

Seasonal phenotypic plasticity and potential trade-offs in wing melanization and adult size in the green-veined white butterfly (*Pieris napi*)

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ABSTRACT

The green-veined white butterfly, *Pieris napi*, is a widely distributed generalist species that thrives in a variety of environments with high seasonality. Seasonal polyphenism of wing melanization in other Pieridae has been suggested to assist in seasonal thermoregulation, but little research has been dedicated to phenotypic plasticity in *P. napi* despite its relative success in areas where other butterfly species have experienced considerable declines due to climate change and human activities. Additionally, very few studies have explored the seasonal plasticity in adult size, the potential role that size may play in butterfly thermoregulation, and whether resource allocation trade-offs occur in environmental conditions that favor the development of certain thermoregulatory traits. This study uses field data to determine whether adult size and/or wing melanization show signs of seasonal phenotypic plasticity and are potentially adaptive to thermoregulation in *Pieris napi* while also addressing whether resource allocation trade-offs may occur in the development of these two traits. Results suggest that seasonal plasticity does occur in size and wing melanization, with adult butterflies in the late summer exhibiting smaller sizes and less melanization compared to early spring adult butterflies. While results indicate that these traits may indeed play a role in thermoregulation, no resource allocation trade-offs were found for size and melanization.

INTRODUCTION

Ectotherm thermoregulation

The physiological processes of ectothermic organisms are strongly affected by spatiotemporal variation in the thermal environment. In this era of global climate change, much research has been dedicated to understanding how ectotherms maintain the optimal internal range of temperatures necessary to perform many physiological processes (Huey & Kingsolver 1989, Dzialowski & O'Connor 2001, Glanville & Seebacher 2006). For ectotherms, reproductive success is strongly temperature dependent (Huey & Berrigan 2001, Savage *et al.* 2004, Martin & Huey 2008); therefore, ectotherms have evolved a variety of mechanisms for thermoregulation—the regulation of internal body temperature within certain optima in spite of fluctuations in the external thermal environment (Hertz *et al.* 1993, Huey *et al.* 2003). Thermoregulation is often achieved by positioning the body in ways that either maximize or minimize the effects of external environmental factors such as solar radiation or wind on body temperature (Stevenson 1985, Kemp & Krockenberger 2002). Thermoregulation in ectotherms may also be influenced by body size; many ectotherms exhibit trends in body size that follow Bergmann's rule¹ (Lindsey 1966, Cruz *et al.* 2005, Tárraga *et al.* 2006, but see Mousseau 1997, Ashton & Feldman 2003)—cooler climates have populations or species with larger body sizes compared to those of warmer climates (Bergmann 1847, Blanckenhorn & Demont 2004). Ectotherm body size is highly dependent on external temperature during the juvenile stages: according to the temperature-size rule, decreasing rearing temperatures leads to slower growth rates but a larger final body size (Diamond & Kingsolver 2010). Additional physiological and morphological adaptations may assist in heat transfer, especially the use of specific color patterns to reflect or absorb solar radiation. For example, laboratory experiments on several ectotherm vertebrates and invertebrates suggest that highly melanized individuals often reach higher internal equilibrium temperatures at a faster rate compared to lighter individuals (Clusella-Trullas *et al.* 2009, Karl *et al.* 2009, Van Rensburg *et al.* 2009). This adaptation, known as thermal melanization, has been shown to lead to fitness advantages for melanic morphs in cold climates (Kingsolver 1987, Solensky & Larkin 2003).

Thermoregulation in butterflies

Butterflies are model organisms by which to explore the evolution of thermoregulatory morphological and behavioral adaptations (Clench 1966, Kingsolver 1985a, Srygley 1994). As heliothermic flying insects, butterflies must absorb heat from solar radiation to achieve sufficiently high body temperature for flight muscles in the

¹ Bergmann's rule initially suggested that, for closely related species within a genus distributed over a broad geographical area, species found in colder environments would be larger than species found in warmer environments (Bergmann 1847). This rule was later extended to address intraspecific variation in body size (Mayr 1956, 1963). Bergmann's rule has received broad support in its applications to endotherms, specifically mammals (Ashton *et al.* 2000) and birds (Ashton 2002), but has been highly debated in ectotherms for many years (Mousseau 1997).

thorax to function (Kingsolver 1983, Dreisig 1995, Van Dyck & Matthysen 1998). The heating and cooling rates of butterflies are often dependent on the interaction between microclimate, wing melanization, solar basking posture/method, and body size (Watt 1968, Kingsolver 1985a, Heinrich 1986). Butterflies select shaded microhabitats to decrease their body temperature and open habitats to increase their body temperature (Kemp & Krockenberger 2002, Kleckova *et al.* 2014). As ectotherms, butterflies typically exhibit body sizes that follow the temperature-size rule, i.e. negative thermal reaction norm in which final body size is larger in cooler temperatures (Atkinson 1994, Diamond & Kingsolver 2010).² Smaller butterflies generally heat up and cool down faster than larger butterflies because larger objects have greater thermal inertia (Heinrich 1993, Gilchrist 1990). This suggests that while larger butterflies heat up more slowly than smaller butterflies, larger butterflies lose heat less quickly than smaller butterflies and may maintain optimal body temperatures for longer periods of time. Additionally, butterflies can orient their wings in several ways to maximize heat absorption at the basal wing regions closest to the thorax while minimizing heat loss through convection: open wing dorsal basking orients dorsal wing surfaces perpendicular to the sun and closed wing lateral basking orients ventral wing surfaces to the sun (Wasserthal 1975). A third method of basking, reflectance basking, reflects solar radiation from the dorsal wing surface directly onto the thorax or to other parts of the wing for direct heat absorption (Kingsolver 1985b, but see Heinrich 1990). To reduce body temperature, butterflies may also use their wings to shield their body from excess solar radiation (Watt 1968, Rawlins 1980). Wing melanization plays an especially important role in the success of these thermoregulatory techniques (Shreeve 1992, Heinrich 1993). In cool environments, melanized wings can increase heat absorption capacity and decrease heat reflection when basking (Watt 1968, Ottenheim *et al.* 1999, Davis *et al.* 2012), while in hot environments, lighter, less melanized wings can reduce overheating (Ottenheim *et al.* 1999, Davis *et al.* 2005). Most importantly, these morphological and behavior adaptations necessary for successful thermoregulation are highly dependent on the surrounding thermal environment—while large, highly melanized butterflies with long periods of basking may be maintain optimal body temperatures in cooler environments with less solar radiation, small, less melanized butterflies with short basking periods may be more successful in hot environments with high solar radiation.

