The adaptive role of melanin plasticity in thermally variable environments

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The authors declare no conflicts of interest.

Author Contributions
SB and GD developed the ideas and concepts for the paper. SB conducted the experiments, data analysis and led the writing of the paper. Both authors contributed critically to the drafts and gave final approval for publication.

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Understanding the evolution of adaptive plasticity is fundamental to our knowledge of how organisms interact with their environments and cope with environmental change. Plasticity in melanin pigmentation is common in response to variable environments, especially thermal environments. Yet, the adaptive significance of melanin plasticity in thermally variable environments is often assumed, but rarely explicitly tested. Furthermore, understanding the role of plasticity when a trait is responsive to multiple environmental stimuli and plays many functional roles remains poorly understood. We test the hypothesis that melanin plasticity is an adaptation for thermally variable environments using *Hyles lineata*, the white-lined sphinx moth, which shows plasticity in melanin pigmentation during the larval stage. Melanin pigmentation influences thermal traits in *H. lineata*, as melanic individuals had higher heating rates and reached higher body temperatures than non-melanic individuals. Importantly, melanin pigmentation has temperature specific fitness consequences. While melanic individuals had an advantage in cold temperatures, neither phenotype had a clear fitness advantage at warm temperatures. Thus, the costs associated with melanin production may be unrelated to thermal context. Our results highlight the importance of explicitly testing the adaptive role of plasticity and considering all the factors that influence costs and benefits of plastic phenotypes across environments.

Introduction

Phenotypic plasticity, the ability of a genotype to produce multiple phenotypes in response to environmental stimuli, is a ubiquitous and diverse phenomenon. Plasticity may be a non-adaptive consequence of the environment. Alternatively, adaptive plasticity may evolve when different phenotypes or trait values are optimal in different environments experienced by the organism (Schmitt et al., 1999; Stearns, 2014). Understanding the evolution of adaptive plasticity is fundamental to our understanding of how organisms interact with their environments and cope with environmental change (Via et al., 1995). Melanin pigmentation is often a plastic trait and is often thought to play a thermoregulatory role (Clusella-Trullas et al., 2007). Differences in melanin pigmentation across species (Heidrich et al., 2018) and populations (Alho et al., 2010) can often be explained by temperature. In thermally variable environments,

plasticity in thermal traits like melanin pigmentation may be important for organisms that have fluctuating thermoregulation needs over their lifetimes or across generations (Kingsolver, 1983a, 1983b; Kingsolver & Watt, 1983). A body of research shows that melanin is often associated with thermal responses including accelerated heating rate and higher body temperatures (e.g. Azócar et al., 2020; Hegna et al., 2013). Yet, the adaptive significance of melanin plasticity in thermally variable environments if often assumed, but rarely tested, and it is possible that melanin can affect the thermal response without translating into performance or fitness differences (e.g. Sandre et al., 2014). How melanin plasticity influences fitness traits via thermal advantages, specifically, the costs and benefits of being melanic versus non-melanic at different temperatures, is still poorly understood (Pinkert & Zeuss, 2018). Furthermore, melanin pigmentation is often plastic in response to multiple environmental stimuli, thus the primacy of the thermoregulatory role can be difficult to confirm.

Melanin pigmentation can be advantageous in cold environments, for example, by increasing activity time, but can also be disadvantageous in warm environments where overheating is a risk (Gunderson et al., 2022). However, it is rare that fitness or performance differences between melanic and non-melanic forms within a single species have been tested across temperatures in the same study. While it is often assumed that there is a benefit for melanic phenotypes at cool temperatures and a cost at warm temperatures, this is not always the case. For example, in the wasp *Meteorus pulchricornis*, melanic forms are more likely to fly than non-melanic forms at cool temperatures, as expected if melanin confers a thermal advantage, but this is true even at warmer temperatures (Abe et al., 2013). Because the expected cost of melanin at high temperatures was not found, this indicates that the costs may be found in traits other than flight, or that melanin plasticity may be an adaptation to another selective pressure. To understand the importance of plasticity and its adaptive value, it is crucial to look at both thermal traits and fitness traits in multiple thermal environments.

Testing the fitness effects of plastic phenotypes is difficult because often the environments used to induce different phenotypes can confound the subsequent experiment (Schmitt et al., 1999). Environments that induce different melanin phenotypes may cause other trait differences as well (Kingsolver, 1995), which can make it difficult to isolate the effect of melanin itself. This is especially problematic with temperature because acclimation plays a known role in success at different temperatures (i.e. beneficial acclimation hypothesis Harrison

et al., 2012). For example, hoverflies have plastic melanin pigmentation in response to developmental temperature: darker morphs develop when larvae are raised in lower temperatures. Darker morphs are disadvantaged at warm temperatures and have lower survival in the summer, but it is unclear whether melanin itself accounts for this disparity (Ottenheim et al., 1999). There are a number of other phenotypic differences between the morphs when raised in different temperatures which may also explain survival differences including body fat, wing length, and bristle number (Ottenheim et al., 1999). One solution to this problem is to directly manipulate melanin by adding dark markings to individuals (Ellers & Boggs, 2004; Kingsolver, 1996; Punzalan et al., 2008). However, not all species or life stages are amenable to this manipulation. Furthermore, this manipulation is not biological realistic and may omit the effects of important processes, such as pigment production and potential associated costs. In some species it may be possible to use genetic manipulation to compare morphs with and without the ability to produce melanin, although this may result in extreme or unrealistic phenotypes or have unforeseen pleiotropic effects on other traits. Another option is to take advantage of a species that is plastic in response to multiple cues to reduce the confounding nature of the inducing environment and the testing environment. This allows the induction of melanism by one environmental factor, then testing the effects of melanism by another environmental factor.

