Beyond thermal melanism: association of wing melanization with fitness and flight behaviour in a butterfly

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Abstract

Cold developmental conditions can greatly affect adult life history of ectotherms in seasonal habitats. Such effects are mostly negative, but sometimes adaptive. Here, we tested how cold conditions experienced during pupal development affect adult wing melanization of an insect ectotherm, the Glanville fritillary butterfly, Melitaea cinxia. We also assessed how in turn previous cold exposure and increased melanization can shape adult behaviour and fitness, by monitoring individuals in a seminatural set-up. We found that, despite pupal cold exposure inducing more melanization, wing melanization was not linked to adult thermoregulation preceding flight, under the conditions tested. Conversely, wing-vibrating behaviour had a major role in producing heat preceding flight. Moreover, more melanized individuals were more mobile across the experimental set-up. This may be caused by a direct impact of melanization on flight ability or a more indirect impact of coloration on behaviours such as mate search strategies and/or eagerness to disperse to more suitable mating habitats. We also found that more melanized individuals of both sexes had reduced mating success and produced fewer offspring, which suggests a clear fitness cost of melanization. Whether the reduced mating success is dictated by impaired mate search behaviour, reduced physical condition leading to a lower dominance status or weakened visual signalling remains unknown. In conclusion, while there was no clear role of melanization in providing a thermal advantage under our seminatural conditions, we found a fitness cost of being more melanized, which potentially impacted adult space use behaviour.

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melanism falls in the realm of responses whereby a detrimental condition early in life is integrated and converted into an advantage in later life, known as predictive-adaptive responses (Nettle & Bateson, 2015; van den Heuvel et al., 2013). However, the ultimate, adaptive explanations of a phenotypic change also depend on the proximate explanations (i.e. the mechanisms underlying it), which can be manifold and complementary. Despite the frequent evidence of melanic forms at higher altitudes and latitudes (reviewed in Clusella Trullas et al., 2007), how consistently central to the thermoregulatory function melanization is across species has recently been revisited in studies showing the complexity of melanization as a trait subject to multiple selective pressures (Stuart-Fox, Newton, & Clusella Trullas, 2017), and by work showing an altitudinal and latitudinal gradient of melanization even in nocturnal moths (i.e. not interacting with solar radiation; Heidrich et al., 2018). Moreover, the impact of melanization on behavioural traits and reproductive performance is less frequently explored.

One fundamental activity of winged insects (Dingle, 1974; Harrison, 1980), which is critically impaired by cold environments, is flight. This is essential for resource acquisition, to escape from predators and to establish new territories, as well as for mate location and courtship (Bell, 1980; Bonte et al., 2012; Dingle, 1972; Eberhard, 1991). Hence, any plastic, genetic or even behavioural modification aiding flight in cold climates is expected to be strongly selected for. Wing melanization has been suggested as one adaptive response in diurnal Lepidoptera which thermoregulate via basking (Guppy, 1986; Kingsolver, 1987; Watt, 1969). For example, a greater proportion of black wing pigments has been shown to increase body temperature during lateral basking (Watt, 1969) in Colias species and flight duration in alpine Colias species (Roland, 1982) and Parnassius phoebeus (Guppy, 1986). However, numerous other behavioural thermoregulatory strategies have also been documented. Insects can use either ectothermic or endothermic thermoregulatory strategies to heat up prior to flight (May, 1979). Resting insects engage in ectothermic thermoregulatory strategies mainly using solar radiation as a source of heat: basking is adopted by species from locusts to butterflies (Clench, 1966; Svensson & Waller, 2013). Even though strategies of endothermic thermoregulation are more commonly observed in moths and dragonflies (May, 1979), wing movements in preparation for flight, such as wing vibration or shivering (Heinrich, 1974), have been also described in diurnal Lepidoptera (Kammer, 1968; Krough & Zeuthen, 1941). Moreover, recent work, using living Vanessa cardui, Satyrium carajaevorus and Parnassius m-album butterflies, has unveiled the mechanisms through which haemolymph flow and androcnial glands support heat transfer across the wing and has confirmed the importance of basking behaviours in thermoregulation (Tsai et al., 2020). Indeed, physiological and behavioural strategies supposedly go hand in hand and are mutually adopted by thermoregulating insects.

Here, we used the Glanville fritillary butterfly, Melitaea cinxia, to test whether cold pupal conditions have long-term consequences for adult wing melanization and behaviour. We closely investigated adult behaviours related to heat absorption preceding flight, reproductive success and space use (i.e. mobilities) under semi-natural conditions. We used a population occurring at the northernmost part of the species’ distribution range, in Finland, where adaptations to cold are expected. The adult butterflies require a body temperature considerably higher than the average ambient temperature to initiate and sustain flight (Nitépold et al., 2009; Saastamoainen & Hanski, 2008), and early life exposure to cold may better prime them to optimize flight. Butterflies typically perform short flight bouts and dorsal basking (Clench, 1966; Kingsolver, 1985; Mattila, 2015) suggesting that modulation of heat absorption plays a role in their flight strategy. We also investigated the consequences of wing melanization for performance traits such as mating behaviour, under the assumption that if thermal melanism aids flight it may also indirectly improve fitness (Saastamoainen & Hanski, 2008). Finally, to shed light on how and when thermally induced melanization takes place, we investigated how melanization varies in different wing parts and the developmental timing of thermally induced melanism (i.e. last larval instar versus pupal stage).

METHODS

Study Species

The Glanville fritillary butterfly has the northernmost limit of its distribution range in the Åland Islands, southwest Finland. At this latitude, the butterfly has a univoltine life cycle with adults flying and reproducing from early June to mid-July, and half-grown larvae diapausing from mid-September to late March (Ojanen, Nieminen, Meyke, Pöyyry, & Hanski, 2013). Snowmelt determines the end of the diapausing phase, but the postdiapause phase (including at least two third larval instars and the pupal stage) can face cold spells delaying development (Kallioniemi & Hanski, 2011) and reducing the activity of adults with implications also for reproductive performance (Saastamoainen, 2007a). The average temperature in mid-June ranges from 8 to 20 °C (Finnish Meteorological Institute, 1981–2010 long-term data), yet adults need to reach a body temperature above 28 °C to be able to take off and perform flight bouts; reproductive performance in a seminatural set-up is also linked to prevailing thermal conditions (Saastamoainen & Hanski, 2008).