Phenotypic plasticity of melanization

Optimal thermoregulatory morphology and physiology may change depending on the specific thermal environment (Kingsolver & Watt 1983, Kemp & Krockenberger 2004). Perpetual spatiotemporal variation in the thermal environment has been argued to explain much inter- and intraspecific diversity (Kingsolver 1995). This diversity, including the diversity in melanized and nonmelanized morphs, can be either determined genetically (Kingsolver & Wiernasz 1991, Ellers & Boggs 2002) or through phenotypic

² But there have been exceptions to the temperature-size rule in Lepidoptera, with species showing positive thermal reaction norms for final body size (i.e. a positive relationship between the phenotypic trait value, size, and the environmental variable, temperature) (Atkinson 1994, Kingsolver *et al.* 2007).

plasticity³ (Lewis 1985, Lindstedt *et al.* 2009). A genetic polymorphism for melanization may occur if there is environmental heterogeneity along a large-scale geographical gradient—for example, if the conditions of two areas favor different amounts of melanization, and the distance between these two areas are beyond the dispersal range of the species, each population will experience one set of conditions and may evolve a fixed local adaptation (Banta *et al.* 2007, Michie *et al.* 2010, Parkash *et al.* 2011). On the other hand, phenotypic plasticity will be more advantageous in environments that vary temporally, especially areas with predictable seasonal changes (Parkash *et al.* 2011, Via *et al.* 1995). Variation in wing melanization in several butterfly species has been interpreted as adaptive seasonal phenotypic plasticity (Van Dyck & Wiklund 2002, de Jong *et al.*, 2010, Daniels *et al.* 2012). Spring morphs may exhibit high melanization, but wing melanization is expected to decrease during hot summer conditions (Feltwell 1982, Kingsolver & Wiernasz 1991, Windig 1999).

Phenotypic plasticity brings about resource allocation trade-offs during development (Moczek 2010)—given a finite amount of resources and high resource requirements, an increase in resource allocation to the one trait (in this case, the trait with high plasticity) should lead to a decrease in resource allocation to other traits (Gadgil & Bossert 1970). Therefore, if variation in melanization results from phenotypic plasticity, we would expect to see a decrease in investments in other traits when morphs are highly melanized. Melanin, a complex nitrogen-rich polymer, is costly to produce because growth in Lepidoptera is nitrogen-limited (Graham *et al.* 1980, Talloen *et al.* 2004, Stoehr 2010). One example of such a trade-off is the reduction in visual signaling capacity in areas of the wing devoted to thermal melanization (Vane-Wright & Boppré 1993 cited in Kemp & Krockenberger 2002). In very small butterflies such as *Polyommatus icarus*, melanization is not needed for thermoregulation. This allows for greater phenotypic diversity in wing color and patterns to serve as signal functions for mate recognition and predator avoidance (Keyser *et al.* 2015). Additional trade-offs have been observed between wing melanization and both development time (Kettlewell 1973) and adult size (Windig 1999). A trade-off has also been shown between high melanization plasticity and female fecundity (Chaput-Bardy *et al.* 2014).

Seasonal plasticity in Pieris napi

Much research on butterfly thermal melanization seasonal plasticity has focused on wing melanization in the family Pieridae, on species of *Colias* (Watt 1969, Roland 1982), and species of *Pieris*, including *Pieris occidentalis* (Kingsolver & Weirnasz 1991, Kingsolver 1995), *Pieris brassicae* (Chaput-Bardy *et al.* 2014), and *Pieris rapae* (Stoehr *et al.* 2008).⁴ Little research, however, has been dedicated to exploring seasonal melanization in *Pieris napi*, the green-veined white, despite the species' widespread distribution across Europe (but see Bowden 1978). Distributed from the Mediterranean to northern Scandinavia and from the Atlantic to the Pacific Ocean, populations of *P. napi* are found in a wide range of environmental conditions. While many other European

³ Phenotypic plasticity is the change of an individual's morphology, physiology, or behavior in response to changes in the environment (Price *et al.* 2003, Kelly *et al.* 2012).

⁴ See also Shapiro 1968 and 1975. However, these studies do not give quantitative measurements of melanization, only qualitative phenotype levels such as “dark” or “light” morphs.

butterfly species that have experienced considerable declines in genetic diversity and population sizes due to climate change and human activities (Van Swaay *et al.* 2008, Krauss *et al.* 2010), *P. napi* populations are considered to be mostly stable, with high genetic diversity within populations (Schmitt & Hewitt 2004). Previous *P. napi* research has focused on heritable variation in melanization (among other morphological characters) along altitudinal gradients (Espeland *et al.* 2007) and latitudinal gradients (Tuomaala *et al.* 2012). But as a thriving generalist species capable of living in a variety of environments with high seasonality, *P. napi* is an ideal species with which we can explore phenotypic plasticity—an extremely advantageous mechanism that allows individuals to quickly adapt to climate change-driven environmental changes within their lifetimes (Williams *et al.* 2008).

This study examines the effects of seasonal temperature changes on two morphological traits in *P. napi*: adult size, as estimated by wing size⁵, and ventral hindwing melanization, which has been shown to vary seasonally in other Pieridae (Kingsolver & Wiernasz 1991). As mentioned, these two traits play key roles in thermoregulation in several butterfly species. Additionally, size and melanization have been shown to affect individual fitness in several ways—as honest signals for mate choices (Andersson 1994, Tuomaala *et al.* 2012) and methods of predator defense/avoidance (Wiklund & Tullberg 2004, Keyser *et al.* 2015), among other fitness-enhancing functions. This study aims to assess the following differing hypotheses:

1. *Pieris napi* shows signs of seasonal phenotypic plasticity in adult size and wing melanization. Individuals that developed in cooler spring temperatures exhibit larger size and more melanization than individuals that developed in warmer summer conditions, because larger, more melanized butterflies would be able to maintain optimal temperatures in cool conditions for longer periods of time.

