

Adaptive plasticity in wing melanisation of a montane butterfly across a Himalayan elevational gradient

SHUBHAM GAUTAM^{1,2}  and KRUSHNAMEGH KUNTE² ¹Masters Programme in Wildlife Biology and Conservation, Wildlife Conservation Society–India Programme and National Centre for Biological Sciences, Bangalore, India and ²National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

Abstract. 1. Traits that are significant to the thermal ecology of temperate or montane species are expected to prominently co-vary with the thermal environment experienced by an organism. The Himalayan *Pieris canidia* butterfly exhibits considerable variation in wing melanisation. We investigated: (i) whether variation in wing melanisation and (ii) activity period of this montane butterfly was influenced by the seasonally and elevationally changing thermal landscape.

2. We discovered that wing melanisation varied across elevation, seasons, sex, and wing surfaces, with the variation strongly structured in space and time: colder seasons and higher elevations produced more melanic individuals. Notably, melanisation did not vary uniformly across all wing surfaces: (i) melanisation of the ventral hindwing co-varied much more prominently with elevation, but (ii) melanisation on all other surfaces varied with seasonal changes in the thermal environment.

3. Observed wing surface-specific patterns indicated thermoregulatory function for this variation in melanisation. Such wing surface-specific responses to seasonal and elevational variation in temperature have rarely been reported in montane insects.

4. Moreover, daily and seasonal thermal cycles were found to strongly influence activity periods of this species, suggesting the potential limits to wing melanisation plasticity.

5. Overall, these results showed that the seasonal and elevational gradients in temperature influence the thermal phenotype as well as activity periods of this Himalayan butterfly. It will be critical to study the phenotypic evolution of such montane insects in response to the ongoing climate change, which is already showing significant signs in this iconic mountain range.

Key words. Colour variation, flight activity, wing colouration, modularity, *Pieris*, phenotypic plasticity, sexual dimorphism, thermal melanism.

Introduction

Harsh thermal landscapes in montane ecosystems pose a particular challenge to cold-blooded organisms such as insects. Thermal conditions impose selection pressures on various traits such as colouration (DeJong, Gussekloo, & Brakefield, 1996; Hegna, Nokelainen, Hegna, & Mappes, 2013), metabolic rates (Addo-Bediako, Chown, & Gaston, 2002), and body size (Ray, 1960; Atkinson, 1994; Angilletta & Dunham, 2003).

Correspondence: Shubham Gautam, Biodiversity and Biocomplexity Unit, Okinawa Institute of Science and Technology, Graduate University, 1919-1 Tancha, Onna, Okinawa, Japan; E-mail: shubham.gautam@oist.jp

Of these, the degree of melanisation is particularly important for local adaptation in a wide variety of species in diverse landscapes (Stuart-Fox, Moussalli, Johnston, & Owens, 2004; Antoniazza, Burri, Fumagalli, Goudet, & Roulin, 2010). Multiple mutually nonexclusive hypotheses may explain the occurrence of melanism in insects, viz., thermoregulation (Kingsolver, 1985b; Clusella Trullas, van Wyk, & Spotila, 2007; Clusella-Trullas, Terblanche, Blackburn, & Chown, 2008), protection from ultraviolet radiation (True, 2003), avoiding desiccation (Brisson, De Toni, Duncan, & Templeton, 2005), escape from predators (Greene, 1996; Karpestam, Merilaita, & Forsman, 2012), protection from pathogens (Fedorka, Lee, & Winterhalter, 2013), and sexual selection (Wiernasz, 1989). Thermoregulation is often considered the most widespread

mechanism driving variation in melanisation that is structured according to the thermal landscape. The thermal melanism hypothesis states that “at a given level of solar radiation, more melanic individuals have an advantage over lighter ones under cooler conditions because they heat up relatively fast and reach an optimum body temperature” (Clusella Trullas et al., 2007). Potential advantages of thermal melanism in colder environments include increased mobility (Ellers & Boggs, 2004), activity periods (Brakefield & Lees, 1987), fecundity (Rhamhal-inghan, 1999), survival (Kingsolver, 1995), and decreased egg maturation time in females (Ellers & Boggs, 2004). On the other hand, lighter morphs may avoid overheating in warmer environments (Gibert, Moreteau, Moreteau, Parkash, & David, 1998; Ottenheim, Wertheim, Holloway, & Brakefield, 1999). Thus, the degree of melanisation is expected to differ among populations that face different environmental conditions, to maximise fitness under specific environments, for example by increasing wing or body melanisation with latitude, elevation, and season (Ellers & Boggs, 2002). Butterflies have been extensively used as model organisms to document these patterns of co-variation in the degree of melanisation and thermal environments (Kingsolver, 1983a; Gibert et al., 1998; Davis, Farrey, & Altizer, 2005; Karl, Geister, & Fischer, 2009; Xing et al., 2018).

Butterfly flight activity is possible under a relatively narrow range of body temperatures (Kingsolver, 1983a). Therefore, butterflies need to regulate body temperature either behaviourally such as by changing basking posture (Clench, 1966), making or avoiding contact with the substrate (Kevan & Shorthouse, 1970) and seeking sun or shade (Watt, 1968), by physiological adaptations such as enzyme polymorphisms (Watt, 1985), or by morphological adaptations such as changes in body or wing colour (Kingsolver, 1985b). Basking behaviour, in particular, plays an important role in insect thermoregulation. *Pieris* butterflies employ different basking postures, for example lateral, dorsal, and reflectance basking behaviour (Watt, 1968).