Ethical Note

The Glanville fritillary butterfly is not endangered or protected. Hence, no federal permits were required to perform this research. The insects were handled carefully through all procedures to minimize individual stress or injury by using round-end tweezers and butterfly nets and by handling the individuals only when strictly necessary. Numbers of individuals were kept low to minimize crowding or any form of stressful rearing conditions. We ensured that they were kept in large containers and cages to avoid additional stress due to growing. Adult butterflies were kept in indoor butterfly cages or in a large outdoor enclosure (see below) until they died, except for 186 individuals which were snap-frozen for wing dissection. We adhered to the ASAB/ABS guidelines for the treatment of animals.

Butterfly Rearing and Experimental Set-Up

Post-diapausing larvae were rearred under controlled laboratory conditions (28:15 °C, 12:12 h light:dark (LD)) and fed ad libitum with leaves of the host plant Plantago lanceolata. For logistical reasons, we used different sets of individuals for different experiments. Wing melanization and development time were recorded for a mixture of individuals collected in the wild in the summer and autumn of 2015 (N = 446) and for F1 individuals bred from wild individuals collected in autumn 2014 (N = 108). The life history experiment in an outdoor enclosure was performed on a subset of 163 of the 2015 wild-collected individuals. The final experiment on when thermally induced wing melanization occurs was based on an additional set of individuals collected as diapausing larvae in the Åland Islands in autumn 2014 (N=186).
**Impact of Cold Pupal Conditions on Adult Melanization and Behaviour**

The average temperature in Åland during the pupal period (between mid-May and mid-June) in the 5 years preceding the experiment (2011–2015) was 12.2 °C, and peaked at 26.8 °C (Finnish Meteorological Institute). With our pupal treatment we wanted to imitate these conditions as opposed to those of individuals in fully sunny areas (i.e. our control treatment) and thus to investigate the impact of pupal temperature on adult wing melanization and behaviour. In April 2016, we exposed 292 pupae to cold cycles (18:12 °C, 12:12 h LD) of 40 h (1 day and 2 nights), alternated by 3 days in standardized rearing conditions (28:15 °C, 12:12 h LD), to allow development to progress. The control group (\(N = 262\)) developed in standardized conditions. The cold cycle was initiated on the second day of the pupal stage and was carried out three times until eclosion. These 554 individuals were all assessed for development time and wing melanization while alive. Wing area and darkness were measured digitally with the software ImageJ (v 1.50i, http://rsbweb.nih.gov/ij). Since it is not known how wing melanization is increased in this species, and since the black patterns are evenly spread across the wings, we measured the darkness of the whole surface represented by the left forewings and hindwings fully open. Additional measures of the increase in melanization in different wing portions are presented below. Pictures were taken with a Canon EOS 30D, with parameters standardized for all images (f/8 focal ratio, 1/25 s exposure time, ISO-100 ISO speed, + 1 step exposure bias, 50 mm focal length, no flash, aperture priority exposure program, manual white balance). Two identical light sources (colour temperature 6500 K, luminous flux 580 lm) were placed 24 cm apart and 12 cm from the focal point in a dark room. Wing area was calibrated on a standard ruler for all images, while darkness consisted of the default grey value provided by the software (255 = white, 0 = black). The values were rescaled so that darker wings corresponded to greater values by subtracting all measures from 255 (i.e. pure white). As the portion of the hindwings close to the thorax is often covered by hair, we also measured the extent of this area (referred to as ‘hairiness’ below) as hairs may be potentially relevant for thermoregulation.

A subset of 163 butterflies was used for assessing the effect of pupal cold exposure on adult behaviours in seminatural conditions in a large outdoor enclosure (32 x 26 m and 3 m high; Hanski, Saastamoinen, & Ovaskainen, 2006). Adult butterflies were individually marked with unique IDs on the underwings with a permanent marker to allow recognition in the enclosure, which was developed in standardized conditions. The central portion hosted 100 potted plants, which were used by the females for oviposition. We synchronized oviposition plants were monitored constantly by at least one person. Lifetime fecundity was estimated as the total number of eggs produced by a female. Number of clutches and their order were also assessed. In most cases females mate once (Saastamoinen, 2007b) and lay on average three egg clutches in their lifetime (Saastamoinen, 2007a). In addition, male paternity was traced from mating and oviposition records; in this species offspring are sired by the last male a female has mated with (Sanhar & Kokko, 2007). The enclosure was divided into an 8 x 8 grid and the location of butterflies was systematically censused every second hour (i.e. 0900–1700 on sunny days; see Rosa & Saastamoinen, 2017 for details) to obtain estimates of individual space use (i.e. ‘mobility’) throughout the experiment (Hanski et al., 2006). Mobility was quantified as the residuals of the linear regression of the number of grid squares where a butterfly was recorded against the number of observations (Hanski et al., 2006). Finally, information on when individuals were last observed alive was available from the daily censuses and was used as a proxy of life span. The experiment ended when all the butterflies died naturally.

To assess long-term effects of pupal cold exposure on adult thermoregulatory potential, we also took thermal images of the 163 adult individuals upon release in the outdoor enclosure. These were taken at midday on four sunny mornings between 0930 and 1230 (temperature range: -20–50 °C; measurement uncertainty: 2% of measured °C). Butterflies were kept in a cool box (temperature ca. 10.5 °C) prior to assessment to minimize the impact of air temperature and placed individually on a white polystyrene platform only when imaged. Since the flight muscles are in the thorax (Mattila, 2015), we measured thorax temperature from the moment a butterfly started basking until it took off. Basking always took place in full sun. Thorax temperatures were measured digitally from the thermal images at any given time by drawing a straight line in the middle of the thorax (PYROSOFT Compact, v 2.12.1). The presence/absence of wing-vibrating activity in preparation for flight was also scored during the thermal imaging, as well as the local ambient temperature at release (Finnish Meteorological Institute).

