial selected lines (DRY-B and WET-C). For pupal development time in days (d) we plotted the mean value as a function of temperature and used bars to represent standard deviation (SD) of WT (A), DRY (B) and WET (C) genotypes. Females (right, pink) and males (left, blue) are represented separately because of the already known sexual dimorphism in *B. anynana* development time. For each sex, we tested for the effect of temperature and genotype using the model *Days ~ temperature * genotype* (see Material and Methods and Annex 5.1). When we found significant effects of temperature or/and genotype on trait value $P < 0.05$, we compared across temperatures (Tukey HSD, $P < 0.01$, see Annex 5.1 for sample sizes and statistical details). Differences between genotypes are mainly due to DRY (B) line with longer pupal development relative to WET (C) and WT (A).

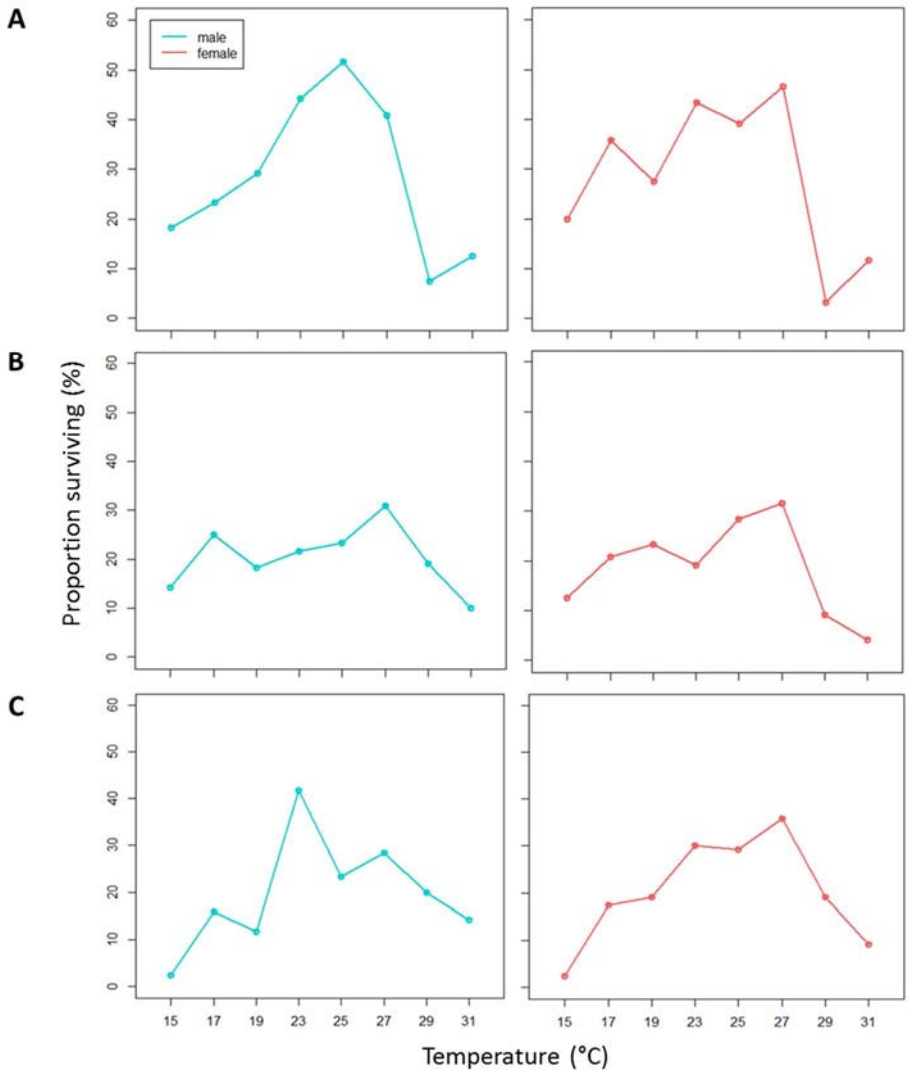


Figure 5.6 - Survival rates for unselected stock (A) and selected DRY (B) and WET (C) artificial selected lines across temperatures. We plotted proportion of survival (%) of 120 individuals (60 per sex) from each genotype, for each temperature (see detailed sample sizes of survival in Annex 5.1). Females (right, pink) and males (left, blue) are represented separately, however because we do not know if sex is a factor that influences survival we included it in the final model. We tested for the effect of temperature, genotype and sex on survival using an ANOVA with a Chi-square test and the model *SurvivalProportion ~ Genotype * Temperature * Sex* assuming a weighted Binomial distribution of the error (see Material and Methods and Annex 5.1). Statistical significance of each factor is represented on the left top corner of the plot with: ^{ns} (non-significant) P>0.05, *P<0.05, ** P < 0.01, *** P < 0.001 (see Annex 5.1 for more details on sample sizes and statistical analysis). We have higher levels of mortality at extreme temperatures especially for the artificially selected lines. We did not plot the survivorship for 21°C because all our lab stocks got a severe fungic infection with notable effects at this temperature, and we needed to use part of the individuals from the experiment to rescue the

stocks. Therefore, because the sample size at 21°C was largely reduced but not due to natural mortality, we decided to not present the results for this temperature.

