Thermal Physiology and Developmental Plasticity of Pigmentation in the Harlequin Bug (Hemiptera: Pentatomidae)

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Abstract

Traits that promote the maintenance of body temperatures within an optimal range provide advantages to ectothermic species. Pigmentation plasticity is found in many insects and enhances thermoregulatory potential as increased melanization can result in greater heat retention. The thermal melanism hypothesis predicts that species with developmental plasticity will have darker pigmentation in colder environments, which can be an important adaptation for temperate species experiencing seasonal variation in climate. The harlequin bug (Murgantia histrionica, Hemiptera: Pentatomidae, Hahn 1834) is a widespread invasive crop pest with variable patterning where developmental plasticity in melanization could affect performance. To investigate the impact of temperature and photoperiod on melanization and size, nymphs were reared under two temperatures and two photoperiods simulating summer and fall seasons. The size and degree of melanization of adults were quantified using digital imagery. To assess the effect of coloration on the amount of heat absorption, we monitored the temperature of adults in a heating experiment. Overall, our results supported the thermal melanism hypothesis and temperature had a comparatively larger effect on coloration and size than photoperiod. When heated, the body temperature of individuals with darker pigmentation increased more relative to the ambient air temperature than individuals with lighter pigmentation. These results suggest that colder temperatures experienced late in the season can induce developmental plasticity for a phenotype that improves thermoregulation in this species. Our work highlights environmental signals and consequences for individual performance due to thermal melanism in a common invasive species, where capacity to respond to changing environments is likely contributing to its spread.

Key words: thermal biology, phenotypic plasticity, vegetable crop pest, development, life history

Many species of insects inhabit wide geographic ranges that span climatic regions and occur in areas that experience seasonal variability. Abiotic factors can influence all aspects of organismal development and impose selection pressure that favors the evolution of flexible life histories that can respond to changing environments (Holloway and Marriott 1997). Phenotypic plasticity is a widespread adaptation to environmental heterogeneity. Although some phenotypes can continually adjust in response to the environment, traits influenced by developmental plasticity are irreversibly determined based on environmentally induced modifications during growth and development (Smith-Gill 1983, Forsman 2015). For insects, developmental plasticity allows individuals and their progeny to develop phenotypes in response to the current growing season (Forsman 2015). Developmental plasticity can result in enhanced establishment success and increased population growth when changes in phenotype during development increase the fitness of reproductive adults in their future environment (Sultan 2000, Pfennig et al. 2010, Forsman 2015). In this study, we examine developmental plasticity in melamin production, where environmental signals have been shown to affect coloration, especially among insects (e.g., Forsman (1997), Kingsolver and Huey (1998), and Harris et al. (2013a)).

For ectothermic organisms, body temperature influences all physiological processes (Hochachka and Somero 2002) and thermoregulation is an important component for maximizing fitness in variable environments (Heinrich 1981, Stoehr and Goux 2008). Body temperature in insects is strongly influenced by coloration through changes in the relative absorption of solar radiation (Digby 1955, Watt 1968, Chappell and Whitman 1990, Forsman 2000, Fielding and DeFoliart 2005). The thermal melanism hypothesis states that individuals with darker coloration and lower surface reflectance are
at an advantage in cool climates because they are able to heat faster and reach higher equilibrium temperatures than lighter-colored individuals with greater surface reflectance (Clusella-Trullas et al. 2007, Clusella-Trullas et al. 2008). This hypothesis predicts that exposure to lower temperatures during development will result in darker pigmentation if the adult coloration is phenotypically plastic (Forsman 2011). Several studies have demonstrated the benefits of thermal melanism in ectotherms (e.g., Kingsolver (1995), de Jong et al. (1996), Ellers and Boggs (2004), and Clusella-Trullas et al. (2008)), with many confirming the relationship among reflectance, absorption of radiation, and body size (Watt 1968, de Jong et al. 1996, Forsman 1997, Harris et al. 2013a). Perhaps the greatest advantage for individuals with darker coloration is the increased activity that results from faster heating rates, which can subsequently lead to benefits such as enhanced feeding, growth, and fecundity (Harrison and Fewell 1995, Lactin and Johnson 1996, Ashby 1998, Ellers and Boggs 2004, Fielding and Defoliart 2005), as well as an increased ability to defend territories, find mates, and escape predators (Clusella-Trullas et al. 2007).