Morphological features such as wing melanisation of butterflies varies with elevation, latitude, and season (Shapiro, 1976; Espeland, Aagaard, Balstad, & Hindar, 2007; Valimaki & Kaitala, 2007). Pierids, especially *Colias* and *Pieris*, have been used as models for studying seasonal polyphenism and overall variation in wing melanisation (Hoffmann, 1973; Tuskes & Atkins, 1973; Hoffmann, 1974; Kingsolver & Wiernasz, 1991; Stoehr & Wojan, 2016). However, wing melanin affects body temperatures in butterflies and other insects alike (Ellers & Boggs, 2004; Hegna et al., 2013), and patterns of increasing wing or body melanisation with colder environments are known in a wide variety of insect species (Kingsolver, 1983b; Ellers & Boggs, 2002; Karl et al., 2009; Tuomaala, Kaitala, & Rutowski, 2012). Therefore, variation in ambient temperature is expected to affect wing melanisation during developmental stages and flight activity during the mobile stages. Both wing melanisation and flight activity are crucial determinants of critical behaviours such as foraging, courtship, and predator avoidance that impact individual fitness in insects in general.

Montane ecosystems offer ideal landscapes to study the impacts of climate on various morphological traits. The Himalaya is the tallest and among the youngest mountain ranges in the world, comprising of a multitude of habitats observed

across Eurasia, with a significant amount of variation in climatic conditions across its approx. 8600 m elevational range (Grytnes & Vetaas, 2002). The elevational gradient in the Himalaya is a significant bioclimatic gradient that supports considerable biodiversity. Many species of plants and animals distributed across the Himalaya occupy a broad elevational range, subjecting the populations to a wide range of climatic conditions.

In this study, we addressed two broad questions: (i) Is wing melanisation in a Himalayan butterfly, *Pieris canidia* (Fig. 1), explained by thermal melanism hypothesis (thermoregulation) as revealed by the spatial and seasonal variation in wing melanin? (ii) Does the elevational and seasonal thermal environment affect flight activity periods, thus indicating the extent of spatio-temporal limits of the thermal environment for this species? With question 1, our goal was to distinguish the thermal melanism hypothesis from two alternative, somewhat overlapping and contradictory hypotheses for wing melanisation: (i) sexual selection: attracting mates with either bright white ground colour or the melanisation of the wing patterns, and (ii) predator avoidance: avoiding predators with cryptic or otherwise protective colouration. Butterflies may partition colour signal components on the two wing surfaces such that they are attractive to potential mates on the dorsal surface that is visible when butterflies are flying, and protectively coloured on the ventral surface that is exposed when butterflies are at rest and most vulnerable to predators (Nijhout, 1991; Endler, 1992; Oliver, Robertson, & Monteiro, 2009).

From these hypotheses, distinct predictions based on specific assumptions follow. The thermal melanism hypothesis predicts a positive correlation of average wing melanin with colder climate (i.e. higher elevations and winters). It is possible that different wing regions vary in their importance for regulating body temperature (Wasserthal, 1975; Kingsolver, 1985a; Stoehr & Goux, 2008; Stoehr & Wojan, 2016), and therefore, we expected to see variation in wing areas and surfaces across these variable climates.

The sexual selection hypothesis predicts that the spatial and seasonal variation in wing melanin should be sexually dimorphic, but under the relatively lesser influence of climatic conditions, that is male butterflies (the courting sex) should show little variation in melanisation of the wings across seasons and elevations if either the bright white ground colour or melanisation of the wings is used in courtship. On the other hand, females may show relatively stronger seasonally and altitudinally variable melanisation, since egg development, fecundity, and activity windows for females are strongly influenced by temperature (Gotthard, Berger, & Walters, 2007; Berger, Walters, & Gotthard, 2008). This prediction, however, hinges on the assumption that mate preference does not correlate with altitudinally and seasonally variable climatic conditions, which may or may not hold true but has not yet been demonstrated in any species.

The predator avoidance hypothesis predicts that darker phenotypes should be associated with warmer climate (i.e. at lower elevations and in summer) since the diversity and abundance of invertebrate as well as vertebrate predators are comparatively higher in a warmer climate (McCain, 2009; Acharya, Sanders, Vijayan, & Chettri, 2011). This hypothesis is based on the



Figure 1. Sexual and wing surface-specific variation in wing melanisation in *Pieris canidia*. Clockwise from top left: dark phenotype (female), pale phenotype (female), pale phenotype (male), and dark phenotype (male). Left and right sides in each image show dorsal and ventral surfaces of the wings, respectively. Coloured dots on the pale male phenotype represent points in relation to wing veins from where RGB values were extracted to measure melanisation. Different colours represent the three wing regions, that is basal (green dots), medial (brown dots), and distal (red dots). [Colour figure can be viewed at wileyonlinelibrary.com].

assumption that darker colouration has a cryptic function since melanised wings will blend with the background better than bright white wings. On the contrary, it is possible that brighter white colouration is an aposematic signal, but an empirical test did not support this hypothesis in pierid butterflies (Lyytinen, Alatalo, Lindström, & Mappes, 1999). Instead, brighter white colouration seems to play a role in sexual selection in the closely related *Pieris rapae*, making males more conspicuous to predators (Morehouse & Rutowski, 2010) and lending support to the notion that darker colouration in females facilitates crypsis in *P. rapae*. Although this cannot be directly extrapolated to *P. canidia*, it is a reasonable assumption that bright white colours play a role in male courtship and darker colouration facilitates crypsis in these closely related *Pieris* species.

In this paper, we tested these hypotheses by measuring the degree of melanisation and comparing population sizes and activity periods across seasons and elevations. Our work provides support for the thermal melanisation hypothesis and reveals a surprising amount of variation in melanisation on different wing surfaces and in the two sexes in line with the ecological expectations.

Materials and methods

Study system and sampling

Pieris canidia is a common species distributed across the Himalaya, NE India, and Myanmar, extending to China to the

north-east, and to the southern Western Ghats in south-western India. In the Himalaya, *P. canidia* occurs at elevations between ca. 350 and 3500 m, flying almost throughout the year, and it is a multivoltine species in most parts of its range (Kunte, Churi, & Bora, 2018). It is predominantly white with variable black markings on the wings, but the black spots on the forewings are sexually dimorphic (females have an extra, large spot along the dorsum, Fig. 1). It exhibits considerable variation in wing melanisation against a white background, which we quantified and characterised by sex, elevation, and season. We chose this species because individuals exhibit extensive variation in wing melanism and because it flies almost throughout the year in the Himalaya (Kunte et al., 2018). Therefore, it is subjected to a wide range of elevational as well as climatic variations in temperature.