B. anynana reactions norms show different shapes across an extended temperature range

We tested different genotypes at a large range of temperatures, including intermediate and extreme values. With our results we would like to see how intermediate and extreme points of the reaction norms respond in relation to the already "well known" points that are common in representations of reaction norms for this species (e.g. Brakefield *et al.* 1996, Wijngaarden *et al.* 2002, Zijlstra *et al.* 2004, de Jong *et al.* 2010, Oostra *et al.* 2011, Mateus *et al.* 2014).

In general, intermediate temperatures show less difference between genotypes and between the effects of environments than more extreme temperatures. These intermediate temperatures are considered as a zone of canalization with the range of environments that have been historically most common in the species (Lewontin 2006), but in new environments much greater variance between genotypes appears.

Eyespot size increases with temperature and it seems that it reaches a plateau at 27°C (Figure 5.3 and 5.4). We did not identify a lower limit plateau for lower temperatures (Figure 5.3 and 5.4). For pupal development time there are also fluctuations across temperatures, again with less pronounced response above 27°C in comparison with cooler temperatures (Figure 5.5). Finally for survivorship (Figure 5.6), despite the higher mortality at extreme low and high temperatures, all genotypes seem to show less resistance to warmer temperatures. Our results suggest *B. anynana* might not be well equiped to respond to higher temperatures, even thought much higher then our highest test temperatures being possible in natural populations (e.g. cf. Fischer *et al.* 2010 during solar radiation temperatures of 45°C are possible). While adults of this species might be able to cope with such higher temperatures, pre-adult stages might not.

Wing background color changes with temperature

Pigmentation is involved in intra- and interspecific communication (e.g. camouflage, mate recognition), structural protection (e.g. temperature and light), and chemical defense (Needham 1974). One of the best examples is butterfly wing patterns (Needham 1974, Nijhout 1991). In butterflies, wing scales show only a single pigment. These monochromatic cells are juxtaposed in parallel rows in a two-dimensional layer of the wing tissue and wing patterns are formed by colored scales arranged to produce pattern elements such as bands or concentric rings (Nijhout 1991, 2010, Koch & Kaufmann 1995). In order to explore differences in plasticity of wing color patterns we plotted the RGB values of *B. anynana* wings for the nine temperatures for both sexes and the three genotypes.

In Figure 5.7, it seems that not only eyespot size changes across temperature increasing with warmer temperatures, but also wing background color changes. In general, while for cool temperatures (15°C-21°C) we see mostly three groups of pigments: "white", "black" and "brown", for warm temperatures (23°C-31°C) we see the four pigment groups (Figure 5.2): "white", "black", "brown", and also the "gold" that was almost not present at lower temperatures. This "gold" pigment corresponds mainly to the large "gold" eyespot ring which almost does not exist at lower temperatures. It also seems there are differences for the group of the "brown" pigment. The "cloud" of "brown" is larger, darker, and with different intensities for low temperatures (15°C-21°C). Finally, it also seems there is heterogeneity between Proximal and Distal sides not only in terms of wing color background, that seems to show different color intensities, but also at the level of the eyespot color rings size. These results are similar for both sexes and for the three genotypes (Annex 5.2).

At lower temperatures, by eye, wings seem more heterogeneous in color, seen by the different intensities of "brown" pigments that cover most of the wing background, in comparison with what seems to be lighter and almost uniform color for the warm temperatures (Figure 5.7). These possible differences in color are probably related to the adaptive strategy of *B. anynana*. The adaptive benefit of the cryptic form in the dry season as response to the lower temperatures has been previously demonstrated (Lyytinen *et al.* 2004, Brakefield & Frankino 2007). In the dry season habitats, adult *B. anynana* butterflies typically express a cryptic wing pattern allowing them to rest undetected among the dried vegetation. In the wet season, vegetation is green and

abundant and the individuals instead express prominent concentric eyespots along the distal margin of their wings to protect the fragile body against the attacks of the predators (e.g. Brakefield & Frankino 2007, Oliver *et al.* 2009, Beldade *et al.* 2011). Adittionaly to the mechanism of defense, it was already shown for other species that according to the thermal budget hypothesis, darker phenotypes are observed in cooler environments to favor the absorption of the light radiations to increase the internal temperature, and light body color prevents overheating in warm environments (David *et al.* 1990, Capy *et al.* 1988, Goulson 1994, Gibert *et al.* 1996, 2000). Previous studies, based on the RGB analysis, also confirm that Dry season adults, both males and females, are generally darker than the wet season form (de Jong *et al.* 2010).

Our imaging of wing background color suggests that the artificial selection fot wet- and dry-season like phenotypes altered that phenotype too. As we can see in Annex 5.2 the DRY line seems to show, at low and high temperatures, a more heterogeneous wing color background in comparison with the WET line and the unselected WT stock. WET line for both temperatures seems to show lighter and more uniform wing color.

We also observed that the "white" eyespot centers are sometimes not really "white", appearing almost "yellow" (e.g. WT 15°C in Figure 5.7), or almost "brown" (e.g. DRY 17°C in Annex 5.2). We do not know the mechanism underlying this, but hypothesize that this different color at the eyespot centers might result from some scales of different color being mixed with the colorless scales. For example, "gold" scales under the "white" scales can make the eyespot center look almost "yellow" rather than "white". The density of cells in the eyespot center might also be different in animals developed at different temperatures being low at cooler temperatures. That being the case could mean that the wing background (non-"white" scales) is more visible and affects "white". In order to confirm either of our hypotheses we should analyse eyespot centers under very high magnification to analize individual scale color and density.