In this study we investigate melanin plasticity in the white-lined Sphinx moth, *Hyles lineata* (Lepidoptera, Sphingidae), which shows extreme variation in degree of melanin pigmentation during the late larval instars (Francois, 2020). We hypothesize that melanin plasticity is an adaptation for thermally variable temperature environments. This is a unique system in which to test this hypothesis because melanin pigmentation is plastic in response to multiple environmental stimuli and individuals that vary greatly in melanin pigmentation are observed to coexist (Francois, 2020, pers. obs.). Unlike previous studies, which often examine morphs from different elevations, latitudes, or seasons, in *H. lineata*, extensive variation in melanin can be observed at the same time and place. The role of melanin plasticity is thus more complex in this species and it is likely that melanin plays multiple functional roles, and that melanin plasticity faces multiple selective pressures.

First, we test whether melanism influences thermal response. In order to show that melanin plasticity plays an adaptive thermal role, it is necessary to first demonstrate that differences in melanization can explain thermal differences among individuals. In a number of

studies, empirical tests have produced inconsistent results on whether melanin affects thermal traits (Umbers et al., 2013). Melanin is not always associated with a thermal response (Matthews et al., 2016; Rivas et al., 2016) because other factors, such as body size (Bittner et al., 2002) or convective cooling (Turner & Lombard, 1990) may swamp the effects of melanin pigmentation. Behavioral thermoregulation can also enhance or mitigate the effects of color (Forsman, 1995). Next, we test whether melanin has an effect on fitness and fitness proxies, and whether this effect depends on temperature (Fig. 1). If melanin plasticity is an adaptation for thermally variable environments, we would expect that melanic individuals have higher fitness than non-melanic individuals in a cold environment but non-melanic individuals have higher fitness than melanic individuals in a warm environment (Fig. 1a). This is true in *Drosophila kikkawai,* where melanic morphs had higher hatching and survival in cold temperatures but lower hatching and survival in warm temperatures (Singh et al., 2022). Alternatively, other factors may be more important in explaining the evolution of melanin plasticity. If this is the case, then we would expect that the fitness of melanic *versus* non-melanic individuals will not be related to temperature (Fig. 1b). Melanic individuals may always outperform non-melanic individuals, regardless of temperature (Fig. 1b, left), indicating that melanin is an advantageous trait but for reasons other than thermal performance. Or non-melanic individuals may always outperform melanic individuals, regardless of temperature (Fig. 1b, center), indicating that melanin is a costly trait, but for reasons other than thermal performance. Finally, melanin may be unrelated to fitness measures across temperature environments (Fig. 1b, right).

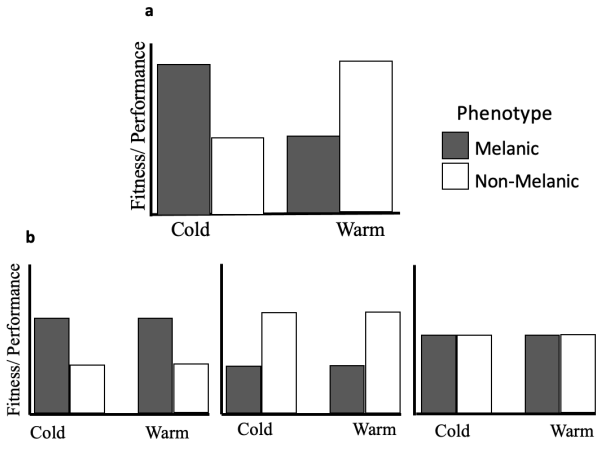

Figure 1. Hypotheses and predictions. If melanin plasticity is an adaptation to thermally variable environments, then fitness consequences of melanic versus non-melanic phenotypes should be temperature dependent (a). If factors other than temperature are more important in explaining the evolution of melanin plasticity, then fitness consequences will not be related to temperature (b).

Methods

Study system

Hyles lineata is a common and geographically widespread moth. In the fourth and fifth instars, larval coloration can vary from little to no melanin, to black dorsal striping, to completely melanic (Fig. 2). In many populations *H. lineata* is multivoltine, often having two to three generations in a year. Thus, individuals within a population may experience a wide range of temperatures across seasons. Temperature affects feeding activity in this species and activity is highest between 20° C and 34° C (Casey, 1976). *Hyles lineata* has a large geographic range in which temperatures regularly fall outside of the ideal temperature range depending on season and time of day. The ability to plastically adjust melanization may be an important adaptation to stay warm at colder times and avoid overheating at other times.

Figure 2. Plastic melanin variation in *H. lineata* larvae during the fifth (final) instar. Larvae can be visually categorized into maximum (left), medium (middle), and minimum (right) melanization.

We used *H. lineata* individuals from a lab colony that has been maintained for multiple generations at the University of Arizona and was originally collected from the vicinity of Colorado Springs and Pueblo, Colorado (von Arx et al., 2013). Larvae are raised in the following conditions: 27° C, 40-50% humidity, 16-hour photoperiod, and ad libitum access to an artificial wheat germ-based diet (Davidowitz et al., 2003). Adults are kept in a cage where they are

provided with ad libitum access to a sponge saturated with a 20% sucrose solution and host plants (*Oenothera caespitosa*) for oviposition.