We sampled butterflies along two trails covering an elevational gradient from ca. 300 to 3500 m in eastern and central Himachal Pradesh in the western Himalaya. We sampled the eastern trail four times and the central trail three times between late November 2015 and early May 2016. We sampled butterflies from areas with agriculture as the predominant land use type interspersed with wooded areas. Out of the total of 4714 individuals counted, we collected 223 females and 233 males. Any individual location was resampled after a period of at least 1 month. This interval was chosen because *P. canidia* completes early stages, from egg to eclosed adult, in ca. 2–3 weeks (Kunte, unpublished observations). Therefore, the sampled specimens and counted individuals from a single location were almost certainly from subsequent generations. Moreover, specific care

Table 1. Monthly average temperatures recorded during field data collection.

Elevation	November 2015	December 2015	January 2016	February 2016	March 2016	April 2016	May 2016	Seasonal range
300–799 m	NA	19.95	20.75	25.67	29.38	36.84	NA	16.89
800–1299 m	24.69	16.9	12.5	21.8	26.33	30.95	NA	18.45
1300–1799 m	22.75	20.8	13	19.57	26.14	30.04	33.67	20.67
1800–2299 m	22.2	18.34	6.2	16.73	22.43	22.47	31.39	25.19
2300–2799 m	14.78	11.53	NA	NA	19.28	20.42	27.42	15.89
2800–3299 m	13.76	NA	NA	9.84	NA	16.14	19.5	9.66
Elevational range	10.93	9.27	14.55	15.83	10.1	20.7	14.17	-

was taken to collect only fresh specimens to avoid sampling individuals in which colours had faded or wings were otherwise considerably damaged. These specimens are deposited in the Research Collections of NCBS (National Centre for Biological Sciences).

Relative abundance

Butterfly counts were conducted following a time-constrained sampling framework, wherein we counted butterflies for 30 min while traversing trails along cultivated fields and surrounding wooded areas. We counted all butterflies seen during the 30 min, which were usually within 10 m of the trail on either side. The counts were taken between 08.30 and 17.00 hours when butterflies were active. We recorded the elevations of the start and endpoints for each count and conducted all the counts only during clear sky conditions. We measured temperature and humidity at the beginning and end of each 30-min count. Monthly average temperatures across all the counts performed in different elevational zones were used to categorise seasons (Table 1). The 30-min counts allowed us to sample populations across a wide elevational gradient in a much more flexible manner in a montane and heterogeneous landscape compared to the popular Pollard Walks.

Measurement of wing melanisation

We pinned and dried specimens following standard museum protocols. We photographed these museum specimens at the field base against a black background using a Nikon D5300 digital SLR camera with a Nikkor 18–55 mm lens. We included a grey standard next to the specimen in each photograph for colour correction and standardisation of images. We corrected colour based on the grey standard and processed all the images in Adobe Photoshop CS6 (Adobe Inc., California) using the batch automate curve adjustment option and setting a grey point on the grey standard. Subsequently, we measured wing melanisation by extracting RGB values from 12 pre-decided points on the forewing and 10 pre-decided points on the hindwing (Fig. 1). We placed these points in relation to the wing venation to ascertain that the same area was sampled from each individual and to get a proper representation of the entire wing surface (Fig. 1). We used ImageJ for the extraction of RGB values from the images. We did this for both dorsal and ventral surfaces of

fore- and hindwings henceforth referred to as dorsal forewing (UPFW), dorsal hindwing (UPHW), ventral forewing (UNFW), and ventral hindwing (UNHW). For the measurement of wing melanisation, we used the right forewing and hindwing unless they were not intact, in which case, left forewing and hindwing were used for measurement. We chose to extract RGB values from specific points over directly estimating the black area of the wing in order to avoid dealing with thresholds of black, white, and grey. Threshold methods inadvertently choose different threshold values creating a range of threshold values across these studies, thus rendering them incomparable. In addition, we decided to extract RGB values in such a fashion so as to include the nuances of differences in the colour intensity, such that more intense black colour will get lower values on the RGB scale than the less intense ones, which cannot be done by setting a threshold value. We tested whether black areas on the wing indeed vary in the intensity of 'darkness', by comparing sexual and seasonal differences from one point on an apical black region of UPFW that is black for all individuals (Figure S3).

In addition, we acquired UV photographs of two specimens each of males and females. We used a Nikon D70 camera with a CoastalOpt 60 mm UV–VIS–IR Apo Macro lens and a Baader U-filter 60 nm HBW/320–380 nm photographic filter that prevented light outside 320–380 nm range from passing through the lens. Furthermore, in order to compare the photographic method with actual reflectance and absorbance of the wing surfaces, we collected reflectance data on the wing surface of 10 specimens using a spectrophotometer. The UV images and a plot of wing reflectance are provided in the supplementary material as a comparison with our Adobe RGB method (Figure S1 and Figure S2).

UV photographs of the specimens revealed that this species has no specialised UV reflectance in any parts of the wings (Figure S1). Results depicting the reflectance of the wing surface based on spectrophotometer readings concur with the RGB results largely (Figure S2). Therefore, we did not sample colouration in the UV range, relying solely on the RGB method for this study.