A wide variety of environmental cues can be important for developmental plasticity in insects as a means for adjusting to alterations in growing conditions (van’t Hof et al. 2011, Yin et al. 2016). The resulting consequences of developmental plasticity range from daily impacts to individuals, to population dynamics and species distributions (Régnière et al. 2012). Although the role of temperature dependence in developmental traits, performance, and ultimately fitness has been well-characterized, the roles of other environmental variables however are less clear. Photoperiod is an example of an important seasonal cue during development, especially in temperate species, but its relationship to developmental plasticity, and specifically thermal melanism, is not as well-established (Hazel 2002, Micke et al. 2011, Yin et al. 2016).

The harlequin bug (Murganta histrionica, Hemiptera: Pentatomidae, Hahn 1834) is an ideal species for exploring the relationship between environmental conditions during development and the phenotypic plasticity of pigmentation given its bold coloration and extensive geographic range. Also known as the harlequin cabbage bug or calico black, this species is native to Central America but has since dispersed across the majority of the continental United States as a major agricultural pest (Ludwig and Kok 1998, Zahn et al. 2008). Although it is not known to overwinter north of 40 degrees latitude (Hodson and Cook 1960), all growth stages of the bug can be found throughout the winter months if environmental conditions, such as appropriate temperature and host plant availability, are favorable (White and Brannon 1933). Colonies typically complete to three generations per year; however, warmer climates in some areas have allowed for faster generation times (Paddock 1915). Nymphs feed for 6 to 8 wk as they develop through five instars, transitioning at the end of the fifth instar from a circular shape to the adult pentatomid shield shape. Adults measure 0.6–1.3 cm in length and are brightly colored yellow to red with black and white markings across the thorax and parts of the abdomen, and color proportions can vary substantially between individuals.

This study tests the effects of photoperiod and temperature on the development of adult M. histrionica. We conducted a two-way factorial experiment design using constant high and low rearing temperatures with short- and long-day photoperiods, and quantified the amount of black pigmentation at the end of development. We then determined whether increased levels of melanism affected the ability of the insect to passively thermoregulate. Consistent with the thermal melanism hypothesis, we predicted that decreased photoperiod and temperature would be associated with darker coloration, smaller body size, and a higher equilibrium body temperature relative to ambient air temperature. Together, these results will demonstrate the impact of seasonally and geographically variable environmental cues on the development of variation in adult melanism.

### Materials and Methods

#### Collection and Rearing

We field collected 200–300 individuals in fall of 2015 (September 23 and October 12) from collard green plants (Brassica oleracea L.) at Virginia Polytechnic Institute and State University’s Kentland Farm in Montgomery County, Virginia (37.199801° N, 80.564519° W). This parental generation was transported to the University of Richmond and maintained in a Powers Environmental Chamber (Model DROS33SD, Powers Scientific Inc., Pipersville, PA) set at 26°C with a photoperiod of 12:12 (L:D) h. The insects were housed in plastic bins with paper towels and a screened lid and fed full collard green leaves every 2 d, which were sourced from local grocery stores and kept in a laboratory refrigerator at 6–8°C. Egg masses were collected every other day between October 4 and November 11 and each egg mass was placed in a 60-mm vented Petri dish until hatching. We kept newly hatched nymphs in their respective Petri dishes and provided them with small pieces of collard stems every other day through their first molt. Upon molting to the second instar, the nymphs were transferred to experimental conditions.