The degree of melanization can be manipulated by changing larval density conditions. Manipulating larval density to generate variation in melanism allows us to separate the effects of temperature from melanin. After hatching, all larvae were moved into individual 1oz (29.5 ml) cups. On the first day of the 4th (penultimate) instar individuals were either moved into a tray of 15 larvae or moved to a fresh 1oz cup. In both the crowded and solitary treatments larvae had access to ad libitum food. All individuals were moved to individual 9 oz (266 ml) cups at the beginning of the 5th instar. The 4th instar rearing manipulation (crowded *versus* solitary) is not expected to bias performance at different temperatures. While some variation can be seen within each density manipulation, minimally melanized individuals are rarely produced in the crowded treatment and maximally melanized individuals are rarely produced in the solitary treatment. For the following experiments we use maximally and minimally melanized larvae, hereafter referred to as melanic and non-melanic, to facilitate clear comparisons between phenotypes. Maximally and minimally melanized larvae are easy to distinguish in a way that is consistent with photographic data (Francois, 2020). We photographed a representative sample of individuals from the following experiments. In ImageJ (NIH, Bethesda, MD) photographs were converted to 8-bit and treshholded at a gray scale value of 30. Larvae were carefully outlined with the freehand selection tool and the percentage of melanin cover (percent of pixels with values below 30) was calculated. Melanic larvae had on average 69.07±8.06% melanin coverage while non-melanic larvae had on average 11.35±4.25% melanin coverage (t (60) = 24.84, p<0.001).

Thermal Response

The goal of this experiment was to test whether melanin has an effect on thermal response. If melanin pigmentation is an effective thermoregulatory trait, then melanic individuals will heat up faster and reach higher body temperatures than non-melanic individuals. We used 46 individuals in the 5th instar, 23 melanic and 23 non-melanic. All larvae were weighed immediately prior to trials since body size is known to influence thermal traits (Clusella-Trullas et al., 2007). A similar range of body sizes was used for both groups so that size did not bias results (melanic average = 4.32±1.27 g, non-melanic average = 4.39±1.27 g, t(44) = -0.1745, p =

0.8622). Before starting the trial, individuals were placed in a chamber at 10° C for 30 minutes to cool to a consistent temperature (average starting body temperature of melanic larvae = 11.47±1.41° C, of non-melanic larvae = 11.15±1.30° C, $t(44) = 0.8156$, $p = 0.4191$). Then, individuals were transferred to an experimental setup where thermal response was measured. In this setup a 150W LED bulb (EcoSmart by Home Depot), which mimics the irradiance and color temperature of midday light (5000K), was suspended 20 cm above a white plastic surface. We chose to use LED lights because they minimize amount of heat given off, thus we could focus on the effects of radiant heat. Two lights were used for this experiment so that two individuals could be tested simultaneously. Lights were turned on 10 minutes prior to thermal trials to reach an equilibrium ambient temperature. The ambient temperature under the lights differed slightly between the lights and among days and was measured directly under the lights prior to every trial with a thermocouple (Taylor, Oak Brook, IL) in order to take this variation into account. The height of the bulbs was chosen to mimic a solar radiation and temperature environment naturally experienced by *H. lineata*. The radiation under the bulbs reached 225 W/m^2, measured with a solar power meter (Amprobe, Solar-100), which falls within a realistic range (Sengupta et al., 2018). Individuals in plastic cups were placed directly below the light. Body temperature was measured with a thermal imaging camera (Teledyne FLIR T-300, Fig. 3). This camera has a sensitivity of 0.05°C and a high resolution of 320 x 240 pixels (Teledyne FLIR). The camera was calibrated by the manufacturer before the experiment. We set up the camera a half meter away from the subjects and carefully manually focused the lens. The camera's instantaneous field of view (IFOV) at a distance of 0.5 meters is 0.068 cm (Teledyne FLIR), giving us a spot size ratio (SSR) of 1:0.136 cm. Since larvae are larger than this (approximately 8 cm in length), the camera can capture multiple pixels on the body of the caterpillar and accurately measure body temperature at this distance. The emissivity was to 0.98, which is consistent with biological materials (Tatterstall et al. 2016). An initial thermal image was taken at minute 0 as soon as caterpillars were placed under the lamp. For eight individuals, pictures were taken every two minutes for thirty minutes and we determined that by 24 minutes body temperatures had stabilized. This was done visually by inspecting heating curves. For the remaining individuals, pictures were taken every four minutes after the initial picture for 24 minutes. The camera's built-in software was used to determine body temperatures. We used the area selection tool to manually select the larva in each image. For minutes 0 and 4 we used the reported minimum

temperature from the selection and for minute 24 we used the reported maximum temperature from the selection. It is important to note that because the selection tool was rectangular, the selection included the larva's body as well as some background. However, each image was visually inspected to ensure that the minimum or maximum temperature, respectively, was on the body of the larva and not the background. ~~Body temperature was determined by manually selecting the larva in the image and finding either the minimum temperature on the body (minutes 0 and 4) or the maximum temperature on the body (minute 24).~~ Body temperature excess was calculated as the body temperature at minute 24 minus the ambient temperature under the lamp. We were interested in how body mass *versus* melanin might differently influence heating early *versus* late in the heating period so we used a simple rate calculation (body temperature minus the initial body temperature per time period) and measured both an initial heating rate (**Δ**T/ 4 minutes) and average heating rate (**Δ**T/ 24 minutes).