Statistical analysis

We used R 3.1.3 for statistical analyses (Team, 2013). For the preparation of plots, we used the ggplot2 package in R (Wickham, 2016). We used generalised linear models (GLMs)

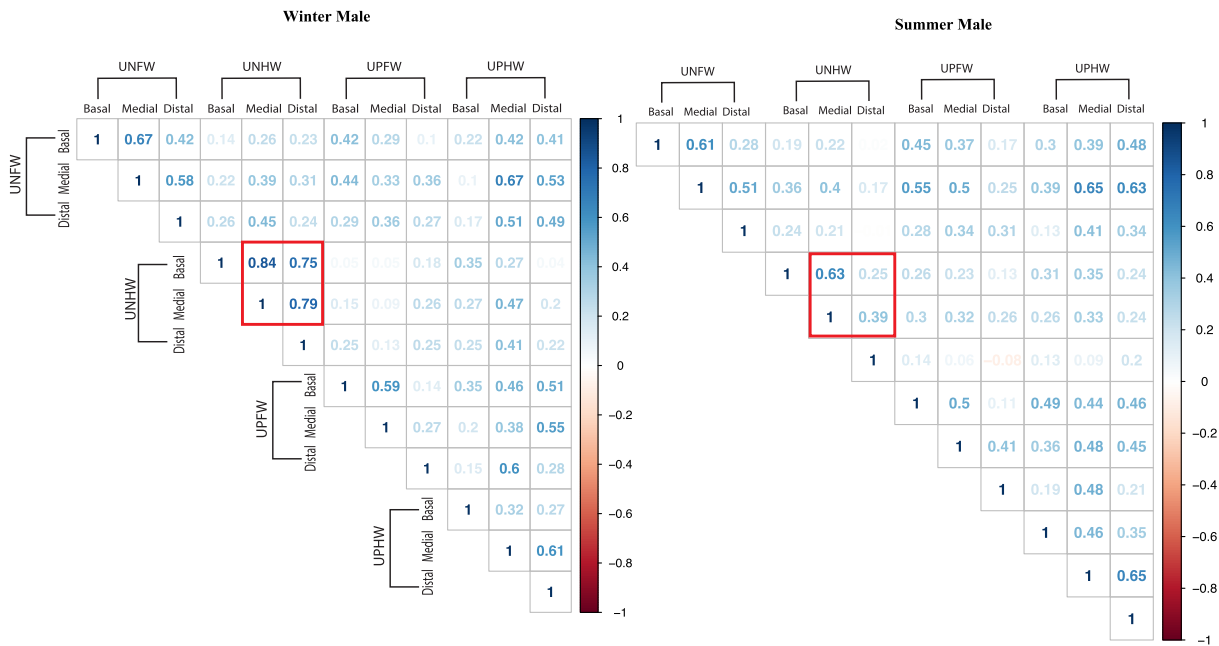


Figure 2. Correlation plots representing seasonal variation in positively covarying wing regions of UNHW in males. Comparisons of correlation coefficients of three regions of UNHW are highlighted with a red square. [Colour figure can be viewed at wileyonlinelibrary.com].

to investigate whether melanisation correlates with elevation as a clinal variation. We calculated wing melanisation by calculating the mean of means of R, G, and B values from all 12 points and then subtracting the mean from the maximum possible RGB value (i.e. 255).

$$\text{Wing melanisation} = 255 - \text{MeanRGB}$$

Therefore, a higher value of wing melanisation signifies a higher degree of darkness. We analysed elevational, sexual, and seasonal differences in wing melanisation using GLMs with gamma error distribution, as the variance of wing melanisation was not constant across elevations. We included wing melanisation as the response variable in our model and included elevation, sex, season, the interaction between elevation and sex, and interaction between sex and season as the explanatory variables, as follows

$$\text{Gamma GLM} = \text{Wing melanisation} \sim \text{Elevation} + \text{Sex} + \text{Season} + \text{Elevation} * \text{Sex} + \text{Sex} * \text{Season}$$

In order to quantify the correlation between melanisation of three wing regions (basal, medial, and distal) on different wing surfaces, we first pooled together RGB sampling points for each of these regions and calculated region-specific melanisation. To confirm that our pooling method was not completely arbitrary, we compared the correlation coefficients of the 12 points on UNFW with the points that we used to define different wing regions (Figure S5). We then performed multivariate correlation analysis and plotted correlational plots for all four wing surfaces (Fig. 2, and Figure S4). To identify and corroborate the

patterns of correlations between different wing regions, we also performed a Principal Component Analysis (PCA) on the same data (Table S1). We have reported PC loadings, eigenvalues, and %variance for each axis explaining more than 5% of the variation in wing melanisation of each group. A high correlation between any of the wing regions or surfaces would imply strong integration, where integration is defined as a propensity of traits to varying jointly, in a coordinated way across a morphological structure (Klingenberg, 2009).

To investigate the change in activity patterns and relative abundance, we used GLMs with a Poisson error distribution. For this analysis, we divided the elevational gradient into six elevational sections, viz., 300–799 m, 800–1299 m, 1300–1799 m, 1800–2299 m, 2300–2799 m, and 2800–3300 m. For all the seasonal analyses, we categorised winter as the months of November, December, January, and February (months with an average monthly temperature value of ~20 °C or less); and summer of March, April, and May (months with an average monthly temperature value of more than ~20 °C). We included the time of day with both a linear and a quadratic term as the trends suggested a quadratic relationship. There was an indication of heteroscedasticity in residuals yet we decided to run models with Poisson error distribution following the principle of parsimony.

Results

Change in wing melanisation with elevation

The amount of wing melanisation increased with elevation only in the case of ventral hindwing (UNHW) for both males