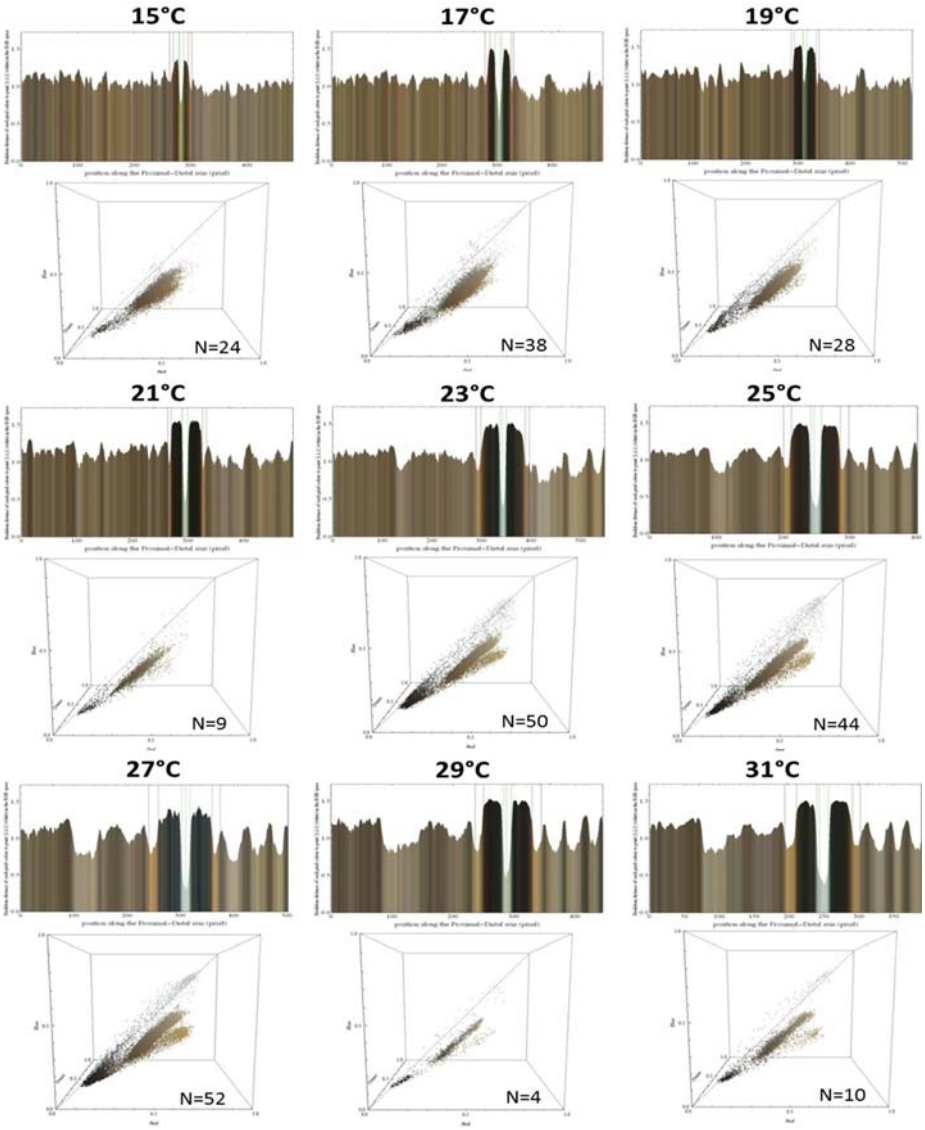


Figure 5.7 - Wing background color changes across temperature. For females of each temperature, we show two different plots representing the pixels along a particular transect of the fifth wing cell of the hindwing. The top plots show distances to white with each pixel along the wing transect. In the xx axis we have "Position along the Proximal-Distal axis (mm)", and in the yy axis we have "Euclidean distance of each pixel to white in RGB space" (axis cf. Figure 5.2C). Each plot represents the typical transect for that temperature. The bottom plots represent the 3D RGB space visualization of the averaged RGB values that allow distinguishing between different wing background pigment groups. Each plot represents the RGB values (see Figure 5.2 to detail on name of axis) of all individuals for that temperature (total N inside each 3D cube). We chose to represent females because wing background is lighter allowing a better visualization of the pigments involved. For higher temperatures samples sizes are very small, in particular at 29°C, due to the low survival observed for these temperatures (see Figure 5.6).

Distal and Proximal wing sides show asymmetry and a novel color seems to appear

Our color analysis also shows clear differences between Distal and Proximal sides of the wing. This goes beyond the light band found only proximally and on the marginal chevrons found distally (for more detail see Figure 5.2). Proximal-distal color asymmetry is more visible for higher than lower temperatures (Figure 5.7). We can clearly see also asymmetry in eyespot ring width between proximal and distal half of the eyespot sides. In fact the rings on the Proximal side seems to be thinner that the ones from the Distal side. This is more obvious for the "gold" ring, but it can also happen for the "black" ring, and it is especially visible at lower temperatures when sometimes the "gold" ring for the Proximal side is almost inexistent (e.g. 19°C in Figure 5.7). We do not know the reason for this asymmetry, but suggest it might be due to the wing developing tissue process. During wing development the distal wing side expands more in surface in comparison with the Proximal side (Nijhout 1991). This could originate that the size of the eyespot rings also follows this process and enlarge also asymmetrically.