#### Experimental Design

We conducted a two-way factorial experiment to examine the direct and interacting effects of photoperiod and temperature on pigmentation plasticity in harlequin bugs. The second-instar nymphs were raised to maturity in four environmental chambers (Model I22VL, Perical Scientific, Perry, IA) using the following treatment programs: Long-day/Warm (15:9 [L:D] h, 30°C), Long-day/Cold (15:9 [L:D] h, 20°C), Short-day/Warm (10:14 [L:D] h, 30°C), and Short-day/Cold (10:14 [L:D] h, 20°C). Siblings were distributed evenly across all four treatments and housed in 16-ounce plastic deli containers with mesh lids. We combined offspring from multiple clutches that molted during the same two-day window, allocating no more than 20 nymphs per container. They were fed small sections of fresh collard green leaves attached to portion of the stem, which were changed every other day to maintain freshness. Individuals that completed all five nymphal instars were removed as adults and frozen for pigmentation analysis.

#### Pigmentation Measurements

Frozen insects were photographed using Panasonic DMC-FZ5 digital camera. Using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland), we quantified the total surface area of each individual’s carapace by outlining the dorsal surface, excluding the soft wings. We measured the amount of black pigmentation on each carapace by summing the surface area of all black regions (Fig. 1) and used these values to calculate the ratio of black to yellow pigmentation. This ratio served as a quantitative measure of cuticle melanization independent of size. Although the non-black pigmentation of adult harlequin bugs can range from a yellow to red hue, a visual examination of our colony showed that adults displayed a consistent yellow color, and thus, we did not quantify total reflectance. Although we acknowledge that this is a possible source of pigmentation variation that we did not account for, we believe that hue variation in this colony was negligible relative to the variation in overall albedo. Nymphs that did not survive to adulthood and adults with malformations were excluded from our analysis.
Thermal Response of Pigmentation

Prior to freezing, we selected 200 adults across all four treatments to have their temperatures monitored during thermal radiation exposure. Warming trials took place in an environmental chamber (Model DR0S33SD4, Powers Scientific, Inc., Perry, IA) set to 13°C. Each insect was suspended 30 mm below a 250-W incandescent heat lamp (Model BR40, Philips) by inserting a 29-gauge Type T needle thermocouple (Model MT-29/1, Physitemp Instruments, Inc., Clifton, NJ) into the posterior abdomen. This maintained the insect in a horizontal position with its dorsal surface oriented towards the light source. To measure the surrounding air temperature, we suspended a second Type-K beaded wire 1.6 mm thermocouple (Fisher Scientific, Waltham, MA) 1 cm below the insect, shielding the exposed wire from any direct radiation. The two probes were connected to a BAT-12 microprobe thermometer (Physitemp Instruments, Inc., Clifton, NJ) and a Traceable infrared thermometer (Fisher Scientific, Waltham, MA), respectively. The heat lamp was turned on after 3 min of acclimation to the dark chamber, and we manually recorded the temperatures of both probes every 20 s throughout the experiment.

A typical warming curve showed a rapid increase in temperature for the insect relative to the ambient air, followed by a period in which both temperatures remained stable relative to each other (Fig. 2). We eliminated 21 samples prior to statistical analysis due to irregular or suspect warming data—e.g., the air temperature decreased after the light was turned on, or the difference between the mean air temperature and mean bug temperature was greater than 1°C during the minute prior to the light being turned on.

**Data Analysis**

Pigmentation analysis included 457 adults: 99 individuals from the long-day/cold (LC) treatment, 136 from the long-day/warm (LW) treatment, 129 from the short-day/cold (SC) treatment, and 93 from the short-day/warm (SW) treatment. All black to yellow pigmentation ratios were log-transformed in order to normalize the data and to avoid the problem of asymmetrical limits (0 if black < yellow, infinity if black > yellow). The normality of the log-transformed data was confirmed using a Shapiro–Wilk goodness-of-fit test. We used a two-way ANOVA to examine the effects of temperature and day length on the ratio of black to yellow pigmentation, including the interaction between temperature and day length. We also used a two-way ANOVA to examine the effects of temperature and day length on the total dorsal surface area of adult bugs. Correlations were used to test for linear associations between pigmentation and surface area across all individuals and within each treatment.