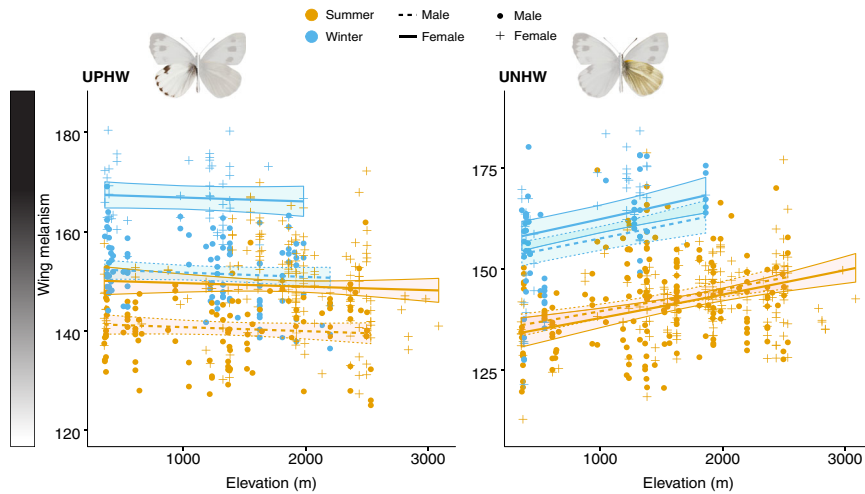


Figure 3. Elevational, seasonal, and sexual differences in wing melanisation of hindwing surfaces (GLM, see Table 2). UPHW, dorsal hindwing and UNHW, ventral hindwing. Seasonal differences in wing melanisation are represented as summer (brown) and winter (blue). Sexual differences in wing melanisation are depicted as female (solid line) and male (dotted line). Data points are depicted as filled circles (males) and plus signs (females). The shaded area around the line depicts standard errors. [Colour figure can be viewed at wileyonlinelibrary.com].

and females (Fig. 3 and Table 2). Melanisation in other three wing surfaces (i.e. dorsal forewing (UPFW), dorsal hindwing (UPHW), and ventral forewing (UNFW)) did not vary with elevation (Figs. 3 and 4 and Table 2). The coefficient estimates from the GLM model are given in Table 2. Overall, these results suggested that variation in wing melanisation showed a wing surface-specific response to change in the elevation.

Sexual and seasonal differences in wing melanisation

Wing melanisation in females was significantly higher than in males for any given season (Figs. 3 and 4 and Table 2). This was true for all wing surfaces except UNHW, for which there was no difference in wing melanisation between males and females (Fig. 3 and Table 2). This indicated that females perhaps have higher thermal requirements in comparison to males. Wing melanisation in both sexes was higher in winter than in summer for all the wing surfaces (Figs. 3 and 4 and Table 2), lending additional support to the relevance of wing melanisation in thermoregulation. Moreover, higher melanisation of constant black wing regions (Figure S3) indicated that not only does the area of melanised wing surface change, but so does the ‘darkness’.

Correlation patterns between basal, medial, and distal wing regions

Correlation analysis suggested seasonal patterns of wing region integration (Fig. 2). These results bring to light relatively high integration between different wing regions of UNHW in winters as compared to summers, regardless of sex. Since seasonal comparisons of positively covarying regions of UNHW were qualitatively similar for both sexes, we have presented the results for females in supplementary figures (Figure S4). Results

from the principal component analyses qualitatively corroborate these patterns (Table S1).

Activity patterns and relative abundance

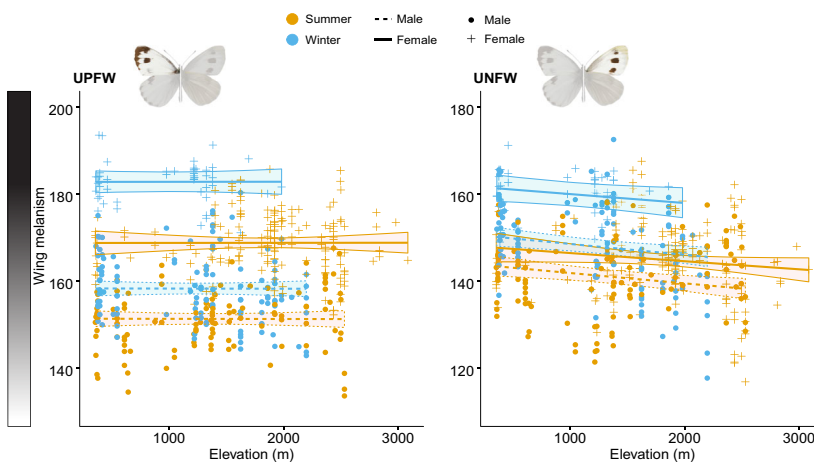
The fitted curve for relative abundances and time of day suggested that the length of activity periods varied with elevations and seasons. During winters, the length of the activity period reduced with increasing elevations (Fig. 5). On the other hand, for a given elevational section, an increased area under the curve in summer indicated an increase in activity periods as compared to the activity periods in winter. A comparison of relative abundances showed that butterflies were generally more abundant in summers than in winters. The lowest elevational section (300–800 m) was an exception where butterflies were more abundant in winter. Patterns of relative abundance across elevation in winters showed that lower elevations supported the highest numbers of butterflies, whereas, in summer, mid-elevations supported the highest number of butterflies. These results suggested that time of day significantly affected the activity in winters, but it had no effect on butterfly activity in summers (Table 3).

Discussion

Here we analysed spatial, seasonal, and sexual variation in wing melanisation in a montane butterfly across a Himalayan elevational gradient. To our knowledge, this is one of the few studies to show wing surface-specific changes in butterfly wing melanisation in response to elevation and season. Consistent associations of darker phenotypes with colder environments suggested the role of melanisation in thermoregulation, but the pattern of variation showed little support for the sexual selection and predator avoidance hypotheses based on the predictions

Table 2. Parameter estimates from GLM analysis with gamma error distribution showing trends in different wing surface melanisation (viz., UNFW, UPFW, UPHW, and UNHW) with elevation, sex, and seasons.