**Pattern and Timing of Melanization**

We measured the melanization of wings and thorax and tested whether cold conditions experienced during the last larval instar also induced more wing melanization (see below). We assessed the melanization specifically within the basal forewing, because it is reported to be the main area involved in heat transfer (Van Dyck & Matthysen, 1998; Wasserthal, 1975). To do so, we measured the mean grey value of this part of the wing between the second black marking close to the thorax and the wing junction to the thorax (Fig. 1a) to ensure repeatability across images on the 554 live individuals. We also measured the remaining (distal) part of the forewing excluding the basal portion and reassembled the mean grey value of the whole surface consisting of the left hindwing and forewing but focusing on the black patterns alone. This was done by converting the orange wing areas to white (Fig. 1b) with Adobe Photoshop CS5.1: the colour scheme of each image was converted to black and white with a default function, and yellow and red components were selectively set to white. Thorax melanization was also assessed. However, butterflies moved their thorax during the measurements and the thorax area openly visible was not the same across different pictures, and hence we used the average darkness of five random points on the central portion of the thorax instead of the full area.
To better understand the temporal determination of thermally induced wing melanization, we performed a similar experiment as above, but exposing individuals to cold either in the last larval instar (N = 78) or in the pupal stage (N = 53). To ensure that the effect of cold-induced melanization would better match the conditions when pupae and larvae overlap in the field (i.e. earlier in the pupal period), we used slightly colder conditions than above (12:8 °C, 12:12 h, LD), while the control group (N = 55) developed in the usual standardized conditions. One-day-old adults were frozen at -80 °C. Wings were dissected and pictures of the dorsal left forewing and hindwing were taken under the same controlled light conditions as above.

Statistical Analyses

All data were analysed with R for Windows (v 3.3.2; package lmerTest, Kuznetsova, Brockhoff, & Christensen, 2016). We used a linear mixed model approach for all response variables, with population or family of origin as random factor for all models. The main fixed effects were pupal treatment, sex and wing melanization and all two- and three-way interactions, and wing area was included as covariate in all models. Additional covariates varied depending on the response variable. Model selection was performed based on the AIC criterion using the step() R function starting from a full model including all interactions and allowing reduction of all factors except for the random effects. Effect sizes for treatment, sex and other binary factors were calculated as Cohen’s d (magnitude of the effect considered ‘negligible’ for |d|<0.2, ‘small’ for |d|<0.5, ‘medium’ for |d|<0.8, otherwise ‘large’). Detailed models are available in Appendix Tables A1–A5.

We assessed the effect of pupal cold treatment on development time, wing area, wing melanization and hindwing hairiness, as well as on the thermal conditions at which important activities such as mating and oviposition were performed. However, there was an initial difference in age and temperature at release between the two treatment groups. As most of the females mated immediately after release, mating temperature varied with release temperature and age (Appendix Table A1); hence, potential effects of treatment could be biased by these confounding factors. To take this bias into account we included age and temperature at release as covariates in the models. In addition, many females (cold: 24/33; control: 12/26) laid their first egg clutch on the same day as their release. Hence, clutches laid on the release day were excluded from the analysis on oviposition temperature, and age was included as a covariate in the models. Additional fixed effects for oviposition temperature were female treatment, wing melanization, clutch rank (i.e. the order of oviposition) and all their interactions, while female ID was included as a random effect (to account for multiple ovipositions by the same female), and an average of daily temperature was also included as a covariate. Adult reproductive performance was assessed with both a binary response variable assessing whether an individual mated or not and lifetime fecundity (i.e. total number of eggs produced by mated adults). We assessed adult mobility and the number of days each butterfly was observed in the enclosure was used as a proxy of life span. In the models for mobility, release age and wing hairiness were also included as covariates. Because of a 3-day cold spell when butterflies were inactive, and after which the daily average temperatures were lower (Appendix Fig. A1), temperature at oviposition and mobility were assessed separately during the period preceding the cold spell, as well as throughout the experiment (see detailed models in Appendix Table A1). We performed a principal component analysis (PCA) on four variables assessing the thermoregulatory ability of basking butterflies (Appendix Tables A2, A3): thorax heating rate during the first 30 s (i.e. linear phase), the duration of the basking period, final thorax temperature at which a butterfly took off and the temperature increment during the basking period. Each PC was then used as a response variable in models including pupal temperature, sex, wing melanization and their interactions, age, wing area, wing hairiness and presence of flight-preparatory wing-vibrating activity as fixed factors. The ambient temperature at take-off was also included as a covariate to account for temperature differences on different days.

Finally, the effect of cold treatment was tested on all additional response variables assessing the increase in melanization and on wing melanization following the slightly colder treatment with larvae versus pupae (Appendix Table A5).