An insect’s thermal response was quantified as the body temperature (T_{bug}) minus the surrounding air temperature (T_{air}). Preliminary studies confirmed that these temperatures stabilized by 500 s post-light exposure, and thus, the mean T_{bug} − T_{air} between 500 and 600 s was used for all statistical analyses. We used this value to represent the point at which an individual could increase its body temperature when exposed to solar radiation in its natural environment based on the assumption that, as heterothermic ectotherms, an insect’s temperature would be the same as the ambient air temperature in the absence of light. We assessed the relationship between T_{bug} − T_{air} and an insect’s degree of melanization using a simple linear regression. Finally, a one-way ANOVA was used to detect differences in the mean thermal response among our four developmental treatment groups. Where differences were significant, Tukey HSD post hoc comparisons were used to identify which treatment groups differed

Fig. 1. Variation in melanization observed in adult *M. histrionica*. (a) Newly molted adults lack a fully melanized carapace, though the pattern that will develop is discernible. The full black pigmentation will develop within the first few hours post-molt (Supplementary Material). (b) An individual with a relatively high degree of melanization (black:yellow = 3.55), and (c) an individual with a relatively low degree of melanization (black:yellow = 0.613). Outlines in (b) and (c) indicate regions of the hard carapace pattern that contributed to measurements of ‘black’ pigmentation.

Fig. 2. Results of a sample warming curve. The insect’s body temperature is indicated by the solid black line, whereas the air temperature inside the environmental chamber is shown by the dashed line. The mean temperature between 120 and 180 s was used as the mean dark temperature for both the ambient air and the insect. The heat lamp inside the chamber was turned on at 180 s, and the mean temperatures between 500 and 600 s (shaded region in figure) were recorded as the air and insect temperatures.
significantly from each other. All statistical tests were run using JMP Pro 13.2.1 (SAS Institute, Inc., Cary, NC).

Results

Adult Patterns of Melanism

We conducted a full-factorial general linear model to compare the main and interacting effects of temperature, photoperiod, and total dorsal surface area on cuticle melanism (Table 1). Adult melanism was strongly influenced by rearing temperatures ($F_{1,450} = 116.12, P < 0.001$), and day length had an interacting effect with temperature ($F_{5,450} = 5.10, P = 0.024$). On average, the largest effect sizes were from temperature, as individuals from cold chambers had a 33% larger ratio of black to yellow pigmentation (Fig. 3a). Developmental photoperiod alone had no main effect on pigmentation ($P > 0.05$), but the significant interaction term reflects that among individuals raised in cold chambers, short-day adults had a slightly higher (4%) degree of melanization relative to long-day adults, whereas among individuals raised in warm chambers, long-day adults were slightly darker in color (7%). Insects reared in warm chambers were larger than those from cold chambers, regardless of day length ($F_{4,450} = 128.23, P = 0.003$; Fig. 3b). Both the effect of developmental photoperiod on body size and the interaction effect of temperature and photoperiod were not significant ($P > 0.05$ for both).

Across all treatments, smaller individuals had a higher degree of melanism, and the linear relationship between total dorsal surface area and the log-transformed ratio of black to yellow pigment was highly significant ($r^2 = 0.10, P < 0.001$). However, this trend was not consistent across all treatments (Fig. 4). The variation in the black to yellow pigmentation ratio in adults that developed in SW chamber had no correlation to total dorsal surface area.

Thermal Response of Pigmentation

When exposed to a source of thermal radiation, darker-colored bugs were able to increase their body temperatures ($T_{\text{bug}}$) more than lighter-colored bugs, relative to the ambient air temperature ($T_{\text{air}}$). Although our model explains a small amount of the variation in the data, pigmentation alone does have a significant relationship to the variability in $T_{\text{bug}} - T_{\text{air}}$ ($r^2 = 0.07, P < 0.001$; Fig. 5).

A one-way ANOVA showed a significant effect of developmental treatment on $T_{\text{bug}} - T_{\text{air}}$ ($F_{1,175} = 52.45, P < 0.001$). Post hoc comparisons using a Tukey’s HSD test indicated that the mean temperature increases for both LC and SC treatments were significantly different from all other treatments (LC: $M = 3.5 \pm 0.3 ^\circ C$; SC: $M = 2.8 \pm 0.4 ^\circ C$), including both warm treatments. Furthermore, both warm treatments were not significantly different from each other (LW: $M = 1.9 \pm 0.5 ^\circ C$; SW: $M = 2.0 \pm 0.4 ^\circ C$; Fig. 6).