Finally, in Figure 5.8, we see the appearance of color pixels of a possibly new color, somewhat more distant from the "brown" pigments group, at the extreme low temperatures mainly for DRY individuals from the line.

For very low developmental temperatures, adult wings seem to display what is possibly a new "orange" color, between the "brown" and "gold" pigment groups, (Figure 5.8). This happens mostly for DRY line (Figure 5.8). During selection for dry-season appearance at intermediate temperature (23°C), we seem to have favored alleles that can now produce a different pigment when at lower temperature. In the forewing this color appears mainly in the Distal part of the wing, next to the margin, and in the hindwing it appears mainly next to the almost inexistent white band (Figure 5.8). We do not know if the orange color corresponds to a pigment from a different type (e.g. ommochromes can be yellow, orange, red), or to a modification of a product of the melanin biosynthesis pathway. We also do not know if there could be any adaptive value for the appearance of this extra color at low temperatures.

In order to explore if this orange color appears in related species that live in the same seasonal environments, we compared this color with that found in other *Bicyclus* and *Heteropsis* species (Annex 5.3). *B. campina*, *B. condamini,* and *Heteropsis perspicua* captured in the wild at their natural temperature, seem to, indeed, display a

similar color (see orange arrows in Figure 5.8 and Annex 5.3). Unfortunately we just had access to one individual from each species and with almost no information about the temperature that they grew in the field and their seasonal form. It would be interesting to analyze color in more individuals of more species, including both seasonal forms.

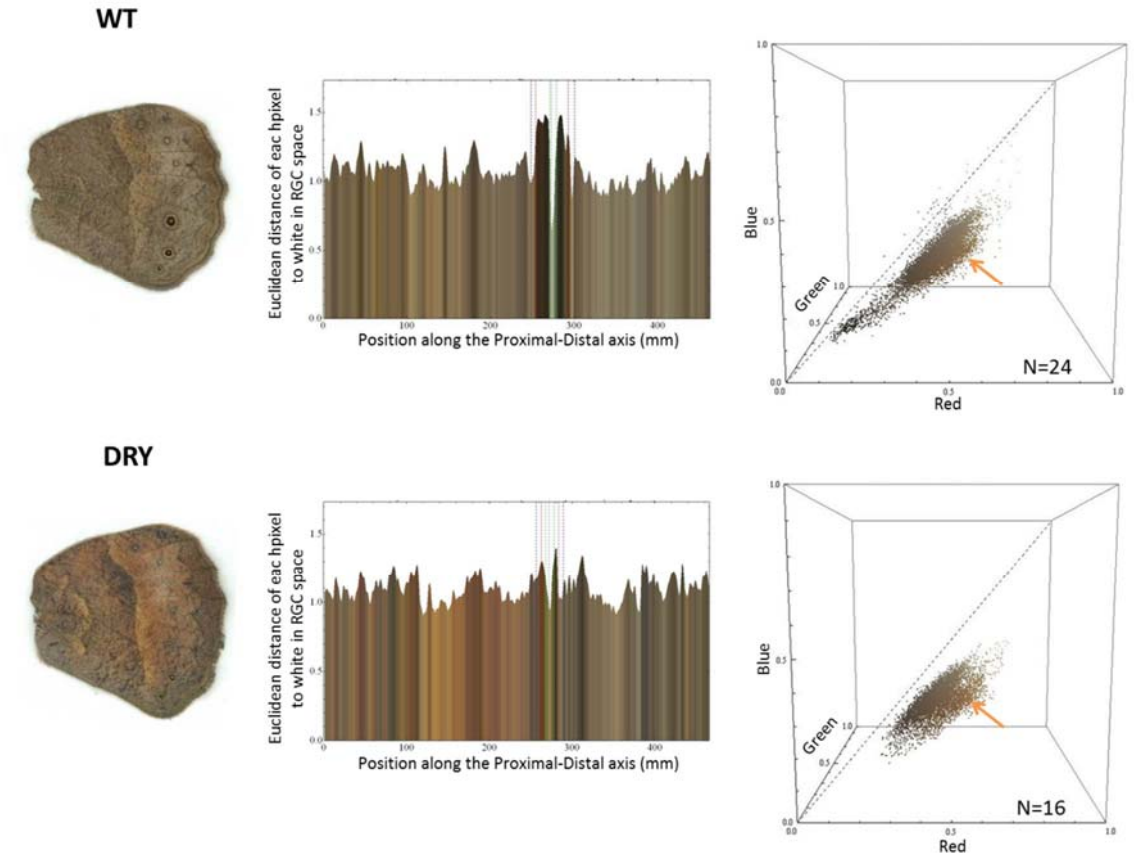


Figure 5.8 - Effect of extreme low temperature in wing background color of different genotypes. In this figure we show differences between genotypes in background color at 15°C. On the top we show the results for the unselected WT stock and on the bottom for the DRY artificial line. From the left to right in the figure we have: the adult hindwing of one individual female that represents the corresponding typical phenotype at 15°C, the plot for that individual showing the distance to white for each pixel along the wing transect (cf. Figure 5.2), and the 3D RGB space visualization of the pixels on the transect for various females together (total N inside each 3D cube). Orange arrows point to orange pixels which we suggest might correspond to different color pigment. Results are similar for both sexes, however we chose to represent females because wing background is lighter allowing a better visualization of the pigments involved (e.g. de Jong *et al.* 2010).