RESULTS

Impact of Cold Pupal Conditions on Adult Melanization and Behaviour

Pupal cold conditions delayed development by over 3 days and led to significantly more melanized wings (Appendix Table A1). Males developed slightly faster and were darker than females (Appendix Fig. A2, Table A1). Wing area was not affected by pupal temperature treatment and differed between the sexes, females being larger than males (Appendix Table A1). In both sexes, hindwing hairiness was higher in the control treatment and males were hairier than females (Appendix Table A1). Moreover, hindwing hairiness correlated positively with wing area (Appendix Table A1). Approximately 93% of females and 54% of males mated. Even though mating success was not affected by treatment, unmated individuals had more melanized wings (Fig. 2a, Appendix Table A1) under the conditions tested. Moreover, females had a
higher mating probability than males (Appendix Table A1). Younger and cold-exposed individuals had their matings at higher temperatures (Appendix Table A1). Note, however, that this is probably because of a bias due to the age difference at release between the treatment groups and the variation in weather on the different release days, also indicated by the correlation between mating temperature and release temperature (Appendix Table A1). Before the cold spell, later clutches were laid at higher temperatures and oviposition temperature correlated with the daily average temperature (Appendix Table A1). When tested throughout the experiment, oviposition temperature correlated with clutch oviposition order and daily average temperature (Appendix Table A1) but not with any of the other variables tested. Almost 90% of the mated females laid one or more clutches and 86% of the mated males sired eggs. Females laid on average 462 eggs during the experiment, in up to six clutches. Each mated male sired on average over 150 eggs more than laid by mated females (Appendix Table A1), since successful males typically mated with multiple females. Notably, more melanized individuals had fewer eggs (Appendix Table A1). Mobility before the cold spell correlated positively with wing melanization (Fig. 2b, Appendix Table A1) and was not affected by the other variables. The positive correlation with wing melanization was present also for mobility over the whole experiment, but only in cold-exposed females (Appendix Table A1). Moreover, females had higher total mobility than males, and cold-exposed females were more mobile than controls (Appendix Table A3). This indicates that cold-exposed individuals and those vibrating their wings had a greater temperature increase, basked for longer and took off with a higher thorax temperature. The effect of cold exposure on PC1 may be an artefact of the bias between ambient temperature at release and pupal treatment discussed above. However, PC1 did not correlate with ambient temperature ($P = 0.6$) or age ($P = 0.5$), so the effect of treatment may be real. PC2 (ca. 26% of variance; Appendix Table A2) was mostly related to thorax heating rate, and was not affected by pupal cold exposure (Appendix Table A3, Fig. 3a). It was higher at higher ambient temperatures and in the presence of wing-vibrating activity prior to flight (Appendix Table A3, Fig. 3b). Furthermore, thorax heating rate (PC2) was higher in males and older individuals (Appendix Table A3). Again, the effect of age on PC2 is probably due to a bias between age and temperature at release.

**Pattern and Timing of Melanization**

In general, pupal cold exposure led to more melanized wings, but the magnitude of the effect varied. An increase in melanization due to the black colour component in the images where the orange areas were digitally set to white on the entire left forewing and hindwing surface was detected on individuals exposed to cold pupal conditions (Appendix Table A4). The basal portion was on average darker than the rest of the forewing (rescaled mean grey value ± SE: basal: 201.74 ± 6.95; distal: 182.39 ± 8.63). Moreover, the increase in melanization was not uniform: the basal portion was less affected by pupal thermal conditions than the distal portion (Appendix Table A4). Thorax melanization was not affected by pupal treatment ($P = 0.3$), while males were generally darker than females for all parameters tested (Appendix Table A4), except for black patterns alone, where there was no sex difference ($P = 0.1$). Finally, slightly colder conditions did not induce wing melanization when experienced during the last larval instar ($P = 0.8$; Fig. 4a, Appendix Table A5), but did when experienced during the pupal stage (Fig. 4b, Appendix Table A5). Wing melanization of the individuals exposed to the larval cold treatment was negatively correlated with wing area ($F_{1,127} = 15.93$, $P = 0.0001$), while in the individuals exposed to the pupal cold treatment males were darker than females (Fig. 4b, Appendix Table A5).

**DISCUSSION**

We were interested not only in the extent of thermally induced melanization in an ectotherm occurring in the temperate zone, but also in the implications melanization has for thermoregulation,
individual fitness and behaviour. While the role of thermal melanism is widely acknowledged, potential effects on fitness and behaviour are less commonly assessed although equally relevant. We found that pupal cold exposure induced greater melanization of adult wings, and that pupal cold exposure was potentially linked with increased basking duration prior to flight take-off. Our analysis showed no obvious thermoregulatory benefits of increased melanization under the seminatural conditions tested, while instead wing-vibrating behaviour clearly increased the efficacy of preflight heating. Increased melanization was also correlated with higher mobility, suggesting a potential role of melanization in flight behaviour, which can be coupled with male dominance and space use. However, greater wing melanization reduced both mating success and lifetime fecundity in both sexes, suggesting a clear fitness cost rather than a benefit.

Pupal cold exposure consistently extended development time of pupae and increased wing melanization of adults. Individuals exposed to cold as pupae basked for longer and had a higher thorax temperature at flight take-off (PC1) independent of melanization, and they also mated at times when ambient temperatures were warmer. However, as the cold-exposed individuals were unintentionally released into the enclosure at an older age and on warmer days, these results should be interpreted with caution. Interestingly, we found that greater wing melanization correlated with higher early mobility under the seminatural developmental conditions led to reduced cold tolerance in adults (de Jong & Saastamoinen, 2018). However, we cannot say whether the potentially altered thermoregulation behaviour found here stems from a physical constraint (i.e. cold-exposed individuals are physically unable to take off with cooler body temperature) or from a motivational effect (i.e. they could take off earlier, but they ‘choose’ to reach higher temperatures before take-off; Krackow, 2003; Bonte, Clercq, Zwertvaegher, & Lens, 2009). Note, however, that pupal cold exposure per se did not influence any of the other adult traits such as mating behaviour, mobility or fitness. Notably, the factor that had the greatest impact on both thorax temperature at take-off (PC1) and heating rate (PC2) preceding flight was wing-vibrating behaviour. Wing vibrating allowed for faster thorax heating rates and higher take-off temperatures. Modulation of wing-vibrating behaviour to either produce heat or aid heat flow towards the thorax is known to facilitate thermoregulation in insects, and can be considered analogous to shivering in mammals (Ducatez & Baguette, 2016; Heinrich, 1974; Kammer, 1970; Srygley, 1994). However, this behaviour has a high metabolic cost (Srygley, 1994) compared to basking, and thus is performed only when necessary such as in the absence of direct sunlight (Kammer, 1970) or at low temperatures (i.e. those experienced here before release).