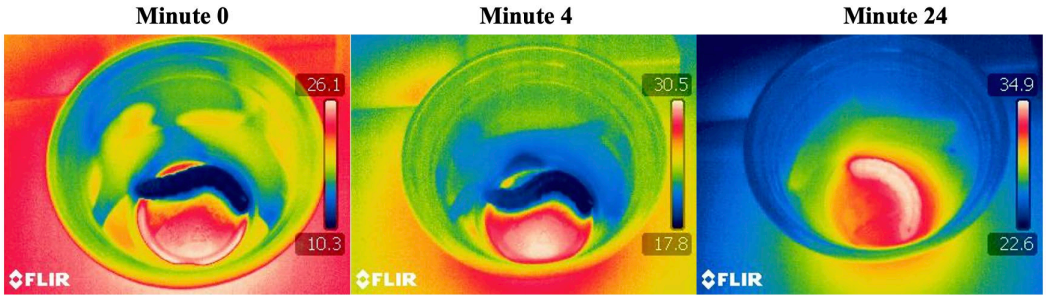

Figure 3. FLIR thermal images of a larva at 0, 4, and 24 minutes under a lamp. The temperature of the larva increases over time (note body color *versus* background color). Note that the temperature scale on the right is different in each image.

Fitness Traits and Proxies

The goal of this experiment was to test the fitness effects of melanin pigmentation by comparing important fitness and life history traits (fitness proxies) between melanic and non-melanic individuals. Specifically, we test whether the effect of melanin on fitness is dependent on environmental temperature, as would be expected if melanin plasticity is an adaptation to temperature variation (Fig. 1a). For this experiment we used a fully factorial 2x2 split brood design. The experiment was run in two blocks due to limited space in environmental chambers. For each block, full siblings were obtained by removing recently emerged ovipositing females from the colony stock and placing them in a 30 cm^3 cage with *O. caespitosa* leaves for

oviposition. After collecting eggs, all females were dissected to ensure the presence of only one spermatophore, signifying each female had only mated with one male. The use of siblings allowed us to control for possible genetic variation. Larvae were grown in individual cups until the 4th instar, at which point siblings were split between the inducing treatments (crowded *versus* solitary). In the 5th instar siblings from each crowding treatment were split again and moved into individual cups and into the temperature treatments: cold environment (21°C) *versus* warm environment (33°C). These temperatures were chosen because they are within the range of temperatures that individuals are still active, which starts to drop sharply below 20° C and above 34° C (Casey, 1976). Furthermore, each temperature treatment was equidistant from the temperature at which individuals were raised (27°C) during the 1st- 4th instars, and that the colony has historically experienced. This was done to minimize the effect of the degree of temperature change on results. However, it is important to note that full performance curves across these temperatures for the traits we measured are not known, thus we cannot confirm the exact effect of the degree of temperature change. The light cycle in the chambers was set to 16 light:8 dark, which is consistent with the photoperiod historically experienced by the colony. The cups were small enough that larvae were not able to behaviorally thermoregulate so that this behavior did not influence results. There were slight differences in temperature during the 4th instar treatments (solitary cup: 27.1-28.5°C, crowded tray: 26.5-27.8°C), although there were no differences in humidity. It is unlikely that this minor difference over a short period, especially compared to the more extreme temperature variation during the 5th instar, had an impact on our results, although it is not possible to rule this out.

Each experimental block contained 72 individual larvae, 36 each in the cold and warm environments, 18 of which were melanic and another 18 non-melanic. Over the two experimental blocks, this resulted in a total of 36 individuals in each of four treatments (melanic/ cold environment, non-melanic/cold environment, melanic/warm, and non-melanic/ warm). Families were evenly distributed among the treatments with four families used in each block.

Each environmental chamber (Percival model 136VL) contained 12 lamps (150W LED bulb, 5000K light), suspended 20 cm above a shelf to provide a source of radiant heat. Three individually labeled cups were affixed to a white piece of foam and placed below each bulb. Cups were arranged under bulbs in such a way that each cup received the same amount of radiation from the bulbs (225 ± 5 W/m^2). Individuals were weighed every day between 5 and 7

PM. Every day after weighing the arrangement of the cups under each light was rotated to remove potential position effects. For each individual we calculated two measures of growth rate: maximal growth rate and total growth rate. Maximal growth rate was measured as percent mass increase from days two to four. This time period represents the linear phase of growth where growth rate is maximal (Nijhout et al., 2006). Total growth rate was measured as percent mass increase from initial body mass (after molt into 5^{th} instar) to peak body mass. Development time (duration of growth period) was the number of days from molting into the 5^{th} instar until peak mass. We also noted survival, and if an individual failed to pupate it was considered that they did not survive.