	Estimate	SE	<i>t</i> value	<i>P</i>
UNFW (Null deviance 2.1950, 453 df. Residual deviance 1.5737, 448 df)				
(Dispersion parameter for Gamma family taken to be 0.003462711)				
Intercept	4.999	1.366e-02	366.080	<2e-16
Elevation	-1.292e-05	7.025e-06	-1.839	0.0665
Sex (male)	-2.783e-02	1.677e-02	-1.659	0.0977
Season (winter)	8.855e-02	1.143e-02	7.744	6.47e-14
Elevation × sex	-4.015e-06	9.146e-06	-0.439	0.6609
Sex × season	-4.249e-02	1.380e-02	-3.079	0.0022
UPFW (Null deviance 2.47153, 456 df. Residual deviance 0.86703, 451 df)				
(Dispersion parameter for Gamma family taken to be 0.001922514)				
Intercept	5.128	9.945e-03	515.593	<2e-16
Elevation	1.071e-07	5.114e-06	0.021	0.983298
Sex (male)	-1.088e-01	1.232e-02	-8.836	<2e-16
Season (winter)	7.993e-02	8.344e-03	9.579	<2e-16
Elevation × sex	-3.413e-07	6.732e-06	-0.051	0.959592
Sex × season	-3.495e-02	1.015e-02	-3.443	0.000628
UPHW (Null deviance 2.1648, 440 df. Residual deviance 1.0994, 435 df)				
(Dispersion parameter for Gamma family taken to be 0.002519943)				
Intercept	5.013	1.143e-02	438.430	<2e-16
Elevation	-4.747e-06	5.883e-06	-0.807	0.4201
Sex (male)	-5.997e-02	1.425e-02	-4.209	3.12e-05
Season (winter)	1.088e-01	9.570e-03	11.371	<2e-16
Elevation × sex	-1.020e-06	7.831e-06	-0.130	0.8964
Sex × season	-3.377e-02	1.177e-02	-2.870	0.0043
UNHW (Null deviance 3.5066, 453 df. Residual deviance 2.2767, 448 df)				
(Dispersion parameter for Gamma family taken to be 0.005068167)				
Intercept	4.885	1.655e-02	295.146	<2e-16
Elevation	4.128e-05	8.550e-06	4.828	1.9e-06
Sex (male)	1.485e-02	2.086e-02	0.712	0.477
Season (winter)	1.635e-01	1.427e-02	11.460	<2e-16
Elevation × sex	-2.805e-06	1.162e-05	-0.241	0.809
Sex × season	-4.196e-02	1.866e-02	-2.249	0.025

**Figure 4.** Elevational, seasonal, and sexual differences in wing melanisation of forewing surfaces (GLM, see Table 2). UPFW, dorsal forewing; UNFW, ventral forewing. Seasonal differences in wing melanisation are represented as summer (brown) and winter (blue). Sexual differences in wing melanisation are depicted as female (solid line) and male (dotted line). Data points are depicted as filled circles (males) and plus signs (females). The shaded area around the line depicts standard errors. [Colour figure can be viewed at wileyonlinelibrary.com].

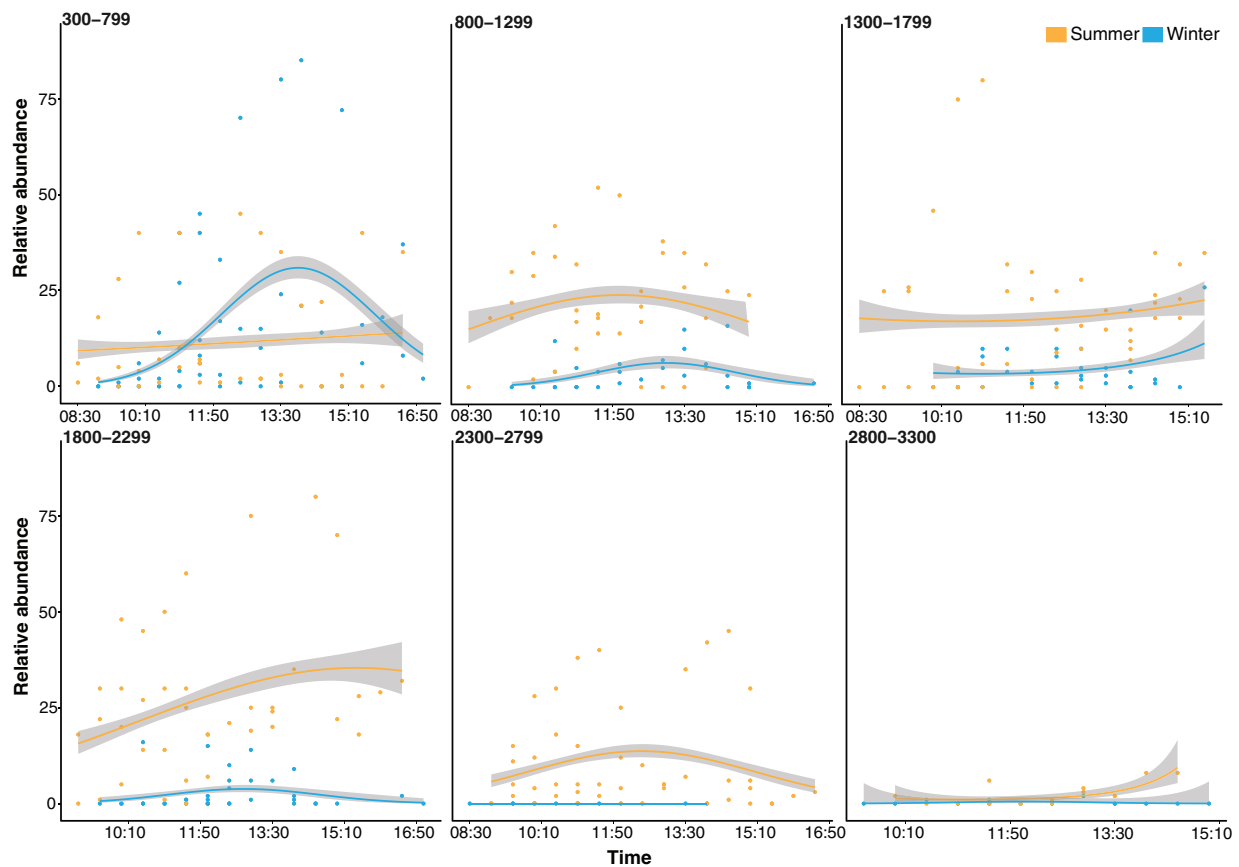


Figure 5. Relative abundance and activity period of *P. canidia* in summer (brown) and winter (blue) for different elevational sections (for results from generalised linear regression, see Table 3). [Colour figure can be viewed at wileyonlinelibrary.com].

outlined above. Thus, the variation in wing melanisation in *P. canidia* supported the thermal melanism hypothesis.