Interestingly, we found that greater wing melanization correlated with higher early mobility under the seminatural

Figure 3. Thorax temperature assessed at intervals of 10 s until take-off. (a) Cold-treated (blue) and control (red) individuals. (b) Individuals that vibrated (orange) and did not vibrate (green) their wings prior to flight. Lines represent model fit and shadings represent the default confidence interval (95%).

Figure 4. Effect of (a) larval and (b) pupal temperature treatments on wing melanization in females (red) and males (blue). Dark red/blue: cold-treated individuals; light red/blue: controls. Mean grey values (see Methods) are rescaled so that higher numbers for wing melanization represent darker wings. Horizontal lines in the box plots represent the median, 25th and 75th percentiles; whiskers include values within 1.5 times the interquartile range and circles represent outliers.
experimental set-up. Melanization may be causally linked to mobility if it facilitates heat transfer during flight, as shown with *P. phoebeus* (Guppy, 1986), or if it increases heating rate prior to take-off thus allowing longer or more frequent flight bouts. Unfortunately, these traits were not assessed in the present study. The impact of melanization on both space use behaviour and mating success may also be linked, as has been found in the speckled wood butterfly, *Pararge aegeria*, in which more mobile patrolling males are darker than more static perching ones (Van Dyck & Matthysen, 1998). In the Glanville fritillary butterfly, patrollers usually have a lower dominance status than perchers, as less fit males are not able to hold mating territories (Niitepõld, Mattila, Harrison, & Hänki, 2011). Consistently, previous work has linked higher mobility with lower physical condition due to larval food stress, especially in males (Rosa & Saastamoinen, 2017). Hence, together our results suggest that more melanized males are of poorer quality and, to compensate for this, potentially need to patrol more to find a mate. Whether the increased patrolling is due to impaired male—male competition or weakened female preference remains unclear. As with females, the increased mobility may be similarly linked with mate-searching behaviour, as more melanized females also had reduced mating success. Alternatively, more melanized/less preferred females may be trying to disperse away from the unfavourable habitat in search of more suitable conditions for their offspring. These ideas should be formally tested. In summary, the correlation between melanization and increased space use may be a direct effect of wing coloration on body temperature in flight. However, melanization also influenced individual condition: first, the more melanized individuals in both sexes also suffered from reduced mating success; second, even when mated, again in both sexes, more melanized individuals had reduced fecundity, calculated as number of eggs produced. Therefore, higher melanization may be impacting space use via condition-dependent changes in space use behaviour.

All the parameters for wing melanization (grey value of total wing surface, grey value when considering only black areas, grey value of distal forewing) were increased by pupal cold treatment to the same extent, with the exception of the basal wing portion, where the effect of treatment was lower. This portion, commonly attributed to butterfly thermoregulation (Wasserthal, 1975), was also in general darker and had lower variance than the dark areas in the rest of the wing. Hence, melanization appeared rather stable in both sexes, more melanized/less preferred females may be trying to disperse away from the unfavourable habitat in search of more suitable conditions for their offspring. This portion, commonly attributed to butterfly thermoregulation (Wasserthal, 1975), was also in general darker and had lower variance than the dark areas in the rest of the wing. Hence, melanization appeared rather stable in both sexes, more melanized/less preferred females may be trying to disperse away from the unfavourable habitat in search of more suitable conditions for their offspring. Whether the increased patrolling is due to impaired male—male competition or weakened female preference remains unclear. As with females, the increased mobility may be similarly linked with mate-searching behaviour, as more melanized females also had reduced mating success. Alternatively, more melanized/less preferred females may be trying to disperse away from the unfavourable habitat in search of more suitable conditions for their offspring. These ideas should be formally tested. In summary, the correlation between melanization and increased space use may be a direct effect of wing coloration on body temperature in flight. However, melanization also influenced individual condition: first, the more melanized individuals in both sexes also suffered from reduced mating success; second, even when mated, again in both sexes, more melanized individuals had reduced fecundity, calculated as number of eggs produced. Therefore, higher melanization may be impacting space use via condition-dependent changes in space use behaviour.

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Melanization of the thorax, as well as of most of the wing traits assessed, was greater in males than in females. Males had also hairier hindwings and a greater heating rate (PC2) than females. All these results indicate sex differences in traits potentially relevant for thermoregulation and may be partially explained by the size difference between the sexes. They may also be a sign of greater flight propensity of males, in line with other work showing that males are typically adapted to take more risks to fly than females, even under suboptimal conditions (Merckx, Karlsson, & Van Dyck, 2006). The abundance of hair or fur on butterfly wings, which has been suggested to increase thermal insulation (Merckx, Van Dongen, Matthysen, & Van Dyck, 2008), was, however, lower in cold-exposed individuals. The reason for this can only be speculated on but may relate to the fact that in colder climates thermal insulation through fur hinders heat absorption via basking, as suggested by Heinrich (1993). At least, ventral fur has been experimentally shown not only to reduce heat transfer in *Colias* butterflies (Kingsolver & Moffat, 1982), but also to minimize convective heat loss during flight (Kingsolver & Moffat, 1982) and to increase with altitude (Kingsolver, 1983). Therefore, there is evidence both in favour of and against our finding. We are unaware of any other studies specifically testing the thermoregulatory function of fur on butterfly wings.

We found that only pupal and not larval cold exposure induced wing melanization in the Glanville fritillary butterfly, despite the two life stages facing similar conditions in the wild. Cold larval conditions act as cues in some butterfly species (e.g. pierid butterflies, *Kingsolver & Wiernasz, 1991*; monarch butterflies, *Danaus plexippus*, Davis, Farrey, & Altizer, 2005), inducing wing melanization and potentially increasing adult thermoregulation. Notably, Lepidopteran wing patterns are generated during the pupal stage (Beldade & Brakefield, 2002), and potentially a direct impact of cold on wing development physiology may explain why we see a pattern only with pupae (Brakefield & French, 1995; Sekimura & Nijhout, 1991). Consistent with this idea, and based on the finding that even nocturnal moths are darker at higher latitudes, Heinrich et al. (2018) suggested a more general relationship between temperature and pigmentation, possibly by means of temperature-dependent developmental traits. As pupal conditions can more reliably predict the conditions adults will face than larval ones (Hoffman, 1978), our finding may also stem from a predictive-adaptive response.