CONCLUSIONS

Even though linear reaction norms are the simplest way to represent graphically phenotypic plasticity, more complex shapes could arise and are also expected to evolve under specific environmental conditions (Gavrilets & Scheiner 1993a, de Jong 1999).

Temperature is of special importance during development of ectotherms because it can pose substantial challenges for survival and development. Thermal plasticity can offer quick and effective ways to cope with environmental fluctuations and even perturbations such as climate change (Chevin *et al.* 2013). Here, we characterized thermal reaction norms in a *B. anynana* wildtype genotype as well as two genotypes artificially selecteded for expression of DRY- or WET-season like wing patterns at intermediate temperature. We used a range of temperature including intermediary values between those typically used to study plasticity in this species (to better assess reaction norm shape), as well as beyond those (to explore extremes). We followed thermal plasticity for an indicative eyespot, pupal development time, and survivorship as well as for wing background color. This could inform about the nature of GxT effects (comparing reaction norms shapes) and allow us to investigate possible novel/extreme phenotypes and increased range of phenotypic variation that might result from exposure of cryptic genetic variation.

Our preliminary analysis show that artificial selection lines for wing patterns at intermediate temperatures resulted in genotypes with different reaction norms, height and possibly also shape. We see evidence of significant GxT effects (Figure 5.3). For both sexes, response to selection seems to have been most extreme for the WET direction. We see that WET reaction norm is heighest and DRY reaction norm is lowest and flattest in comparison with the WT (Figure 5.3). Previous studies targeting *B. anynana* eyespot plasticity were able to change reaction norms height but not shape (Brakefield *et al.* 1996, Wijngaarden & Brakefield 2001).

We show that our artificial selection on wing pattern also could be indicative of differencesin other traits such as pupal development time and survivorship. These correlated responses to selection could possibly reflect genetic pleiotropy. For all genotypes pupal development time decreases with increasing developmental temperature similarly for both sexes (Figure 5.5); in agreement with previous work (e.g. Zijlstra *et al.* 2004, Oostra *et al.* 2011). For both sexes, both temperature and genotype factors had significantly affected development time. The significantly GxT interaction

effect on development time indicates differences between the unselected stock and artificial selected lines in how they respond to temperature. We see that the DRY line shows the highest reaction norms (i.e. longer pupal development across temperature) in comparison with the WET line and the unselected stock that are similar (Figure 5.5). For survivorship DRY, WET and WT genotypes show differences in survival that depend on temperature, with higher levels of mortality at extreme temperatures especially for the artificial selected lines at warmer temperatures (Figure 5.6). Because we had a microspordian infection in our laboratory populations, we only had one replicate line of each selection direction. Therefore, all the correlated responses between the wing pattern (target of our selection) and life-history traits should be interpreted very carefully, and seen as possibilities to explore rather than definitive.

For wing background pigmentation, our results show for low temperatures three groups of pigments and for high temperatures four well distinct groups, with "gold" pigment detected pnly for the latter. There also seems to be a difference in the group of "brown" pigment that is darker and with different tones for lower temperatures. Finally, we found differences between Proximal and Distal sides not only in terms of wing color background but also at the width of eyespot color rings (Figure 5.7). Our analysis also revealed what is possibly a new color appearing at the most extreme low temperatures and mainly for DRY artificial line (Figure 5.8). We do not know what causes these differences, but suggested that the orange color corresponds to a pigment from a different type (e.g. ommochromes can be yellow, orange, red), or to a modification of a product of the melanin biosynthesis pathway.

The results present here are still from preliminary analyses and future work needs to be done in order to explore the data in more detail. This will include: 1) quantitative analysis of plasticity in background color, 2) formal mathematical treatment of the influence of external environment on development to characterize shape of thermal reaction norms, 3) mechanisms underlying the plasticity we document, and 4) extend analysis to other species.

ACKNOWLEDGEMENTS

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ANNEX 5.1

Summary of the statistical results for survival rate to test the effect of temperature, genotype and sex (S), for the total size of the fifth eyespot on the hindwing, corrected for wing size (WA), and pupal development time to test the effect of temperature (T) and genotype (G) for females and males (c.f. Figures 5.3, 5.4, 5.5 and 5.6). Statistical significance for the effects of T, G, S and their interactions is indicated as: *P < 0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of each factor on trait value P < 0.05, we compared across temperatures (Tukey HDS, P < 0.01).