Broadly, in agreement with the findings in other pierids (Kingsolver & Wiernasz, 1991; Stoehr & Goux, 2008; Stoehr & Wojan, 2016), *P. canidia* showed elevational as well as seasonal plasticity in melanisation on ventral hindwing without any sex-specific differences, whereas melanisation on other wing surfaces showed only seasonal plasticity and variation in a sex-specific manner. Surprisingly, our results showed seasonally varying positive correlations between different wing regions, which stands in contrast to the patterns described in other species where melanisation of distal dorsal portions are shown to be negatively correlated with the basal portions. Notably, distal regions had lower correlation coefficients in summer butterflies, suggesting weakly correlated variation between distal and basal regions in summer months. However, it is not clear whether these differences stem from the fundamental disparities in our methods or from actual biological differences between these species. Further studies may shed light on this.

Plasticity in wing melanisation of lepidoterans is driven by photoperiod and/or temperature to which early life stages are exposed (Shapiro, 1976). Shorter photoperiods and/or lower temperatures result in greater wing melanisation, that is in darker phenotypes (Kingsolver, 1996; Stoehr & Wojan, 2016). Wing

melanisation improves thermoregulation, thus increasing the fitness of resident individuals within a given thermal condition (Kingsolver & Watt, 1983; Kingsolver & Wiernasz, 1991; Ellers & Boggs, 2004; Hegna et al., 2013). Our results showed surface-specific melanisation patterns in response to elevation and season, and suggested two possibilities: first, there is perhaps a threshold of photoperiod or temperature beyond which all four wing surfaces exhibit an increase in melanisation; or second, that temperature may be the cue for change in melanisation for UNHW, as temperature changes considerably with an increase in elevation but the photoperiod more or less remain unchanged. It is possible that a seasonally changing photoperiod serves as a cue that drives wing melanisation in UPFW, UPHW, and UNFW. Detailed experiments are needed to tease apart these possibilities in the future.

Thermoregulation is not only a function of morphology but a combination of morphology and behaviour. *Pieris* butterflies use lateral (Kingsolver, 1995), dorsal, and reflectance basking behaviour (Watt, 1968). Of these, lateral basking was observed to be frequently used by *P. canidia* during early mornings and late evenings when sunlight was mild. Across seasons, when there is a considerable change in temperature, such a behavioural strategy is perhaps not adequate to cope with a broader temperature change. This may lead to increased

Table 3. Results from GLM with Poisson error distribution with relative abundance as dependent variable and time of day as an independent variable (including both linear and a quadratic term).

	Estimate	SE	Z value	P
300–799 m, winter (Null deviance 1274.95, 49 df. Residual deviance 937.62, 47 df)				
Intercept	–6.696e-01	2.431e-01	–2.754	<0.01
Time of day	2.521e-02	1.785e-03	14.126	<0.0001
Time of day ²	–3.874e-05	3.057e-06	–12.675	<0.0001
300–799 m, summer (Null deviance 686.17, 37 df. Residual deviance 680.36, 35 df)				
Intercept	2.229	1.403e-01	15.883	<0.0001
Time of day	9.405e-04	1.361e-03	0.691	0.49
Time of day ²	–1.933e-07	2.812e-06	–0.069	0.94
800–1299 m, winter (Null deviance 149.76, 28 df. Residual deviance 122.82, 26 df)				
Intercept	–2.153	8.054e-01	–2.673	<0.01
Time of day	2.906e-02	6.570e-03	4.422	<0.0001
Time of day ²	–5.284e-05	1.255e-05	–4.211	<0.0001
800–1299 m, summer (Null deviance 387.71, 35 df. Residual deviance 378.78, 33 df)				
Intercept	2.713	1.395e-01	19.451	<0.0001
Time of day	4.442e-03	1.525e-03	2.912	<0.01
Time of day ²	–1.059e-05	3.748e-06	–2.824	<0.01
1300–1799 m, winter (Null deviance: 184.14, 28 df. Residual deviance 167.45, 26 df)				
Intercept	1.609	6.448e-01	2.495	<0.05
Time of day	–4.841e-03	5.228e-03	–0.926	0.3544
Time of day ²	1.620e-05	9.853e-06	1.645	0.1001
1300–1799 m, summer (Null deviance 726.38, 43 df. Residual deviance 721.16, 41 df)				
Intercept	2.888	1.228e-01	23.515	<0.0001
Time of day	–7.545e-04	1.245e-03	–0.606	0.545
Time of day ²	3.124e-06	2.768e-06	1.129	0.259
1800–2299 m, winter (Null deviance 221.08, 35 df. Residual deviance 202.81, 33 df)				
Intercept	–1.677	8.275e-01	–2.027	<0.05
Time of day	2.320e-02	6.707e-03	3.459	<0.001
Time of day ²	–4.464e-05	1.313e-05	–3.400	<0.0001
1800–2299 m, summer (Null deviance 556.15, 38 df. Residual deviance 497.54, 36 df)				
Intercept	2.619	1.243e-01	21.068	<0.0001
Time of day	4.544e-03	1.097e-03	4.141	<0.0001
Time of day ²	–5.453e-06	2.092e-06	–2.606	<0.01
2300–2799 m, summer (Null deviance 682.11, 46 df. Residual deviance 648.67, 44 df)				
Intercept	1.474	1.914e-01	7.701	<0.0001
Time of day	9.563e-03	1.781e-03	5.370	<0.0001
Time of day ²	–2.004e-05	3.640e-06	–5.504	<0.0001
2800–3300 m, winter (Null deviance 13.347, 14 df. Residual deviance 11.433, 12 df)				
Intercept	–5.6692547	4.7876507	–1.184	0.236
Time of day	0.0470903	0.0453029	1.039	0.299
Time of day ²	–0.0001101	0.0001018	–1.081	0.280
2800–3300 m, summer (Null deviance 51.009, 14 df. Residual deviance 29.902, 12 df)				
Intercept	1.32	1.614	0.817	0.4137
Time of day	–1.647e-02	1.370e-02	–1.202	0.2292
Time of day ²	5.287e-05	2.735e-05	1.933	0.0532