### Table 1. Results of general linear model examining effects of day length and temperature on the log-transformed black to yellow pigment ratio (melanization) and on the total dorsal surface area

| Dependent                  | Model parameters | df    |      F  |  P     |
|----------------------------|------------------|-------|---------|--------|
| Melanization               | Day length       | 1     | 0.119   | 0.730  |
|                            | Temperature      | 1     | 116.12  | <0.001 |
|                            | Day length*temperature | 1 | 5.10  | 0.024  |
|                            | Error            | 450   |         |        |
| Total surface area         | Day length       | 1     | 0.265   | 0.607  |
|                            | Temperature      | 1     | 128.23  | 0.003  |
|                            | Day length*temperature | 1 | 0.0176 | 0.972  |
|                            | Error            | 450   |         |        |

*P*-values in bold text denote significance.

Discussion

The thermal melanism hypothesis predicts that individuals reared in lower temperatures will develop darker adult pigmentation to support passive thermoregulation (e.g., Clusella-Trullas et al. (2007) and Harris et al. (2013a)), but less is known about the role of other environmental triggers for developmental plasticity in coloration. Our study revealed that temperature was indeed an important factor in determining both body size and melanization in the harlequin bug. Individuals exposed to a colder environment during development had smaller, darker-colored body types than those reared in the warmer environment. Interestingly, the directional effect of photoperiod was only significant within the context of temperature: a shorter photoperiod resulted in darker-colored adults in the cold developmental treatment and lighter-colored adults in the warm treatment. Although this interaction may warrant further examination in future studies, the magnitude of photoperiod effects on pigmentation was much smaller than the effect of temperature. We found that bugs with more melanization reached a higher equilibrium temperature relative to the ambient air than those with lighter coloration. Black to yellow pigmentation ratio was the only factor that significantly influenced an individual’s equilibrium body temperature when exposed to a light source, thus supporting the hypothesis that pigmentation is an important trait affecting thermoregulation in ectotherms.

We found evidence of pigmentation plasticity in the harlequin bug, with the amount of melanization dramatically increasing and
body size significantly decreasing under colder developmental temperatures. Temperature has long been considered an important environmental cue for developmentally plastic traits, and our results are consistent with other studies addressing the thermal melanism hypothesis and its assumptions regarding the relationship between thermoregulation and size (e.g., Harris et al. (2013a)). Although previous work has found evidence for developmental plasticity in other members of Heteroptera in the context of predator avoidance (Tullberg et al. 2008) and iridescence (Fabricant et al. 2013), evidence supporting the impact of temperature on wing or body size and coloration is more prevalent in the literature for Lepidoptera...
in colder environments, they may experience benefits from being in cold environments, insects benefit from higher heating rates due to cooler environments through more efficient conversion of solar radiation to body heat. Insects that can respond to varying temperatures by becoming more or less melanistic during development have been shown to better regulate thermal energy absorption as adults (Kingsolver and Wiernasz 1991, Davis et al. 2005, Fielding and Defoliart 2005), thus providing an evolutionary advantage for thermal melanism and phenotypic plasticity in coloration. Differences in thermoregulation efficiency between low and high surface reflectance individuals, characterized by faster heating rate and higher equilibrium temperatures, can therefore influence the evolutionary fitness of a population (Clusella-Trullas et al. 2007, Clusella-Trullas et al. 2008). Species that exhibit such developmental plasticity are therefore expected to better adapt to variable environmental conditions potentially resulting in wider geographical ranges (Whitman 1988, Bryant et al. 2002, Ellers and Boggs 2004, Fielding 2004, Fielding and Defoliart 2005).