SURVIVAL ANALYSIS

Model: SurvivalProportion~Genotype*Temperature*Sex, weight=totalN

SAMPLE SIZE[a]										
	Females			**Males**				Df	Dev	Pr(>Chi)
	DRY	WET	WT	DRY	WET	WT				
15°C	15	3	24	17	3	22	**G**	1	54.1	1.78e-12 ***
17°C	25	21	43	30	19	28	**T**	2	0.04	0.82
19°C	28	23	33	22	14	35	**S**	1	0.1	0.75
23°C	23	36	52	26	50	53	**G:T**	2	25.7	2.62e-06 ***
25°C	34	35	47	28	28	62	**S:T**	1	2.62	0.1
27°C	38	43	56	37	34	49	**G:S**	2	1.07	0.58
29°C	11	23	4	23	24	9	**G:S:T**	2	0.13	0.93
31°C	5	11	14	12	17	15				

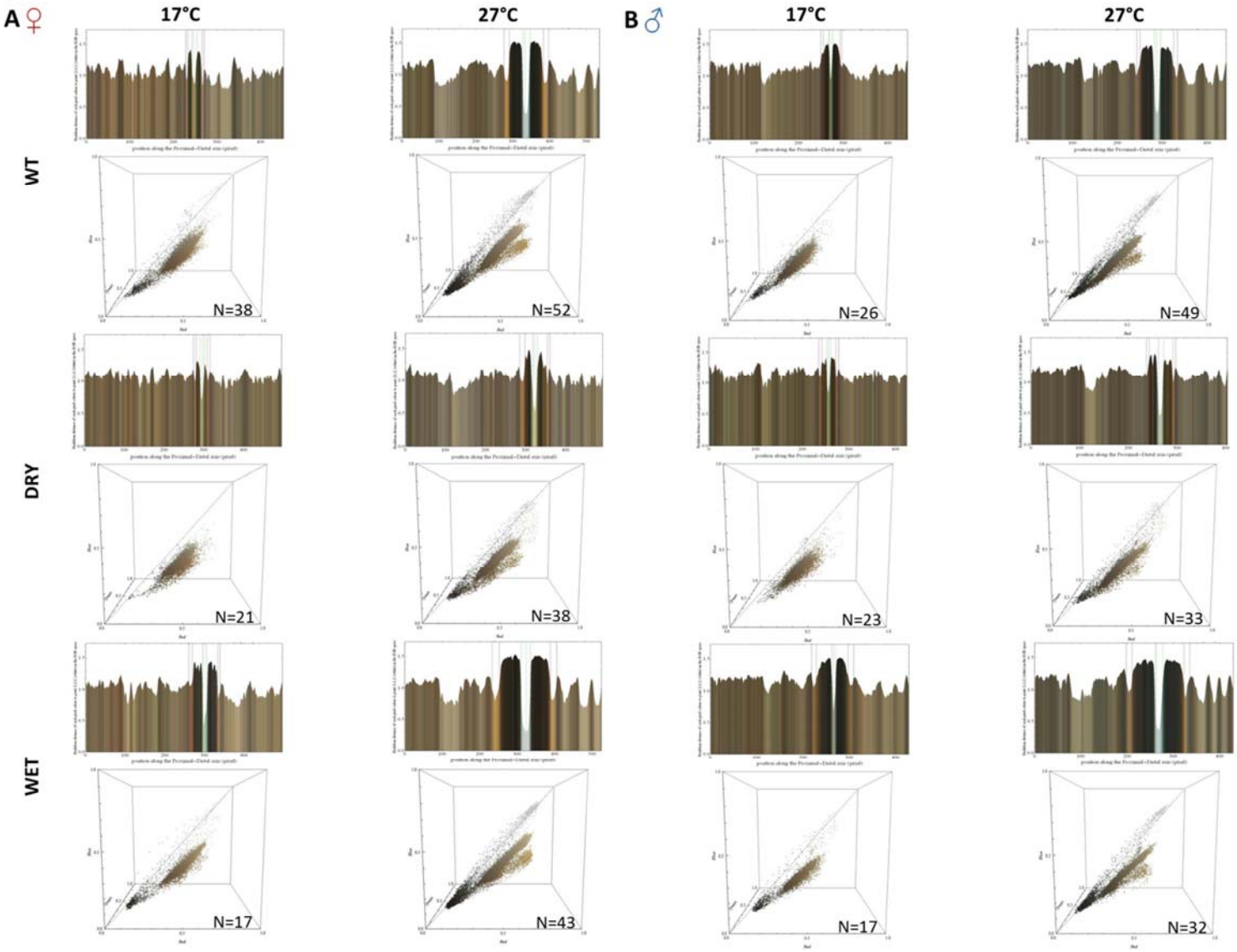
[a] Sample size at 21°C was largely reduced not due to natural mortality, but because we used the individuals to rescue the lab stock. Therefore, we decided to not present the results for survivorship at this temperature.

		EYESPOT SIZE Model: EyespotSize~TransectSize+Genotype*Temperature						DEVELOPMENT TIME Model: log(Days)~Genotype*Temperature					
		A-FEMALES			B-MALES			A-FEMALES			B-MALES		
	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	9.79	135.01	<2.2e-16 ***	1.6	31.11	<3.7e-08 ***	-	-	-	-	-	-
G	2	195.54	898.43	<2.2e-16 ***	114.6	743.1	<2.2e-16 ***	3.73	192.5	<2.2e-16 ***	2.00	98.96	<2.2e-16 ***
T	8	138.42	238.49	<2.2e-16 ***	92.6	225.07	<2.2e-16 ***	1.87	144.1	<2.2e-16 ***	1.43	106.3	<2.2e-16 ***
G:T	16	9.24	8.49	<2.2e-16 ***	6.37	8.26	<2.2e-16 ***	1.04	26.9	<2.2e-16 ***	0.28	7.10	4.7e-07 ***