2. After correcting for the effects of seasonal temperature changes on size and melanization, variation in one trait may be negatively correlated⁶ to variation in the other trait due to resource allocation trade-offs during development.

METHODS

Sampling

From 2012 to 2014, over 1500 adult butterflies were collected at six sites across Catalonia throughout the duration of the species' annual flight period during the spring, summer, and early fall. The six sites were chosen because they reflect the diversity of the species' southern European habitat ranges: two wetland sites (Parc Natural dels Aiguamolls de l'Empordà [hereafter Empord] and Cal Tet) located at sea level, two mid-elevation sites (Parc Natural del Montseny [hereafter El Puig, 1031 m] and Parc Natural de la Zona Volcànica de la Garrotxa de Can Jordà [hereafter Can Jord, 539 m]), and two high-elevation sites in the Pyrenees mountains (hereafter Malniu [1997 m]) and Sant Maurici National Park (hereafter Sant Maurici [1914 m]). Daily temperature readings at

⁵ Wing size is often used to estimate total adult size in studies examining the development of butterflies and other winged insects (Berwaerts *et al.* 2001)

⁶ Negative correlations are expected to be observed among fitness-enhancing life-history traits that cannot be maximized simultaneously, leading to a “trade-off” (Zera & Harshmann 2001, King *et al.* 2011).

each site were retrieved from local meteorological stations. The date of collection and the butterfly's sex were recorded for each individual.

Estimating temperature during larval/pupal development

P. napi have been shown to have an adult lifespan of 25-30 days in laboratory conditions (Stjernholm & Karlsson 2008) and around 20 days in situ (Stefan Constanti, personal communication). The *P. napi* larval stage is estimated to last 28 days and the pupal stage to last about 10 days (Carter & Hargreaves 1986). Age estimations were therefore based on the approximations that the larval/pupal stage lasts 38 days and the adult lifespan about 20 days. Age was estimated by wing wear assessments of forewings and hindwings on a 4 category scale based on the fading of wing regions due to scale loss (see Kemp 2000, Miller *et al.* 2012): 1 = recently emerged fresh wings, 2 = few scales lost, 3 = some scales lost, 4 = scale loss and some wing transparency, some tearing at edges (for examples of wing wear categories, see ANNEX Figure A1). Wing wear categories were used to estimate the age of the butterfly at the date of collection. Individuals with wing wear categories of 1 were estimated to be 4 days old, 2 to be 8 days old, 3 to be 12 days old, and 4 to be 16 days old. The date of collection and the approximate age of the butterfly were used to determine the approximate dates of larval/pupal development. The average daily temperature was calculated for the dates of larval/pupal development for each individual. Individuals belonging to the first annual generation at each site were not included in this study; accurate approximations of temperature during larval/pupal development could not be estimated for these individuals, as the first generation undergoes several months of winter diapause in the pupal stage. Subsequent data analyses were therefore performed with a total of 921 individuals belonging to the second, third, or fourth annual generation.

Wing size and melanization measurements

All four wings were removed from each individual and photographed alongside a standard color calibration chart (CameraTrax 24 ColorCard 2x3) and millimeter scale on a standard blue background using a Nikon D7100 with a SigmaMacro lens. Both the ventral and dorsal sides of the wings were photographed. Wing melanisation and wing size measurements of the ventral hindwing were estimated using a graphical user interface in MATLAB version R2012b (The Mathworks, Inc. 2012). First, three "landmarks" were selected on each ventral hindwing: two landmarks where veins M1 and M3 meet the edge of the wing and one landmark at the bifurcation of veins M3 and CuA₁ (see ANNEX Figure A2 for venation diagram). These three landmarks form a triangle zone whose area varies between individuals. Wing size measurements were estimated by counting the number of pixels in the triangle zone. To assess the validity of this approximation, total wing size of a subsample of butterflies were calculated by hand and compared with these measurements. As the wing size approximations were correlated with the exact wing size measurements ($R^2=0.84$), the wing size approximations were considered accurate estimates for use in subsequent analyses. To assess wing melanisation, the wing image was converted to gray scale. The image was standardized for contrast by establishing the full tonal range of the image using a scale from 0 to 255,

with pure black the lowest value and pure white as the highest value (Wilkie & Finn 1996). Each pixel in the hindwing triangle zone was assigned a value on this scale, and the average pixel melanization in the triangle was calculated as an estimate of the level of ventral hindwing melanization.

Determining the effect of temperature on wing size and wing melanization

The effects of temperature on wing size and wing melanization were analyzed by restricted maximum likelihood (REML) using linear mixed effect models. REML was used because this method is less sensitive to unbalanced data than methods such as maximum likelihood (Meyer 1987). Bayesian approximations generated through MCMC model fitting were used to complement the REML estimates from the linear mixed effect models for two main reasons. First, Bayesian MCMC models can be used to verify the statistical significance of fixed effects in linear mixed effect models (Friberg *et al.* 2013), even if the data are unbalanced (Haugen *et al.* 2012). The validity of *p*-values as measures of statistical significance for fixed effect parameters in linear mixed effect models is a topic of much debate (see Baayen *et al.* 2008). Analysis of Bayesian posterior distributions of fixed effect parameters is often considered to be a more reliable approach than using frequentist approaches to test fixed effect significance (Fong *et al.* 2010, Browne & Draper 2006, Hong 2013). Comparisons of the results of both analyses can provide a more accurate picture of how fixed effects influence a given response variable. Second, using Bayesian MCMC models can reliably estimate covariance components between multiple response variables while estimating parameter values and confidence intervals for fixed effects and variance components. This allows us to examine the relationship between the fixed/random effects and each response variable individually as well as collectively to estimate correlation between response variables.

Linear mixed effects models and multi-response MCMC models were implemented in this current study to determine whether: 1. temperature during larval/pupal development influences wing size and/or wing melanization, 2. wing size and wing melanization are negatively correlated, consistent with a trade-off hypothesis. Both linear mixed effect models and Bayesian models were implemented in R version 3.2.1 (R Development Core Team 2014). Linear mixed effect models were constructed with the lmer function in the LME4 package (Bates *et al.* 2015), and Bayesian MCMC models were performed using the MCMC_{GLMM} package (Hadfield 2010).