Growth rate and development time are important fitness proxies for a number of reasons. First, these two traits together determine body size (Davidowitz & Nijhout, 2004), an important trait that influences fitness in insects via the number of offspring that can be produced, mating success, and survival (Blanckenhorn, 2000; Kingsolver & Huey, 2008). Second, the ability to shorten development time is important because it shortens the time in a vulnerable stage where individuals are at risk of predation and parasitism (Benrey & Denno, 1997). Furthermore, at the end of the season larvae may be under time constraints to develop before food resources expire or conditions become unfavorable (Homeny & Juliano, 2007). Arguably, being able to maximize growth rate while minimizing development time is advantageous in many contexts. In this species, peak larval mass correlates strongly with adult mass (unpl. data), and thus is a good proxy for adult size. We chose to focus our fitness results on the 5^{th} instar because this is the longest instar, in which the most growth and development occurs (pers. obs.). Additionally, melanin plasticity does not occur in earlier instars (1-2) and is not as dramatic in mid-instars (3-4) (pers. obs.).

Statistical Analysis

All statistical tests were conducted in R version 4.2.2 (R Core Team 2022). For the thermal response experiment, we used ANOVAs to test for the effect of melanin on thermal traits: body temperature excess, initial heating rate, and average heating rate. In this experiment body mass was included as a cofactor, as this is known to influence thermal traits. There was no significant interaction between melanin and body mass, so this term was removed from the final models and a Type II sum of squares was calculated using the Anova function from the car

package (Fox and Weisberg, 2019). For the fitness traits experiment we constructed linear mixed models with the lmer function in the lmerTest package (Kuznetsova et al., 2017). We used these models to test whether there was a significant interaction between melanin and temperature on fitness traits: maximal growth rate, total growth rate, development time, and peak body mass. For all models, random effects included family nested within block and fixed effects included melanin (crowding treatment), temperature environment, and melanin by temperature interaction. An F-test approximation using Satterthwaite's method was used to calculate p-values using the anova function (Type III) from the lmerTest package (Kuznetsova et al., 2017). Post-hoc analyses were conducted using the Kenward-Roger approximation with a Tukey p-value adjustment using the emmeans function in the emmeans package (Lenth, 2023). To analyze survival, we used a z-test of proportions for each temperature environment. A significant temperature by melanin interaction indicates that the effect of melanin differs across temperature treatments, as predicted by the hypothesis that melanin is an adaptation for thermally variable environments (Fig. 1a).

Results

Thermal Response

Melanin pigmentation had a significant effect on all thermal response traits. Melanic individuals had higher initial heating rates (**Δ**T/ 4 minutes) than non-melanic individuals (melanic = 2.28 ± 0.33°C/min, non-melanic = 2.11± 0.36°C/min, Fig. 4a, Table 1). Melanic individuals also had higher average heating rates (**Δ**T/ 24 minutes) than did non-melanic individuals (melanic = 1.13± 0.06°C/min, non-melanic=1.05± 0.08°C/min, Figure 4b, Table 1). Melanic individuals reached higher excess body temperatures than non-melanic individuals (melanic = 5.88 ± 1.24°C, non-melanic = 3.77± 1.29°C, Figure 4c, Table 1). For all thermal response variables body mass also had a significant effect (Table 1).

Table 1. Model results from Thermal Response Experiment.

Model	F-value $_{1,43}$	p-value
Initial heating rate ~		
Melanin Phenotype	4.991	**p = 0.031**

Body Mass	62.19	**p < 0.001**
Average heating rate ~		
Melanin Phenotype	16.926	**p < 0.001**
Body Mass	9.922	**p = 0.003**
Body temperature excess ~		
Melanin Phenotype	40.137	**p < 0.001**
Body Mass	14.369	**p < 0.001**

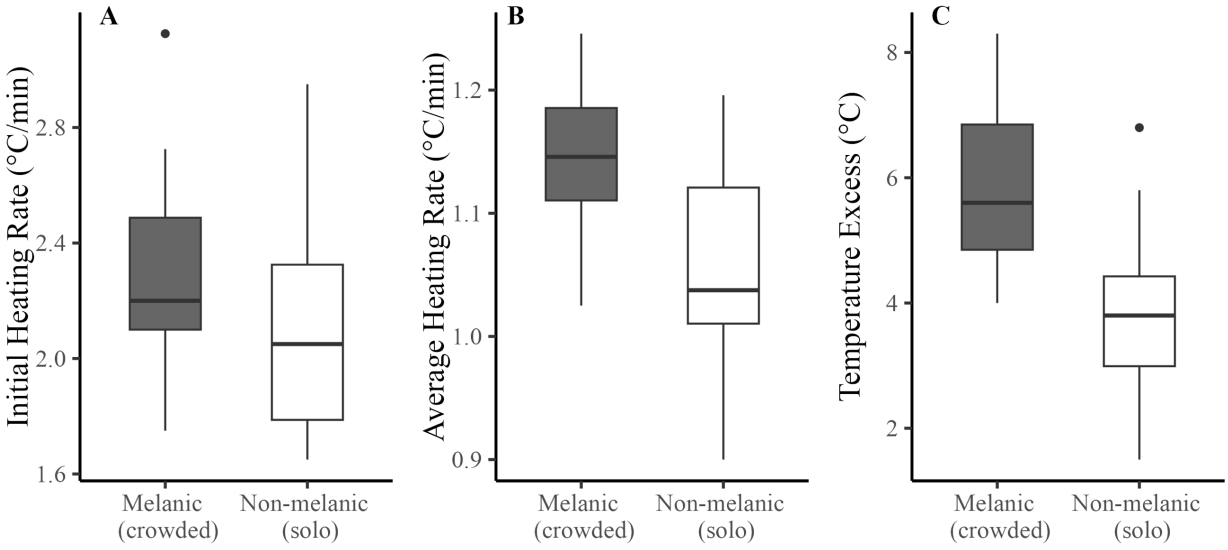

Figure 4. Effect of melanin on thermal response. The effect of melanin on thermal traits including (a) initial heat rating (over the first four minutes), (b) average heating (over the entire 24 minutes), and (c) body temperature excess above ambient temperature.