HSD	EYESPOT SIZE						DEVELOPMENT TIME					
	A-FEMALES			B-MALES			A-FEMALES			B-MALES		
	Sample size (N)	Means	Groups	Sample size (N)	Means	Groups	Sample size (N)	Means	Groups	Sample size (N)	Means	Groups
DRY_15	16	0.635	h	13	0.664	h	15	3.458	a	17	3.468	a
DRY_17	21	0.934	h	23	0.918	gh	14	3.178	bc	12	3.142	bc
DRY_19	24	0.959	h	19	0.979	gh	19	2.953	de	15	2.863	de
DRY_21	18	0.919	h	6	1.076	gh	10	2.620	fg	5	2.576	fg
DRY_23	22	1.101	h	20	1.118	fg	22	2.260	hi	15	2.264	h
DRY_25	28	1.414	gh	22	1.473	ef	20	2.192	ij	26	2.096	ij
DRY_27	38	1.718	fg	33	1.474	ef	38	1.991	k	36	1.962	jk
DRY_29	10	1.417	gh	22	1.395	ef	11	1.793	l	15	1.822	kl
DRY_31	1	1.485	fgh	6	1.522	ef	2	1.946	kl	3	2.079	ijk
WET_15	1	0.948	h	2	2.055	bcde	3	3.454	ab	2	3.450	ab
WET_17	17	1.758	efg	17	1.668	e	11	3.084	cd	8	3.065	bcd
WET_19	22	1.909	def	13	1.734	de	11	2.800	ef	10	2.795	ef
WET_21	17	2.113	cde	20	1.815	de	9	2.442	gh	12	2.558	g
WET_23	34	2.684	ab	43	2.385	b	30	2.228	ij	45	2.202	hi
WET_25	29	2.951	a	24	2.652	a	32	2.111	j	27	2.100	i
WET_27	43	3.054	a	32	2.622	a	43	1.826	l	33	1.831	kl
WET_29	21	2.945	a	19	2.651	a	5	1.713	l	17	1.714	lm
WET_31	6	3.067	a	12	2.709	a	8	1.599	l	17	1.611	m
WT_15	24	0.929	h	22	0.887	gh	24	3.434	ab	22	3.419	ab
WT_17	38	1.147	h	26	1.084	g	17	3.061	cd	9	3.045	cd
WT_19	28	1.423	gh	31	1.402	ef	30	2.765	f	29	2.766	ef
WT_21	9	1.583	fg	12	1.448	ef	7	2.633	fg	19	2.624	fg
WT_23	50	2.161	cd	52	2.037	cde	51	2.223	ij	53	2.217	h
WT_25	44	2.225	c	58	1.944	cde	27	2.132	ij	33	2.113	hi
WT_27	52	2.673	ab	49	2.342	b	56	1.841	l	48	1.837	kl
WT_29	4	2.094	cdef	9	2.067	bcd	3	1.708	l	9	1.711	lm
WT_31	10	2.395	bc	11	2.169	bc	13	1.622	l	14	1.611	m