Linear mixed effect models

Separate models were constructed for wing size and wing melanization. Wing size models had wing size as a normally distributed response variable and wing melanization models had wing melanization as a normally distributed response variable. Both wing size and wing melanization models had identity link functions. The following independent variables were included in both wing size and wing melanization models: year of collection (categorical random effect), sampling site (categorical random effect), sex (categorical fixed effect), and average temperature during larval/pupal development (continuous fixed effect, hereafter referred to as “temperature”). Year of collection and sampling site were included as crossed, random intercept terms to account for any

possible correlation among observations within the same variable level (for example, possible correlation between wing size/melanization for individuals collected during a particular or possible correlation between wing size/melanization for individuals found in a specific sampling site). Sex was included in both wing size and wing melanization models to account for any sexual dimorphism. Full models for both wing size and wing melanization included all independent variables and all possible interactions between independent variables. All possible nested model iterations were constructed by a stepwise removal of each interaction and each main effect. With the R package MuMIn (Bartoń 2013), the second order Akaike Information Criterion was used to select the most parsimonious models while minimizing the probability of overfitting. Several model diagnostics were performed on the selected models, including calculating variance inflation factors and kappa numbers to check for multicollinearity and generating residual plots to visually confirm the normality and homoscedasticity of residuals. Visualizations of the linear mixed effect models were constructed using the R package visreg (Breheny & Burchett 2013). Statistical significance of fixed effects was determined using a Kenward-Roger corrected test implemented in the R package lmerTest (Kuznetsova *et al.* 2013).

Bayesian MCMC models

When implementing the Bayesian MCMC models, wing size and wing melanization were treated as a bivariate response variable. Full Bayesian MCMC models included all independent variables present in the linear mixed effect models, including sex and temperature as fixed effects and year of collection and sampling site as crossed random intercept terms. All possible nested model iterations were constructed and compared with full models using the deviance information criterion (DIC). Models were implemented with several prior distributions to confirm that the models' posterior probability distributions were valid and not heavily impacted by a given prior distribution (see ANNEX Table A1). Trace plots were examined visually to check for convergence, and autocorrelation values were checked to ensure weak autocorrelation. Models were run for enough iterations to ensure a large effective sample size of >4000 (see ANNEX Note A1 for prior information, number of iterations, burn-in, and thinning values). Posterior density curves were checked for symmetry and any aberrations of unimodality. The Gelman-Rubin diagnostic and the Geweke diagnostic were calculated as formal diagnostic tests. For random effects and fixed effects, the modes of the variables' posterior distributions and their high posterior density intervals⁷ were calculated for the variance and covariance between wing size and wing melanization. *p*MCMC values were calculated for each fixed effect as measurements of confidence in whether the relationship of the fixed effect with the response variable(s) is different from zero.⁸ The

⁷ High posterior density interval: a Bayesian credible set that describes the interval in which most of the distribution lies. The parameter falls inside this interval with a measurable probability, i.e. 95% probability in the case of a 95% high posterior density interval.

⁸ *p*MCMC values are twice the probability that the true value of the parameter coefficient is above or below zero, and therefore is simply a measurement of confidence in the sign of the coefficient. Standard *p*-values do not make sense in a Bayesian framework because significance testing is strictly frequentist. Many studies, however, interpret *p*MCMC values in a way comparable to *p*-value, especially when comparing Bayesian model results with the results of linear mixed effect models (see Friberg *et al.* 2013)

variance explained by each random effect was estimated by dividing the posterior mode of a random effect by the sum of the posterior modes of all random effects, and the HPDI of this value was generated by dividing the HPDI of a random effect by the sum of the HPDIs of all random effects [V_I / V_T (HPDI), see Hadfield 2010 Course Notes for further details).

RESULTS

Linear mixed effect models

The most parsimonious linear mixed effect model analyzing wing size included sex and temperature as fixed effects and sampling site and year of collection as random effects (see Table 1a for full/null model AICc values). The analyses showed a significant negative effect of temperature on wing size and a significant difference between male and female wing size, as males typically have larger wings than females (Wiklund & Forsberg 1991, see also Table 2a in this study). The most parsimonious linear mixed effect model analyzing wing melanization was determined by comparing two models with the lowest, nearly identical AICc values by refitting the models using maximum likelihood and performing an Analysis of Variance (ANOVA) to compare the models' sums of squares of residuals (Table 1b). The most parsimonious model chosen ($p = 0.029$) included temperature as a fixed effect and sampling site and year of collection as random effects. The analyses showed a significant negative effect of temperature on wing melanization (see Table 2b). "Heat maps" generated for both melanization and wing size models show differences in the respective response variable according to sex and changing temperature (Figure 1a and 1b). Caterpillar plots representing the 95% confidence intervals for each level of the random effects were examined to validate the inclusion of the random effects in the linear mixed effect models. As not all random effect intervals comfortably overlapped zero (ANNEX Figure A3), the inclusion of the random effects explained enough variance to validate their presence in both wing size and wing melanization models. Linear mixed models displayed no collinearity and residuals displayed normality and homoscedasticity. Residual plots and other model diagnostics for both wing size and wing melanization models can be found in the ANNEX Figure A4.

Bayesian MCMC models

The most parsimonious Bayesian MCMC model included sex and temperature as fixed effects and sampling site and year of collection as random effects (see Table 3 for full/null model DIC values). Trace plots confirming model convergence and the results of other model diagnostics can be found in the ANNEX Figure A5. Analyses of posterior distributions (see Table 4) revealed a negative effect of temperature on both wing size and wing melanization, with highly confident p MCMC values (with a p MCMC measurement of less than 0.001). Analyses also showed a difference in wing size between the sexes, but no difference in melanization. Analyses also showed slight positive correlation between wing size and wing melanization after accounting for the effects of the fixed and random variables on these traits ($r = 0.22$). However, the HPDI of this

correlation included both negative and positive values (-0.58 – 0.71); this estimate of correlation between wing size and wing melanization is therefore highly uncertain. Table 5 shows estimates of random effect variance. Examination of the variance in wing size and wing melanization due to random effects revealed that the variable year explains more of the random effect variance than site; analyses of the variance in wing size showed especially high estimates of random effect variance due to year [V_I / V_T (HPDI) = 0.74 (0.21 – 0.99)].