Fitness Traits

For all five fitness-related response variables (survival, maximal growth rate, total growth rate, development time, and peak body mass), there was a significant interaction between melanin and temperature, indicating that the effect of melanin differed in the two temperatures. In the cold treatment there was a significant difference in survival between melanic and non-melanic larvae (melanic = 100%, non-melanic = 63.9% $z = 3.98$, $p<0.001$), whereas in the warm treatment there was no difference (melanic = 75%, non-melanic = 63.9% $z = 1.02$, $p=0.44$, Figure 5a). Melanic larvae had higher maximal growth rates (percent mass increase) in both cold and warm

environments, but the difference between melanic and non-melanic was more extreme in the cold treatments, as indicated by a significant interaction term (cold/melanic = 112.97% ± 35.81%, cold/non-melanic = 55.61% ± 32.47% *vs* warm/melanic = 104.34% ± 43.84%, warm/non-melanic = 77.29% ± 42.89%, Figure 5b, Table 2). Similarly, melanic individuals had higher total growth rates in both cold and warm environments, but the difference between melanic and non-melanic was more extreme in the cold treatments, as indicated by a significant interaction term (cold/melanic = 444.15% ± 117.83%, cold/non-melanic = 189.66% ± 61.05% *vs* warm/melanic = 458.55% ± 134.67%, warm/non-melanic = 323.30% ± 102.73%, Figure 5c, Table 2). In the cold treatment melanic individuals had a longer 5th instar than non-melanic larvae (melanic = 10.2 ± 1.9 days, non-melanic = 8.1 ± 1.4 days), but in the warm treatment development time was similar (melanic = 6.5 ± 1.4 days, non-melanic = 6.4 ± 0.8 days, Figure 5d, Table 2). In the cold treatment melanic larvae reached a higher peak body mass than non-melanic larvae (melanic = 3.64g ± 1.13g, non-melanic = 2.89g ± 0.95g), whereas in the warm treatment non-melanic larvae reached a higher peak body mass than melanic larvae (melanic = 3.52g ± 1.12g, non-melanic = 4.17g ± 1.16g, Figure 5e, Table 2).

Table 2. Model results from Fitness Traits Experiment.

Response Variable	F-value	p-value
Maximal Growth Rate	$F_{1,133}$	
Melanin	44.499	**p < 0.001**
Temperature	1.098	p = 0.297
Melanin x Treatment	5.645	**p = 0.019**
Total Growth Rate	$F_{1,125}$	
Melanin	124.673	**p < 0.001**
Temperature	18.576	**p < 0.001**
Melanin x Temperature	9.751	**p = 0.002**
Development Time	$F_{1,130}$	
Melanin	20.462	**p < 0.001**
Temperature	125.460	**p < 0.001**
Melanin x Temperature	11.644	**p < 0.001**
Peak Body Mass	$F_{1,125}$	

Melanin	0.131	p = 0.718
Temperature	12.255	**p < 0.001**
Melanin x Temperature	14.320	**p < 0.001**