**Conflict of Interest**

None declared.

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Appendix

Table A1: Statistical model selection as output of the step-by-step function and final models for the impact of cold pupal treatment on pupal and adult life history traits

| Model selection | Numerator df | Denominator df | $F^2$ | $P(>F^2)$ | Effect sizes | Magnitude |
|-----------------|--------------|----------------|-------|-----------|--------------|-----------|
| Pupal development time (days) | | | | | | |
| Full model | treatment + sex + treatment*sex + wing area | AIC = 1371.9 | BIC = 1401.6 |
| Final model | treatment + sex | AIC = 1370.6 | BIC = 1391.8 |
| Treatment | 1 | 480.73 | 1758.78 | <0.0001 | 3.24 | Large |
| Sex | 1 | 500.08 | 7.20 | 0.008 | 0.21 | Small |
| Wing area (cm) | | | | | | |
| Full model | treatment + sex + treatment*sex | AIC = 164.1 | BIC = 138.5 |
| Final model | treatment + sex | AIC = 166.3 | BIC = 149.3 |
| Treatment | 1 | 517.18 | 1147.3 | <0.0001 | 2.90 | Large |
| Hindwing hairiness | | | | | | |
| Full model | treatment + sex + treatment*sex + wing area | AIC = 1740.6 | BIC = 1710.8 |
| Final model | treatment + sex + wing area | AIC = 1741.9 | BIC = 1716.3 |
| Treatment | 1 | 485.13 | 4.26 | 0.040 | -0.19 | Negligible |
| Sex | 1 | 516.99 | 101.88 | <0.0001 | -0.52 | Medium |
| Wing area | 1 | 516.67 | 58.52 | <0.0001 | -0.28 | -- |
| Total wing melanization* | | | | | | |
| Full model | treatment + sex + treatment*sex + wing area | AIC = 3409.9 | BIC = 3439.7 |
| Final model | treatment + sex | AIC = 3407.9 | BIC = 3429.1 |
| Treatment | 1 | 482.42 | 24.73 | <0.0001 | 0.30 | Small |
| Sex | 1 | 500.25 | 91.87 | <0.0001 | -0.74 | Medium |
| Mating success (mated/unmated) | | | | | | |
| Full model | treatment + sex + melanization + treatment*sex + treatment*melanization + sex + treatment*sex*melanization + wing area | AIC = 151.0 | BIC = 181.2 |
| Final model | treatment + sex + melanization + sex | AIC = 143.6 | BIC = 158.7 |
| Melanization | 1 | 10.17 | 0.001 | -0.43 | | |
| Sex | 1 | 8.71 | 0.003 | 0.39 | Medium |

(continued on next page)
### Table A1 (continued)

|                                | Numerator df | Denominator df | \(F_{1,2}^2\) | \(P(>F_{1,2}^2)\) | Effect sizes | Magnitude |
|--------------------------------|--------------|----------------|----------------|-----------------|--------------|-----------|
| **Temperature at first mating** |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization + wing area + age + release \) |               |                |                |                 |              |           |
| AIC ~ 272.9                    | BIC ~ 306.3  |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- treatment + release temperature + age \) |               |                |                |                 |              |           |
| AIC ~ 268.8                    | BIC ~ 284.2  |                |                |                 |              |           |
| **Oviposition temperature (before cold spell)** |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + clutch + melanization + treatment*clutch + treatment*melanization + melanization*clutch + treatment*clutch*melanization + wing area + age + daily temperature \) |               |                |                |                 |              |           |
| AIC ~ 235.3                    | BIC ~ 268.9  |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- clutch + daily temperature \) |               |                |                |                 |              |           |
| AIC ~ 224.2                    | BIC ~ 237.1  |                |                |                 |              |           |
| **Oviposition temperature (whole experiment)** |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + clutch + melanization + treatment*clutch + treatment*melanization + melanization*clutch + treatment*clutch*melanization + wing area + age + daily temperature \) |               |                |                |                 |              |           |
| AIC ~ 454.5                    | BIC ~ 496.3  |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- clutch + daily temperature \) |               |                |                |                 |              |           |
| AIC ~ 445.1                    | BIC ~ 461.2  |                |                |                 |              |           |
| **Lifetime eggs produced**     |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization + wing area \) |               |                |                |                 |              |           |
| AIC ~ 1548.3                   | BIC ~ 1577.8 |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- melanization + sex \)      |               |                |                |                 |              |           |
| AIC ~ 1546.0                   | BIC ~ 1559.4 |                |                |                 |              |           |
| **Mobility (before cold spell)** |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization + wing area + hair + age \) |               |                |                |                 |              |           |
| AIC ~ 487.0                    | BIC ~ 526.3  |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- melanization \)           |               |                |                |                 |              |           |
| AIC ~ 476.7                    | BIC ~ 488.7  |                |                |                 |              |           |
| **Mobility (whole experiment)** |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization + wing area + hair + age \) |               |                |                |                 |              |           |
| AIC ~ 784.2                    | BIC ~ 823.4  |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization \) |               |                |                |                 |              |           |
| AIC ~ 783.5                    | BIC ~ 813.7  |                |                |                 |              |           |
| **Mobility (observational data)** |              |                |                |                 |              |           |
| Model selection                |              |                |                |                 |              |           |
| Full model                     |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization + wing area + hair \) |               |                |                |                 |              |           |
| AIC ~ 1013.7                   | BIC ~ 1046.9 |                |                |                 |              |           |
| Final model                    |              |                |                |                 |              |           |
| \(- treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex*melanization + wing area \) |               |                |                |                 |              |           |
| AIC ~ 1003.6                   | BIC ~ 1012.6 |                |                |                 |              |           |