Because of this high estimate of random effect variance due to the variable year, additional tests were performed to determine whether all years have similar posterior distributions or if the high variance could be attributed to differences between certain years. Therefore, two-way ANCOVAs and post hoc analyses were used to detect differences in the levels of the random effect. ANCOVAs and post hoc analyses examined both random effects, site and year, and examined wing size and wing melanization separately as dependent variables. Assumptions of homogeneity of variances were confirmed with a variance ratio test, ($\sigma^2_{MAX} / \sigma^2_{MIN} < 2$). Temperature was included as a covariate and site and year were included as factors. Sex was included as an additional independent variable in the ANCOVA with wing size as the dependent variable. The ANCOVA with wing size as a dependent variable revealed significant effects for temperature, sex, site, year, and the interaction between site and year ($p < 0.001$). Significant effects for temperature, site, and the interaction between site and year ($p < 0.001$) were also found for wing melanization. Group means (the means for each site and each year) were adjusted for the effect of the covariate (temperature for the ANCOVA analyzing wing melanization, temperature and sex for the ANCOVA analyzing wing size) using the R package effects (Fox *et al.* 2013). The `glht` function of the R package multcomp (Hothorn *et al.* 2015) was used to perform post hoc Tukey tests to determine differences between the adjusted means. Results of the Tukey tests are displayed in Figure 2. Tukey tests revealed that the wing sizes of individuals collected at Malniu were significantly different from those of the El Puig ($p = 0.003$, Figure 2a) and the wing sizes of individuals collected in 2014 were significantly different from those collected 2012 and 2013 ($p < 0.001$, Figure 2a). Tukey tests also showed that the wing melanization of individuals collected from El Puig was significantly different from those of Cal Tet, Can Jord, Empord, Malniu, and Sant Maurici ($p < 0.001$, Figure 2b) and the wing melanization individuals collected in 2012 were significantly different from those collected in 2013 and 2014 ($p < 0.001$, Figure 2b).

DISCUSSION and PERSPECTIVES

Male and female *Pieris napi* butterflies showed evidence of seasonal phenotypic plasticity in the melanization and size of the ventral hindwing at all six sampling sites for every year of collection. Seasonal variations of both wing size and wing melanization appear to be potentially adaptive for thermoregulation: individuals captured in cool spring temperatures had larger, more melanized ventral hindwings than those caught in warmer temperatures. No evidence was detected of a potential tradeoff between these two traits, as wing size and wing melanization were positively correlated.

Unbalanced sampling effort among different years could also explain differences in the wing sizes of individuals collected in 2014 compared to those of 2012 and 2013, and differences in wing melanization in individuals collected in 2012 compared to those of 2013 and 2014. However, further investigation of the climatic conditions in each of these years is necessary to determine whether any climatic anomalies could account for this variation among years. While the same general trends in seasonal plasticity (specifically, a decrease in size and melanization as external temperatures increase) are present at all sites for every year, the effects of additional environmental factors, such as photoperiod and humidity, on these traits remain to be explored.

Results also show geographical variation in the two traits. Differences in wing size and wing melanization at different sites may be due to unbalanced sampling efforts; however, while the wing melanization of individuals collected at El Puig followed the same seasonal trends as that of the other sampling sites, the melanization of El Puig individuals was also significantly different from those of all other sampling sites. This variation in melanization of different populations is not altogether surprising, as between-population and between-family variation in thermoregulatory traits have been observed in other Pieridae (Chapt-Bardy *et al.* 2014, Kingsolver *et al.* 2007). This geographical variation may be due to some heritable variation in the El Puig population (as seen for *P. napi* populations along altitudinal gradients, see Espeland *et al.* 2007). Rather than a simple mechanistic relationship between temperature and wing melanization across all *P. napi* populations, quantitative genetic variation can lead to population divergence in thermal melanization (Chapt-Bardy *et al.* 2014). On the other hand, this geographical variation may not be due to genetic divergence. Phenotypic plasticity is expected to lead to optimal phenotypes that are specific to local environmental conditions. Therefore, we may observe differences in the optimal phenotype in sites with differing local conditions. While it has been argued that geographic variation in *P. napi* wing color is unlikely to be due to phenotypic plasticity in response to local environmental conditions (Tuomaala *et al.* 2012), geographic variation due to phenotypic plasticity has been found in *P. napi* (Shapiro 1977, quoted in Tuomaala *et al.* 2012) and other butterflies (Daniels *et al.* 2012, quoted in Tuomaala *et al.* 2012).

Seasonal phenotypic plasticity of the melanization of the ventral hindwing in these Catalanian *P. napi* populations is consistent with that of other Pieridae, whose ventral hindwing melanization patterns have been shown to be adaptive for thermoregulation (Shapiro 1976, Kingsolver & Wiernasz 1991). This trait follows a similar pattern of seasonal plasticity as the melanization of the ventral hindwing in the closely related, sympatric species *Pieris rapae* (Stoehr *et al.* 2008), with spring butterflies having more heavily melanized wings than summer butterflies. Increased ventral hindwing melanization in cooler temperatures assists in lateral basking—by positioning the ventral hindwing perpendicular to the sun, the heavily melanized wings would absorb more sunlight than unmelanized wings (Watt 1968, Ellers & Boggs 2004). Pierids may also exhibit seasonal plasticity in melanization of the basal portion of the dorsal forewing for dorsal and/or reflectance basking (Wasserthal 1975, Kingsolver 1985a). While ventral hindwing melanization has been shown to be more plastic than dorsal wing melanization in *P. rapae*, this species also exhibits dorsal wing melanization plasticity with high basal melanization and low distal melanization—signatures of reflectance basking (Stoehr *et al.* 2008). Dorsal wing melanization should also be examined in *P. napi* in order to assess

whether reflectance basking also occurs in this species or if these two coexisting species have evolved different thermoregulatory behaviors.