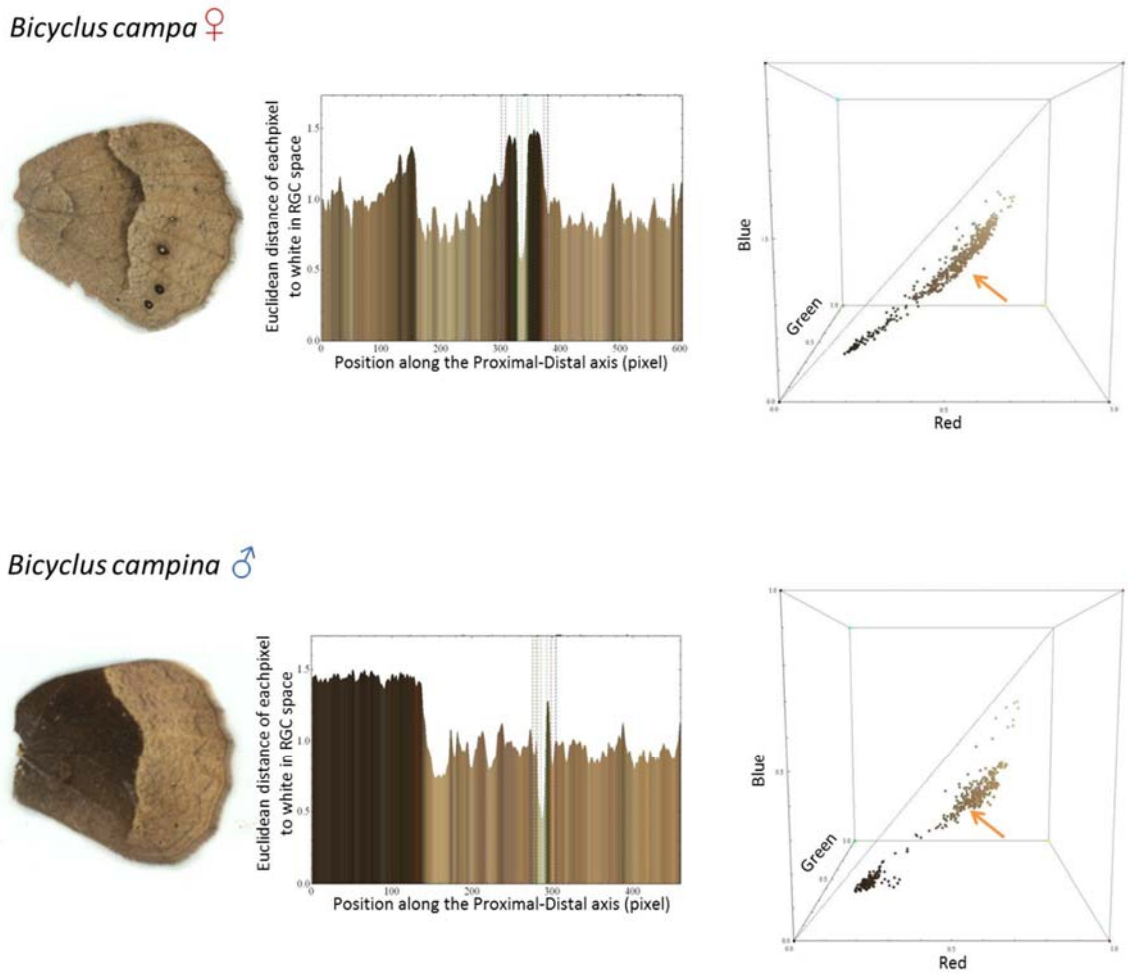
ANNEX 5.2

Wing background color at 17°C and 27°C for the DRY, WET and WT genotypes for females (A) and males (B). We chose these two temperatures of 17°C to represent low and 27°C to represent high, because these were temperatures with large sample sizes. When sample sizes are small some of the pigment groups (see Figure 5.2C) are difficult to distinguish. For each temperature, we show two different plots to characterize the transect through a hindwing (cf. Figure 5.2B): 1) distances to white of each pixel is color along the transect of one typical individual for that temperature, 2) 3D RGB space visualization of the RGB values of all pixels on transect of various individuals (sample size in each 3D) that allow distinguishing between different pigment groups.

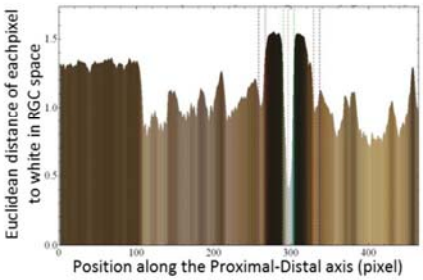


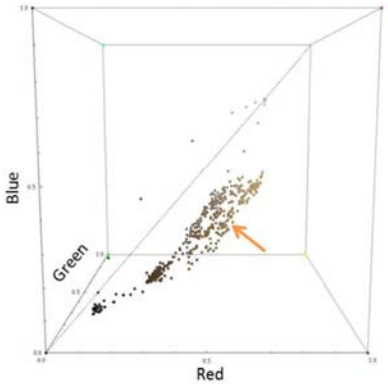
ANNEX 5.3

Wing background color for four different species of *Bicyclus* and *Heteropsis perspicua*. From the left to right in the figure we have: the adult hindwing of one individual that represents the typical phenotype, the plot for that individual that shows distances to white with each pixel along the wing transect illustrated by the average RGB value of that pixel, and the 3D RGB space visualization of the averaged RGB values that allow distinguishing between different wing background pigment groups for all individuals. Orange arrows point to the correspondent position where we find the orange pigment for *B. anynana* found at this temperature (Figure 5.8). For each species we just measured one individual that represents each characteristic phenotype, as it is very difficult to capture these species in the field. We used females and males because for some species females were not available.

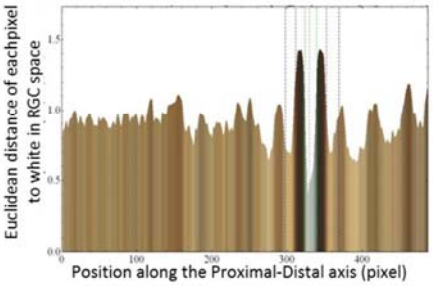


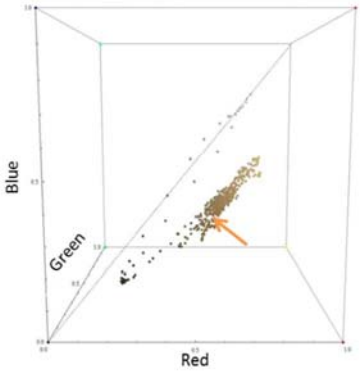
Bicyclus condamini ♂



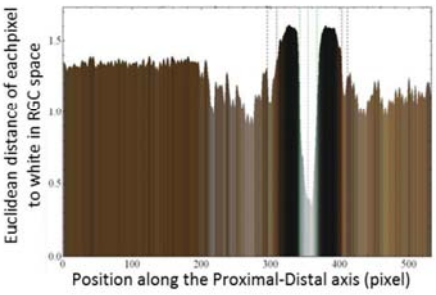


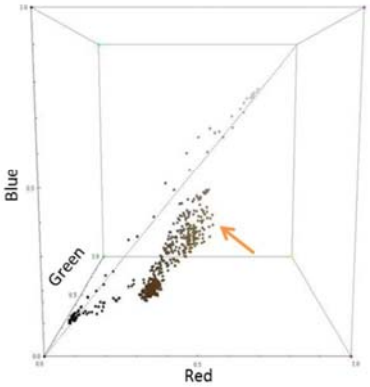
Heteropsis perspicua ♂





Bicyclus smithi ♀





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