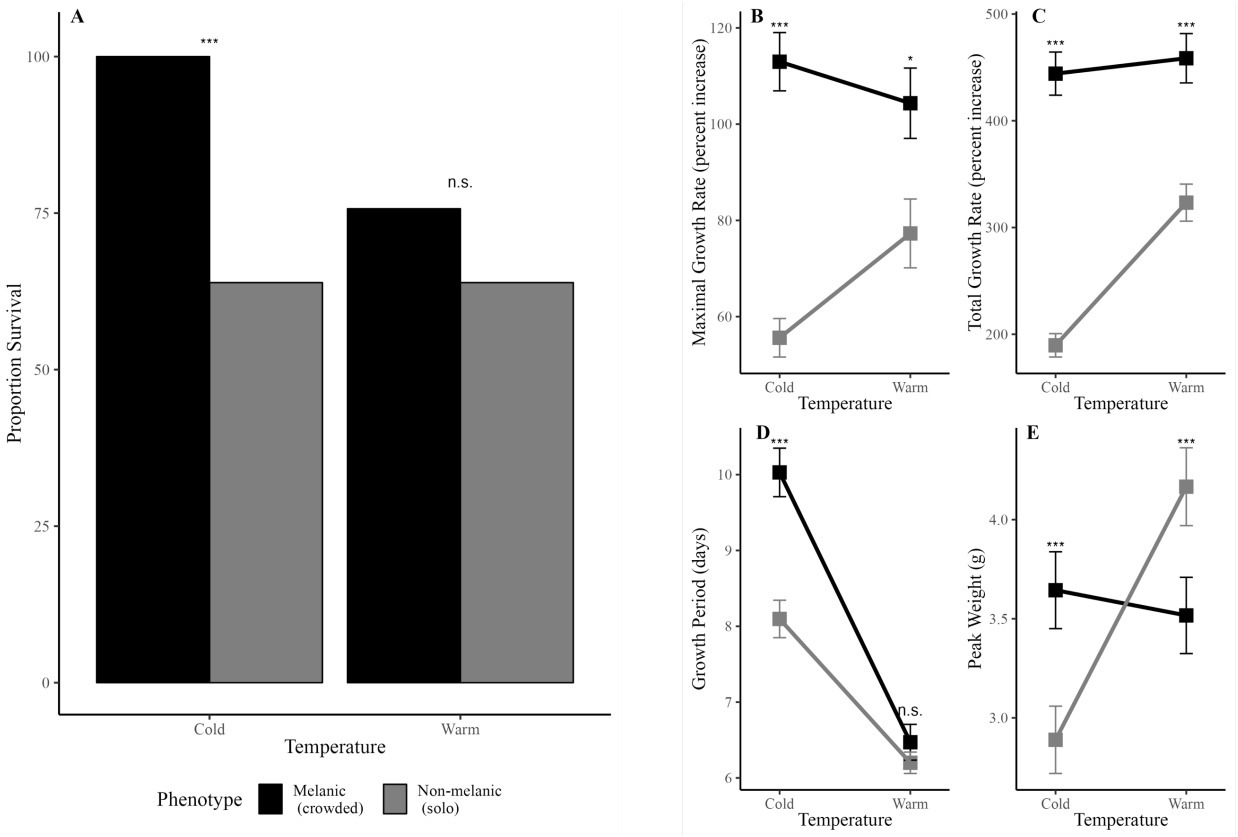

Figure 5. Effect of melanin on fitness traits differs between temperature environments, including (a) survival, (b) maximum growth rate, (c) total growth rate, (d) growth period and (e) peak larval mass. Melanic larvae from the crowded treatment are represented in black while non-melanic larvae from the solitary treatment are represented in gray. In graphs b-e squares represent group means +/- standard error.

Discussion

In this study we investigated melanin plasticity in *H. lineata* by testing its adaptive value under thermally variable environments (Fig. 1). First, we compared thermal traits in melanic *versus* non-melanic individuals to test whether melanin can influence thermoregulation. Then,

we tested whether these thermal differences are translated into fitness differences, an often untested assumption. Overall, our data are consistent with the hypothesis that melanin plasticity is an adaptation for thermally variable environments, although other traits and environmental contexts need to be considered to fully understand the costs and benefits of melanin and why this trait is plastic.

Melanic individuals heated up faster and reached higher body temperatures than non-melanic individuals (Fig. 4, Table 1). This is consistent with a number of studies in other insects and ectothermic animals (Clusella-Trullas et al., 2007). Body size also had an effect on thermal traits, as has been found in other systems (e.g. Bittner et al., 2002). This pattern can be explained by thermal inertia, when larger individuals heat up and cool down more slowly than smaller individuals (Karl et al., 2009). Body size seems to be a better predictor of initial heating rate, while melanin is a better predictor of overall heating rate and body temperature (Table 1). Thus, for small larvae, body temperatures may fluctuate more rapidly with the environment and melanin may be an especially important thermoregulatory factor. Interestingly, in the earlier instars of *H. lineata*, melanin pigmentation is not plastic; all second and third instars are fully melanic (although darkness can vary in the third instar) and it is not until the fourth instar that plastic variation is seen (pers obs.).

For all five fitness response variables (maximal growth rate, total growth rate, development time, peak body mass, and survival), there was a significant interaction between melanin and temperature environment, indicating that the effect of melanin depends on temperature (Fig. 5, Table 2). In the cold treatment, all melanic individuals survived, whereas only 63.9% of non-melanic larvae survived, while in the warm treatment there was no evidence for a difference in survival. Kingsolver (1995) found the opposite pattern in western white butterflies (*Pontia occidentalis*) with higher survival for light winged morphs in the summer (warmer environment), but no difference in survival between light and dark winged morphs during the spring (cooler environment). However, it was unclear whether melanin wing patterns themselves were responsible for survival differences, or whether it was other traits also induced by the photoperiod treatment used in the experiment. It is also possible that the effects of melanin on survival also differ between larvae (this study) and adults (Kingsolver 1995).

Growth rate and development time are important fitness related traits and are both temperature dependent in insects. Since melanic individuals can maintain higher body

399 temperatures than non-melanic individuals, we might predict that growth rate would increase
400 while development time would decrease. In both temperatures, we found that melanic larvae
401 grew faster than non-melanic larvae, whether considering maximal growth rate or total growth
402 rate (Fig. 5, Table 2). However, this difference was larger in the cold treatment (significant
403 melanin x temperature term), indicating that melanin plays a more important thermal role at cold
404 temperatures. Interestingly, we found that in the cold treatment melanic individuals had a longer
405 development time than non-melanic individuals. One explanation for this may be that by
406 increasing both growth rate and development time melanic individuals will achieve larger body
407 sizes. In fact, melanic individuals were larger than non-melanic individuals in the cold treatment
408 (Fig. 5, Table 2). In a lab setting with no threat of predation or parasitism and ad libitum food
409 access, it is possible that there was no pressure to accelerate development. Development time
410 was much shorter in the warm treatment, as expected for a temperature-dependent trait, and we
411 found no evidence for a difference between melanic and non-melanic larvae. In the warm
412 environment, non-melanic individuals reached higher peak body masses than melanic larvae.
413 It is important to note that because we used a density manipulation to induce melanin
414 variation, it is difficult to disentangle the effects of the manipulation itself and melanin
415 pigmentation per se. This would be true for any conditions used to induce melanin variation.
416 Because we have no evidence to suggest that the density manipulation differentially prepares
417 individuals for one temperature over another, this manipulation does a better job of isolating the
418 effect of melanin itself compared to manipulations involving temperature. Using one set of
419 environmental factors to induces plastic changes and then test fitness effects under a different set
420 of environmental factors may be a useful way to test hypotheses about the adaptive role of
421 plasticity by better isolating the effects of a specific trait.
422 In this study we group melanin phenotypes into two categories (melanic *versus* non-
423 melanic), but in this species melanin pigmentation can be continuous with intermediate forms,
424 depending on the environment (Fig. 1). In future work it will be important to understand the
425 thermal and fitness consequences of different temperature environments for these intermediate
426 forms as this may further explain the complexities of plasticity in this species. Furthermore,
427 investigating continuous variation may also help disentangle the effects of melanin versus the
428 effects of the inducing treatment.