Even though size has been shown to have some effect on the thermoregulatory capabilities of butterflies (Heinrich 1986, Gilchrist 1990), there have been relatively few studies that have examined adult size in varying thermal environments. Size has been shown to affect thermoregulation in several Lepidopteran (sub)families, including Nymphalidae (*Pararge aegeria*, Berwaerts *et al.* 2001, *Hypolimnas bolina*, Kemp & Krockenberger 2004) and Polyommatae (*Polyommatus icarus*, Keyser *et al.* 2015). In these studies, heightened rates of heat exchange have been found in smaller individuals, who can use wing positioning to control heat gain and heat loss more effectively than larger individuals. Larger individuals lose heat at a slower rate than smaller individuals, and may be more successful at thermoregulation in cooler temperatures (Diamond & Kingsolver 2010). This current study, however, is the first time we see evidence of adult size seasonal plasticity in Pieridae. Results show that these *P. napi* populations appear to follow the temperature-size rule because they exhibit larger sizes in cooler environments. This suggests that size may play a role in thermoregulation.

Research has shown, however, that differences in size and/or melanization in butterflies may be strongly dependent on other factors besides temperature. Diamond & Kingsolver 2010 showed in *Manduca sexta* that larvae reared on high quality plants followed the temperature-size rule, with larger final size in cooler temperatures, while larvae reared on low quality plants followed a reversal of the temperature-size rule. Additionally, Talloen *et al.* 2004 showed that both melanization and size in adult *Pararge aegeria* decreased when larvae were reared on host plants undergoing drought-stress treatments at a constant temperature. As larval host plant quality has been shown to affect both size (Gotthard *et al.* 2008, Davidowitz *et al.* 2004, Diamond & Kingsolver 2010) and melanization (Talloen *et al.* 2004, Janmaat & Myers 2005, Diamond & Kingsolver 2010) in adult butterflies, further investigation into the seasonal conditions of host plants in situ can determine whether variations in melanization and size are indeed responses to seasonal changes in temperature, and/or if host plant quality influences melanization and size of *P. napi* adults.

Choice of developmental pathway, including direct development to adulthood or larval/pupal diapause, has also been shown to affect size and melanization. Developmental pathway is known to affect size in *P. napi*, as diapausing individuals have been shown to have lower growth rate and longer larval time compared to directly developing individuals, resulting in great pupal weight (Nylin & Gotthard 1998). Van Dyck & Wiklund 2002 showed that decrease in wing melanization in *Pararge aegeria* is potentially canalized: directly developing and diapausing butterflies were reared at the same temperatures, yet direct developing butterflies were always paler than their diapausing conspecifics. Several other species, including *Polygonia c-album* (Nylin 1992), *Polygonia c-aureum* and *Polygonia egea* (Nylin *et al.* 2005), show increased wing melanization in diapausing morphs and decreased wing melanization in direct developing summer morphs. The coloration of diapausing morphs may serve as cryptic coloration (Wiklund & Tullberg 2004), as these species undergo winter reproductive diapause in the adult stage, and decreases in wing melanization in summer morphs may result from nitrogen resource allocation away from wing coloration towards higher reproductive output (Karlsson *et al.* 2008). *P. napi* undergoes diapause in the pupal stage, so the

increased melanization of diapausing morphs may not necessarily be adaptive for predator avoidance. The choice of developmental pathway may, however, influence the allocation of nitrogen and other resources and therefore potentially affect wing melanization.

Regardless of the influences of other environmental and developmental factors, if these *P. napi* populations are indeed exhibiting a positive reaction norm to the seasonal thermal environment, this does not necessarily mean that all populations of *P. napi* exhibit the same thermal response. Kingsolver *et al.* 2007 illustrated that thermal reaction norms can evolve rapidly in natural field conditions by examining thermal reaction norms for size in two *P. rapae* populations from northwestern and a southeastern North America. The rapid evolutionary divergence of these two populations suggests that the relationship between temperature and size in *P. rapae* may not be controlled solely by simple, mechanistic constraints. While this study does not show divergent thermal reaction norms in the closely related *P. napi*, analyses comparing the thermal reaction norms of other *P. napi* populations across Europe may show variation in thermal reaction norms. As suggested earlier, quantitative genetic variation may influence variation in melanization; this may also be the case for variation in size. Quantitative genetic variation could account for differences within thermal reaction norms seen among populations exposed to different selective pressures (e.g. varying thermal environments) that have consequently evolved different life history and ecological strategies (Chapt-Bardy *et al.* 2014). It would be highly informative to study inter- and intra-family and population variation in size and melanization thermal reaction norms.⁹

⁹ At Universitat de Barcelona, experimental procedures based on Popkin & Murphy 2013 breeding protocols are currently underway to explore the effects of constant and varying rearing temperature on size, melanization, and growth rate in butterflies from different families collected from several sampling site populations. Results will provide further insight into the role of heredity and developmental temperature on thermal reaction norms in *P. napi*.

TABLES and FIGURES

Table 1. Selection of linear mixed models to estimate (a) wing size and (b) melanization. The most parsimonious models are marked in bold.

	Random Effect	Fixed Effect	AICc
(a)			
Response Variable:	Sampling Site	---	23036.8
Wing Size	Sampling Site	Sex	22951.8
	Year	---	22920.4
	Sampling Site	Temperature	22886.2
	Year	Temperature	22878.9
	Year	Sex	22851.1
	Year	Temperature:Sex	22808.7
	Sampling Site	Temperature:Sex	22796.7
	Sampling Site + Year	---	22787.7
	Sampling Site + Year	Temperature	22736.4
	Sampling Site + Year	Sex	22713.5
	Sampling Site + Year	Temperature:Sex	22661.1
	Sampling Site + Year	Temperature + Sex	22656.3
(b)			
Response Variable:	Year	Sex	252
Wing Melanization	Year	Temperature:Sex	249.9
	Year	---	246.3
	Year	Temperature	234.9
	Sampling Site + Year	Temperature:Sex	144.8
	Sampling Site	Temperature:Sex	144.8
	Sampling Site + Year	Sex	138.8
	Sampling Site	Sex	136.9
	Sampling Site + Year	Temperature + Sex	134.5
	Sampling Site + Year	---	130.8
	Sampling Site	---	128.8
	Sampling Site + * Year	Temperature	126.4
	Sampling Site	Temperature	126.3

* The following wing melanization mixed models showed very similar AICc values: (1) site as random effect, temperature as fixed effect and (2) both site and year as random effects, temperature as fixed effect. Therefore, an ANOVA was used to refit the models using ML and compare them using a Chi-square test. The model with both sampling site and year as random effects was selected as the most parsimonious model ($p = 0.029$)

Table 2. Estimates of the effects of sex and average temperature during larval/pupal development on (a) wing size with sampling site and year captured as random effects, and average temperature during larval/pupal development on (b) melanization. Kenward-Roger corrected tests p -values ($\alpha = 0.01$) are marked in bold when significant.