The effect of photoperiod on melanization in the literature is less consistent, and our results indicated that day length alone had little effect on pigmentation. Although developmental temperature showed a clear influence on adult coloration, the difference in melanization found between long- and short-day groups among the warm and cold treatments was of a much lesser magnitude. Our results therefore implicate temperature as a major factor influencing melanization plasticity, whereas similar studies have found mixed support for the relative impact of temperature and photoperiod across species. Not unlike the harlequin bug, temperature was the major determinant of coloration in the bean bug, Riptortus clavatus (Kobayashi and Numata 1995) and the bella moth, Utetheisa ornatrix (Sourakov 2015); however, Fedorka et al. (2013) found day length to be the primary variable influencing pigmentation in the cricket Allonemobius socius. Photoperiod and temperature are both major seasonal cues for biological processes in insects and understanding responses to these variables has become increasingly important as more extreme environmental conditions become more common under climate change.

Our results also revealed differences in body size across treatments: harlequin bugs reared at 20°C obtained smaller adult body sizes than individuals reared at 30°C. In contrast, prior analyses exploring the effects of temperature on body size in heteropterans found that decreased temperatures were associated with larger adult body size in 78% of studies (Atkinson 1994, Atkinson 1995). This pattern is consistent with the temperature-size rule, which describes the theory that an inverse relationship between body size and temperature occurs in ectotherms (Atkinson 1994, Angilletta et al. 2004, Davidowitz and Nijhout 2004, Nijhout et al. 2014). However, this pattern is not universal and the temperature-dependence of growth and development can lead to a smaller body size in colder environments for species with limited periods of seasonal growth (Roff 1980, Walters and Hassall 2006). We also found that smaller harlequin bugs were darker in coloration in three of our four treatments. Although larger ectotherms may be better able to conserve body heat in cold environments, insects benefit from higher heating rates due to their large surface area compared with their relatively small size. Our results suggest that although harlequin bugs may be growth-limited in colder environments, they may experience benefits from being able to heat quickly when hatching.

We demonstrate empirically that variation in melanism translates to increases in body temperature for harlequin bugs. Individuals with darker pigmentation achieved higher stable body temperatures when exposed to an artificial light source, a result consistent with previous thermal experiments on butterflies (Karl et al. 2009), grasshoppers (Forsman 1997), beetles (de Jong et al. 1996), and moth larvae (Goulson 1994). This response may be highly beneficial to harlequin bugs in colder environments, as darker-colored individuals can subsequently engage in thermally dependent behaviors, such as feeding and reproduction, earlier than conspecific competitors with a lesser degree of melanization. Recent work on leaf beetles demonstrated that at low ambient temperatures darker-colored males not only moved faster than lighter-colored males but also copulated more frequently (Zverev et al. 2018), suggesting a link between melanization and thermally dependent behaviors that ultimately influence reproductive fitness.

The harlequin bug is a crop pest throughout the United States and Mexico and before the emergence of synthetic insecticides, this species was considered highly destructive to crucifers such as cabbage, broccoli, and collards (Ludwig and Kok 1998, Zahn et al. 2008). Phenotypic plasticity, particularly in passive thermal regulation, is a key trait of successful pest species and can affect the duration and extent of damage to crops and seasonal limits on activity. Plasticity in thermal traits has been shown to improve the fitness of invasive species such as Ceratitis capitata (Mediterranean fruit fly) and Ceratitis rosa (Natal fruit fly) (Nyamukondiwa et al. 2013), the slug Arion lusitanicus (Donnelly et al. 2012), and species of collombolan springtails (Pogonomognathus and Isotomurus spp.; Chown et al. 2007, Slabber et al. 2007). Ultimately, temperature is one of the most important factors influencing geographic range in insects and plasticity in thermal traits has significant potential to allow native and invasive species to cope with more variable temperatures and expand distributional limits (Uvarov 1931, Harris et al. 2013b). The ability for phenotypic plasticity in melanization to influence passive thermoregulation is a key example of the potential for insects to respond to changing climates (Kearney et al. 2009, Harris et al. 2013b). Understanding how agriculturally important species respond to seasonal environmental cues such as temperature and photoperiod is crucial for understanding the potential for future shifts in geographic range.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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