Significant \(P\) values are shown in bold. Akaike (AIC) and Bayesian information criteria (BIC) are presented for the initial full and final models. \(F\) values are shown for all traits except for ‘Mating success’ for which chi-square values are given. Effect sizes for treatment and sex are expressed as Cohen’s \(d\) for continuous response variables and as Cliff’s \(r\) for binary variables. The magnitude of the effect size follows the thresholds: \(|d|<0.2\) ‘negligible’, \(|d|=0.5\ ‘small’, \(|d|\leq 0.8\ ‘medium’, otherwise ‘large’. Effect sizes for continuous effects are expressed as Pearson \(r\). *See Table A4 for additional measures of melanization and Fig. A2 for the interrelations among wing melanization, area, sex and pupal treatment.*
Table A2
Principal components (PCs) describing thermoregulatory patterns of basking butterflies

| Thermoregulatory variable (%) | PC1 | PC2 | PC3 |
|-------------------------------|-----|-----|-----|
| (60.88)                      | (26.46) | (8.60) |
| Heating rate                 | -0.219 | 0.891 | -0.312 |
| Basking duration             | -0.504 | -0.444 | -0.677 |
| Temperature increment        | -0.605 | 0.086 | 0.043 |
| Take-off temperature         | -0.577 | -0.040 | 0.665 |

Correlations of the original variables are shown together with the percentage of variance explained by each PC (in parentheses). Correlations higher than |0.5| are shown in bold and presented in the main text.

Table A3
Statistical model selection as output of the step() function and final models for each thermoregulation-related PC

| PC1 model selection | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|---------------------|--------------|----------------|---|----------|--------------|-----------|
| Full model          | ~ treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex + melanization + wing area + air temperature + wing vibrating + age + hair | 124 | 6.99 | 0.009 | -0.46 | Small |
| Final model         | ~ treatment + wing vibrating | 124 | 19.25 | <0.0001 | 0.81 | Large |

| PC2 model selection | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|---------------------|--------------|----------------|---|----------|--------------|-----------|
| Full model          | ~ treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex + melanization + wing area + air temperature + wing vibrating + age + hair | 124 | 5.68 | 0.019 | -0.30 | Small |
| Final model         | ~ sex + air temperature + wing vibrating + age | 1 | 17.09 | <0.0001 | 0.29 | - |
|                  | Wing vibrating | 1 | 15.04 | 0.0002 | -0.54 | Medium |

| PC3 model selection | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|---------------------|--------------|----------------|---|----------|--------------|-----------|
| Full model          | ~ treatment + sex + melanization + treatment*sex + treatment*melanization + melanization*sex + treatment*sex + melanization + wing area + air temperature + wing vibrating + age + hair | 124 | 5.54 | 0.019 | 0.14 | Negligible |
| Final model         | ~ air temperature | 1 | 95.13 | <0.0001 | -0.85 | Large |

Table A4
Statistical model selection as output of the step() function and final models for effect of pupal temperature on additional melanization features in live individuals.

| Black patterns | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|----------------|--------------|----------------|---|----------|--------------|-----------|
| Model selection | ~ treatment + sex + treatment*sex + wing area | AIC=4012.7 | AIC=4042.5 |
| Final model    | ~ treatment | AIC=4009.9 | AIC=4026.9 |
| Treatment 1    | 490.65 | 8.06 | 0.005 | 0.19 | Negligible |

| Basal wing melanization | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|-------------------------|--------------|----------------|---|----------|--------------|-----------|
| Model selection         | ~ treatment + sex + treatment*sex + wing area | AIC=3411.7 | AIC=3441.5 |
| Final model             | ~ treatment + sex | AIC=3409.4 | AIC=3430.7 |
| Treatment 1             | 496.35 | 5.54 | 0.019 | 0.14 | Negligible |
| Sex 1                   | 516.69 | 95.13 | <0.0001 | -0.85 | Large |

| Distal wing melanization | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|--------------------------|--------------|----------------|---|----------|--------------|-----------|
| Model selection          | ~ treatment + sex + treatment*sex + wing area | AIC=3621.3 | AIC=3651.1 |
| Final model              | ~ treatment + sex | AIC=3618.0 | AIC=3639.3 |
| Treatment 1              | 487.14 | 15.62 | <0.0001 | 0.24 | Small |
| Sex 1                    | 511.25 | 90.78 | <0.0001 | -0.79 | Medium |

| Thorax melanization      | Numerator df | Denominator df | F | Pr(>|F|) | Effect sizes | Magnitude |
|--------------------------|--------------|----------------|---|----------|--------------|-----------|
| Model selection          | ~ treatment + sex + treatment*sex + wing area | AIC=3621.3 | AIC=3651.1 |
| Final model              | ~ treatment + sex | AIC=3618.0 | AIC=3639.3 |
| Treatment 1              | 487.14 | 15.62 | <0.0001 | 0.24 | Small |
| Sex 1                    | 511.25 | 90.78 | <0.0001 | -0.79 | Medium |

(continued on next page)
are expressed as Cohen’s magnitude of the effect size for Cohen’s Pupal treatment

All individuals underwent the same cold pupal treatment described in the main text, plus an immune challenge as adults with either lyophilized bacterial cells (Micrococcus luteus) or phosphate-buffered saline (PBS, as control). The data show no direct correlation between melanization of wings and PO activity, but a negative impact of high PO activity on male life span, only under cold pupal treatment (Fig. A3). Effect sizes for binary factors are expressed as Cohen’s $d$ and for continuous variables as Pearson $r$. The magnitude of the effect size for Cohen’s $d$ follows thresholds $|d|<0.2$ ‘negligible’, $|d|<0.5$ ‘small’, $|d|<0.8$ ‘medium’, otherwise ‘large’.