	Parameter	Estimate	SE	df	F	P
(a)						
Response Variable:	Intercept	5.07E5	2.47E4			
Wing Size	Sex	3.29E4	4.05E3	1	65.96	<0.0001
	Temperature	-5.3E3	785.8	1	45.48	<0.0001
(b)						
Response Variable:	Intercept	0.1717	1.14E-6			
Wing Melanization	Temperature	-2.12E-7	5.05E-8	1	126.3	<0.0001

Table 3. Selection of bivariate MCMCglmm to estimate wing size and melanization. The most parsimonious model is marked in bold.

Random Effect	Fixed Effect	DIC
Year	---	23163.4
Sampling Site	---	23148.9
Year	Temperature	23118.5
Year	Sex	23113.4
Sampling Site	Sex	23084.5
Year	Temperature:Sex	23063.5
Sampling Site	Temperature	22987.2
Sampling Site	Temperature:Sex	22913.7
Sampling Site + Year	---	22888
Sampling Site + Year	Temperature	22836.2
Sampling Site + Year	Sex	22833.8
Sampling Site + Year	Temperature:Sex	22777.5
Sampling Site + Year	Temperature + Sex	22774.8

Table 4. Bayesian estimates of the posterior modes and their 95% highest posterior density intervals (HPDI) of the effects of sex and temperature during larval/pupal development on (a) wing size and (b) melanization with sampling site and year captured as random effects. Significant *pMCMC* values ($\alpha = 0.01$) are marked in bold.

	Fixed Effect	Posterior Mode	HPDI	<i>pMCMC</i>
(a)				
Response Variable:	Sex	3.24E4	2.55E4 – 4.11E4	<0.001
Wing Size	Temperature	-5E3	-6.6E3 – -3.46E3	<0.001
(b)				
Response Variable:	Sex	6.67E-3	-3.16E-2 – 4.37E-2	0.72
Wing Melanization	Temperature	-1.61E-2	-2.29E-2 – -8E-3	<0.001

Table 5. Bayesian estimates of the posterior modes and their 95% highest posterior density intervals (HPDI) of the random effects of sampling size and year of collection on (a) wing size and (b) melanization, with sex and average temperature during larval/pupal development as fixed effects.

	Random Effect	Posterior Mode (HPDI)	V_I / V_T (HPDI)
(a)			
Response Variable:	Site	9.23E8 (3.78E8 – 4.14E9)	0.06 (6.35E-4 – 0.36)
Wing Size	Year	1.058E10 (3.58E8 – -2.12E11)	0.74 (0.21 – 0.99)
(b)			
Response Variable:	Site	1.86E-2 (5.78E-3 – 6.07E-2)	0.14 (8.1E-4 – 0.37)
Wing Melanization	Year	4.86E-2 (4.529E-3 – 0.84)	0.37 (0.08 – 0.92)

a.

Figure 1. Heat maps showing the change in (a) wing size and (b) wing melanization in both females and males as external temperature increases. Red coloration indicates larger wing size and increased melanization, while blue coloration indicates a decrease in size/melanization. For both sexes, melanization and wing size decrease with increased temperature. Males have larger wing sizes than females, while there is no difference between the sexes in the area of the wing analyzed for melanization.

b.

a.

Figure 2. Results of ANCOVAs and post hoc analyses examining both random effects, site and year, as factors, temperature as a covariate and (a) wing size and (b) wing melanization separately as dependent variables. Letters above box plots indicate a significant difference in wing size/melanization at a given site or during a particular year.

1.