Overall, our results from the fitness experiment are partially consistent with the prediction for adaptive melanin plasticity in response to temperature (Fig. 1a) and partially consistent with the prediction of no fitness differences (Fig. 1b): while melanin was advantageous in the cold treatment, there was no clear advantage to either phenotype in the warm treatment. In the cold treatment melanic larvae had higher survival, and grew faster for longer, reaching larger peak body masses. In the warm environment we found no evidence for differences in survival or development time. While melanic larvae grew faster than non-melanic larvae, non-melanic larvae reached larger sizes.

This pattern begs the question, why not be melanic all the time? What is the benefit of plasticity? These results are similar to a handful of other studies investigating temperature specific fitness effects of melanin. In male ambush bugs (*Phymata americana*), for example, darker males have reduced mate searching time (the limiting factor in male fitness), but this was only true in cool temperatures (Punzalan et al., 2008). Conversely, in western white butterflies, lighter morphs had higher survival in warm temperatures but there was no difference in survival at cool temperatures (Kingsolver, 1995, 1996).

Why have a plastic phenotype, especially given that plasticity itself can be costly (Murren et al., 2015; Snell-Rood et al., 2010), including melanin plasticity (Chaput-Bardy et al., 2014)? One explanation may be that there are additional costs of a melanic phenotype that we did not measure or are not related to the thermal environment. For example, in tiger leaf beetles *(Chrysomela lapponica)* darker morphs run faster than lighter morphs, but only at low temperatures (Zverev et al., 2018). However, lighter morphs lay more eggs regardless of temperature. This reproductive cost for dark morphs may be outweighed by the ability to run faster at lower temperatures to escape predators or hunt prey. This example highlights the importance of considering multiple fitness and performance traits under different selective contexts to understand the full cost-benefit ratio of melanic *versus* non-melanic phenotypes across temperatures. In *H. lineata,* for example, it is possible that the non-melanic form is better at avoiding predation because it is less conspicuous to predators. Similarly, in the wood tiger moth, adults that have more wing melanin are at a thermal advantage in cold temperatures, but their aposematic signal is weaker, and they suffer higher predation (Hegna et al., 2013).

Another hypothesis is that the production of melanin is costly when it leads to resource allocation trade-offs (Stoehr, 2006). In insects, melanin plays an important role in immunity and

there may be a trade-off between cuticular melanin and the melanization immune response. Several studies have shown trade-offs between these two traits (Cotter et al., 2008; Debecker et al., 2015; Goulson & Cory, 1995), and that diet may influence these trade-offs (Cotter et al., 2011). Thus, it is possible that in the presence of parasitoids or disease, melanin pigmentation is costly because it detracts from the immune response. Furthermore, because *H. lineata* is folivorous, the amino acid precursors needed for melanin production may be limited. In a context where melanin precursors are limited and needed elsewhere (e.g., for immune response, for building muscle, etc.), melanin production may costly. However, in cold environments, this cost may be outweighed by the thermal benefits we demonstrate here.

Other studies, however, have found a positive correlation between cuticular melanin and measure of immune response (Armitage & Siva-Jothy, 2005; Bailey, 2011; Fedorka et al., 2013; Reeson et al., 1998), thus, this is not necessarily a cost associated with melanin production. A number of other mechanisms have been proposed to explain the cost melanin production including pleiotropy (Ducrest et al., 2008) and the toxic byproducts that are created during melanogenesis (Nappi & Vass, 1993; Sadd & Siva-Jothy, 2006). Across insects, many studies have documented life history costs associated with melanin (Busso & Blanckenhorn, 2018; Lindstedt et al., 2019; Ma et al., 2008; Roff & Fairbairn, 2013). Because melanin plays many roles and is plastic in response to numerous factors, this is a good system for considering the complex costs and benefits of plastic phenotypes.

Considering the evidence from our two experiments, our data are consistent with the hypothesis that melanin is an adaptation for thermally variable environments, although it is important to consider costs of melanin pigmentation that are unrelated to temperature. Plasticity in melanin pigmentation is widespread and warrants explicit investigation, especially because it is thought to facilitate adaptation to climate change (Kingsolver & Buckley, 2017). Furthermore, melanin plasticity is likely to be an important thermal process in non-animal taxa, as well (Cordero et al., 2018). Understanding adaptive plasticity is critical because it helps us understand and explain how organisms interact with their environment and cope with environmental change. Our results highlight the importance of explicitly testing the adaptive role of plasticity and considering the factors that influence costs and benefits of phenotypes across environments.

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493 **Literature Cited**

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