### Table A4 (continued)

| Effect sizes | Magnitude |
|--------------|-----------|

| Full model | treatment + sex + treatment*sex + wing area | AIC=3825.6 | BIC=3855.4 |
| Final model | sex | AIC=3821.6 | BIC=3838.6 |
| Final model | sex | AIC=768.5 | BIC=780.1 |
| Final model | Wing area | 1 | 127.2 | 15.93 | 0.0001 | -0.33 | – |
| Pupal treatment | treatment + sex + treatment*sex + wing area | AIC=625.7 | BIC=644.5 |
| Final model | sex | AIC=622.8 | BIC=636.2 |
| Final model | sex | 1 | 101.3 | 13.23 | 0.0004 | -0.78 | Medium |

Significant $P$ values are shown in bold. Akaike (AIC) and Bayesian information criteria (BIC) are presented for the initial full and final models. Effect sizes for treatment and sex are expressed as Cohen’s $d$. The magnitude of the effect size for Cohen’s $d$ follows thresholds $|d|<0.2$ ‘negligible’, $|d|<0.5$ ‘small’, $|d|<0.8$ ‘medium’, otherwise ‘large’.

### Table A5

| Effect sizes | Magnitude |
|--------------|-----------|

| Larval treatment | Model selection |
| Treatment | treatment + sex + treatment*sex + wing area | AIC=773.0 | BIC=791.3 |
| Final model | wing area | AIC=768.5 | BIC=780.1 |
| Final model | Wing area | 1 | 127.2 | 15.93 | 0.0001 | -0.33 | – |

| Pupal treatment | Model selection |
| Treatment | treatment + sex + treatment*sex + wing area | AIC=625.7 | BIC=644.5 |
| Final model | sex | AIC=622.8 | BIC=636.2 |
| Final model | sex | 1 | 101.3 | 13.23 | 0.0004 | -0.78 | Medium |

Significant $P$ values are shown in bold. Akaike (AIC) and Bayesian information criteria (BIC) are presented for the initial full and final models. Effect sizes for treatment and sex are expressed as Cohen’s $d$. The magnitude of the effect size for Cohen’s $d$ follows thresholds $|d|<0.2$ ‘negligible’, $|d|<0.5$ ‘small’, $|d|<0.8$ ‘medium’, otherwise ‘large’.

### Table A6

Data from an experiment assessing the role of melanization in terms of immune response, measured as phenoloxidase activity in adult haemolymph (PO)

| Effect sizes | Magnitude |
|--------------|-----------|

| PO after immune challenge | Model selection |
| Treatment | treatment + bacteria + sex + melanization + treatment*bacteria + treatment*sex + bacteria*sex + treatment*melanization + bacteria*melanization + sex*melanization + treatment*bacteria*sex + treatment*bacteria*melanization + treatment*sex*melanization + bacteria*sex*melanization + treatment*bacteria*sex*melanization + wing area | AIC=1449.1 | BIC=1485.2 |
| Final model | bacteria*sex + treatment*bacteria*sex | AIC=1461.7 | BIC=1530.2 |
| Final model | Treatment | 1 | 255.78 | 1.03 | 0.31 | 0.079 | Negligible |
| Final model | Sex | 1 | 260.37 | 29.17 | <0.0001 | 0.59 | Medium |
| Final model | treatment*bacteria | 1 | 256.44 | 6.75 | 0.010 | 0.28 | Small |
| Final model | treatment*sex | 1 | 257.61 | 0.20 | 0.65 | 0.15 | – |
| Final model | bacteria*sex | 1 | 255.34 | 4.03 | 0.046 | – | – |
| Final model | treatment*bacteria*sex | 1 | 258.64 | 0.58 | 0.45 | – | – |
| Final model | Life span | 1 | 257.58 | 9.72 | 0.002 | – | – |

Significant $P$ values are shown in bold. Akaike (AIC) and Bayesian information criteria (BIC) are presented for the initial full and final models. Effect sizes for treatment and sex are expressed as Cohen’s $d$. The magnitude of the effect size for Cohen’s $d$ follows thresholds $|d|<0.2$ ‘negligible’, $|d|<0.5$ ‘small’, $|d|<0.8$ ‘medium’, otherwise ‘large’.

All individuals underwent the same cold pupal treatment described in the main text, plus an immune challenge as adults with either lyophilized bacterial cells (Micrococcus luteus) or phosphate-buffered saline (PBS, as control). The data show no direct correlation between melanization of wings and PO activity, but a negative impact of high PO activity on male life span, only under cold pupal treatment (Fig. A3). Effect sizes for binary factors are expressed as Cohen’s $d$ and for continuous variables as Pearson $r$. The magnitude of the effect size for Cohen’s $d$ follows thresholds $|d|<0.2$ ‘negligible’, $|d|<0.5$ ‘small’, $|d|<0.8$ ‘medium’, otherwise ‘large’.
Figure A1. (a) Number of females present in the cage at any time indicating the pool of potentially ovipositing individuals. (b) Box plots show the average daily oviposition temperature per female treatment throughout the experiment. Horizontal lines in the box plots represent the median, 25th and 75th percentiles; whiskers include values within 1.5 times the interquartile range. The grey dotted curve depicts how daily temperature between 0900 and 1800 varied throughout the experiment. Blue: cold-treated females; red: controls.

Figure A2. Interrelations among wing melanization, wing area (cm²), sex and pupal temperature treatment. Blue: cold-treated males; green: control males; red: cold-treated females; yellow: control females. Circles represent individual butterflies, lines represent model fit and shadings represent the default confidence interval (95%). A general negative correlation exists between wing melanization and wing area ($F_{1,501.3} = 65.80, P < 0.0001$; Pearson correlation: $r = -0.33$). This correlation is due to the sex differences, with males (blue and green) being smaller and more melanized, and females (red and yellow) larger and less melanized.
Figure A3. Female and male (a, b) adult life span and (c, d) phenoloxidase (PO) activity, represented by the slope of increase in absorbance during the linear phase measured at 490 nm, by pupal temperature treatment after an immune challenge with bacteria or phosphate-buffered saline (PBS). Dark grey: cold-treated individuals; light grey: controls. Horizontal lines in the box plots represent the median, 25th and 75th percentiles; whiskers include values within 1.5 times the interquartile range and circles represent outliers.