ANNEX

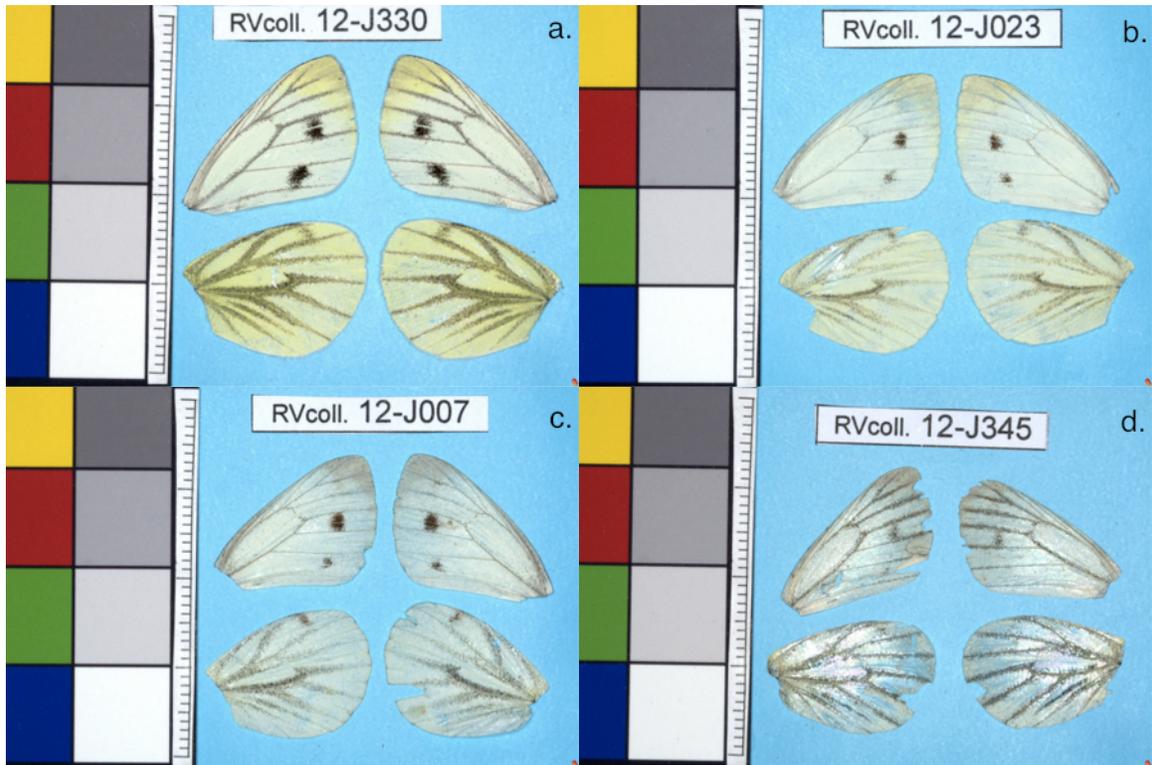


Figure A1. Examples of 4 wing wear categories determined by the fading of wing regions due to scale loss. Wings are photographed alongside color calibration chart and millimeter scale. (a) Category 1: recently emerged fresh wings (b) Category 2: few scales lost (c) Category 3: some scales lost (d) Category 4: scale loss and some wing transparency, some tearing at edges.

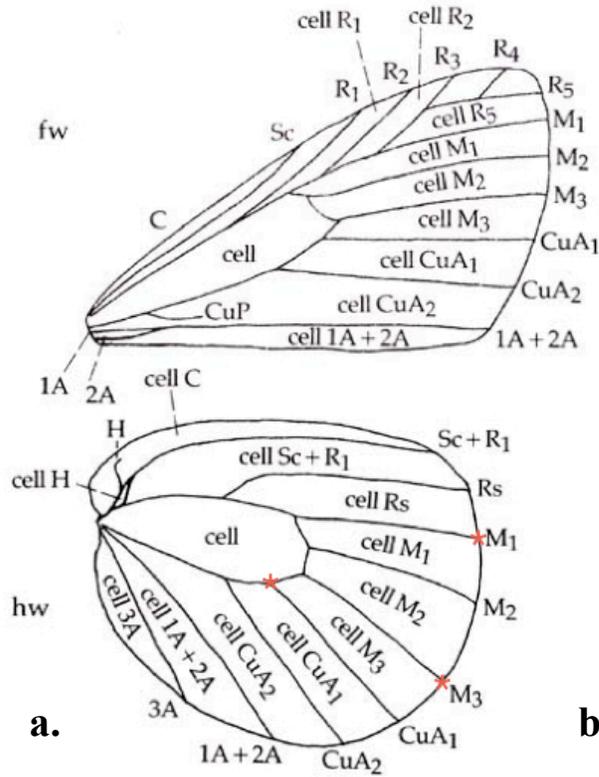
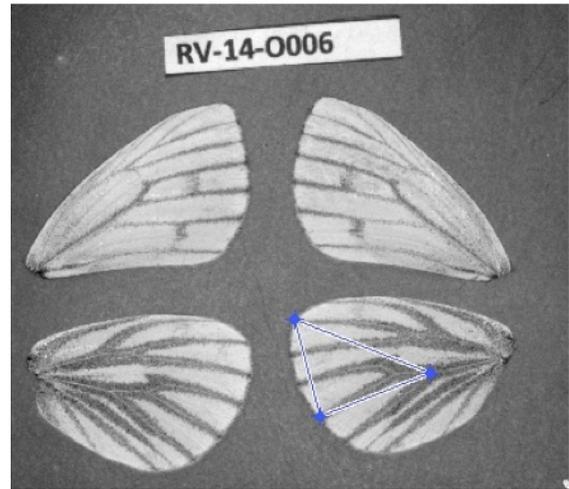


Figure A2. (a) Venation diagram¹⁰ of butterfly forewing and hindwing. Red dots represent the three “landmarks” that form the wing triangle zone used in wing size/melanization assessments. (b) Gray scale image depicting the selection of the three landmarks on ventral hindwing of *P. napi*.



¹⁰ Wing venation diagram by James Scott, 2011.

Note A1.

Bayesian models were implemented with three different priors: one flat prior and one less flat prior based on a bivariate response variable covariance matrix (see WAMwiki <http://www.wildanimalmodels.org/tiki-index.php>), and one inverse Wishart prior (Hadfield 2010) (see `pnapiscriptfinal.R` for further details). All MCMCglmms were run for at least 500 000 iterations, with a burn-in of 5000 iterations and a thinning interval of 1000.

Figure A3. Caterpillar plots representing the 95% confidence intervals for each level of the random effects were examined to validate the inclusion of the random effects in the linear mixed effect models.

Table A1. Bayesian estimates of the posterior modes and their 95% highest posterior density intervals (HPDI) of the effects of sex and temperature during larval/pupal development on (a) wing size and (b) melanization with sampling site and year captured as random effects. Significant pMCMC values ($\alpha = 0.05$) are marked in bold.

Prior	Fixed Effect	Posterior Mode	HPDI	pMCMC		
Less Flat Prior	(a)	Response Variable: Sex	3.26E4	2.53E4 – 4.14E4	<0.001	
		Wing Size Temperature	-4.84E3	-6.6E3 – -3.46E3	<0.001	
	(b)	Response Variable: Sex	6.84E-3	-3.35E-2 – 4.17E-2	0.71	
		Wing Melanization Temperature	-1.63E-2	-2.32E-2 – -8.45E-3	<0.001	
	Wishart's Prior	(a)	Response Variable: Sex	3.3E4	3.30E4 – 3.31E4	<0.002
			Wing Size Temperature	-5.57E3	-5.573E3 – -5.574E3	<0.002
(b)		Response Variable: Sex	4.73E-2	-1.30E-1 – 1.65E-1	0.74	
		Wing Melanization Temperature	-1.42E-2	-3.67E-2 – -5.10E-4	0.04	

a.

b.

Figure A4. Residual quantile plots of linear mixed models with response variable (a) wing size and (b) wing melanization. Wing size model shows normal residuals and no collinearity ($\kappa = 10.5$, variance inflation factor [VIF] = 1, and wing melanization model shows normal residuals and no collinearity ($\kappa = 10.65$, VIF = 1).

Figure A5. Trace plots of Bayesian MCMC model confirming model convergence. Gelman and Rubin multiple sequence diagnostic of model showed a multivariate potential scale reduction factor of 1.1.

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