GLM was not performed for 2300–2799 m winter, because there were no butterflies at these elevations during winter.

investment in morphology (increased wing melanisation in all four wing surfaces), which may explain the observed seasonal patterns. Morphological changes, however, might be a costly investment for dealing with relatively smaller changes in temperatures, which may perhaps be dealt with slight modifications in time allocation rules (e.g. increased time for basking vs feeding). Therefore, investing in a costlier strategy may only be worthwhile when coping with larger changes in temperatures, that is with a change in seasons. Such time and resource allocation tradeoffs are well known in life-history trait evolution (Lima & Dill, 1990; DeWitt, Sih, & Hucko, 1999; Steiner &

Pfeiffer, 2007), and their role in the context of the evolution of thermal reaction norms is also reasonably known (Angilletta, Wilson, Navas, & James, 2003).

Correlation coefficients between different wing regions (basal, medial, and distal) and wing surfaces suggested a seasonally varying degree of integration between basal and distal regions of the wing. Importantly, higher integration of wing regions on UNHW in winter may indicate that challenges posed by a broad range of seasonal changes in temperature require an increase in the melanised area of the entire wing. On the other hand, lower integration in summers perhaps indicates that thermoregulatory

requirements may be met without changing the melanisation of the distal wing region. Seasonally varying positive covariation, combined with seasonal and elevational differences in wing melanisation, suggest the presence of two loosely dependent “modules”: (i) under hindwing (exhibits seasonal ‘modularity’), and (ii) the remaining three wing surfaces (upper forewing and hindwing, and under forewing). It is possible that each of these ‘modules’ might have been selected for different kinds of variation in temperature. Melanisation in UNHW may have been selected to be responsive to a narrow range of variation in temperature or for crypsis, whereas melanisation of the other three wing surfaces may represent selection to respond to a broader range of temperature variation, perhaps assisting in thermoregulation.

We observed greater melanisation in females as compared to males in any given season. These results were consistent with other studies, which showed that higher wing melanisation in females caused higher body temperature, which in turn was associated with increased oviposition and egg maturation rates (Ellers & Boggs, 2004). However, the level of sexual dimorphism that we demonstrated in *P. canidia* is striking and offers opportunities to study how natural selection may mediate sexual dimorphism via thermal ecological demands on the biology of the sexes. Whether differences in other wing regions are driven by sexual selection or some other factors remain to be seen.

Daily flight activity is a crucial determinant of whether individuals are able to track resources, escape from predators, and search for mates, which affect individual fitness (Ellers & Boggs, 2004; Clusella Trullas et al., 2007). Observed activity patterns suggested that in the winter months, the optimal activity period was highly constrained at mid and high elevations. On the other hand, daily activity was not affected during the summer, perhaps because the daytime temperature was not a constraint. Such broad seasonal differences suggest that the flight activity limitations imposed by the thermal environment cannot be fully compensated by the existing plasticity of wing melanisation, therefore, suggesting limits to wing melanisation plasticity. However, the direct link between wing melanisation and flight activity remains untested in this species.

Relative abundance patterns across elevations suggested that seasonality played an important role in determining the suitability of habitats. In the winter months, lower elevations seemed to be the most suitable based on a higher abundance of butterflies. In the summer months, however, butterfly abundance in the lower elevations plummeted, suggesting that lower elevations may be too hot during the summer for this species. Such a pattern suggests that global climate change might have major implications for the elevational distribution of this species. Similar trends have been shown in other insects, for example uphill shifts in the lower elevational limits of *Agollinus lapponum*, a species of dung beetle, owing to its lower maximum and minimum thermal tolerance (Birkett, Blackburn, & Menendez, 2018). Given that this species is also considered as an agricultural pest, insights from these patterns may potentially inform on the risk of crop damage.

It remains to be seen whether the extent and type of phenotypic plasticity in wing surface-specific melanisation that we

showed in *P. canidia* may have helped it adapt to the past climatic changes (MacLean, Kingsolver, & Buckley, 2016). It is conceivable that such considerable phenotypic plasticity may physiologically prepare a wide-ranging species such as *P. canidia* to quickly adjust to changing environments. Interestingly, our results also suggest the existence of a limit to plasticity in wing melanisation as pointed out by the sharp decline in populations and flight activity of this butterfly at lower elevations during the hot summer months. A better understanding of such limits to plasticity and evolutionary potential across traits may be useful in crop pests such as *P. canidia*.

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Author Contributions

SG and KK conceived and designed the study. SG collected and analysed the data. SG and KK wrote the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information

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

Figure S1 UV photographs of the upper side (panels on the left) and the underside (panels on the right) of a female (panels on the top) and a male (panels on the bottom) *P. canidia*.

Figure S2 Elevational and sexual differences in wing reflectance of the pale and dark wing areas of the UPFW of specimens from spring, based on the spectrophotometer method. FSH, females in spring from high elevations; FSL, females in spring from low elevations; MSH, males in spring from high elevations; and MSL, males in spring from low elevations.

Figure S3 Sexual and seasonal differences resulting from melanism of one point on the apical black region of UPFW. This region is black for all individuals, regardless of sex and season, hence the resulting differences in darkness are due to varying intensity of darkness.

Figure S4 Correlation plots representing seasonal variation in positively covarying wing regions of UNHW in females. Comparisons of correlation coefficients of three regions of UNHW are highlighted with a red square.

Figure S5 Correlation plots representing correlation coefficients between 12 sampling points on UNFW. Correlation between points within a pre-decided wing region is highlighted with coloured boxes. This plot shows a high correlation between the points that were manually chosen to define different wing regions.

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