

MORPHOLOGY AND PHYSIOLOGY OF LARVAL COLOR VARIATION IN
THE WHITE-LINED SPHINX MOTH, HYLES LINEATA

by

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I can do hard things! However, I am lucky to not have to do them alone.

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This dissertation would have been written, eventually, but for the last several months, the entire world stopped for a bit. and I wrote, a lot. I can't help but feel horrifically guilty about appreciating a global pandemic, but I cannot even fathom how long this would have taken me to finish without it. While I am celebrating the completion of my dissertation, it is not lost on me how universally difficult just *being* is at this moment. It's ironic, since the last eight years have been filled with trying to find appropriate boundaries with this work that, amid an unprecedented time of COVID, civil unrest, national political rock-bottom, gross uncertainty and heightened social anxiety, that this work was a bit of a respite.

DEDICATION

To the Kitten Bandit and her friend.
Thank you both for holding my hand.

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ABSTRACT

Animal color patterns occur for a variety of reasons and can have a broad impact on fitness. Additionally, the presence of multiple color phenotypes in a species can be ecologically advantageous. One must first understand the physiological mechanism behind color patterning and variable morphs to understand how this variation affects fitness or provides an ecological advantage. Multiple phenotypes produced by one genotype, including multiple color morphs, can be genetic or plastic and may evolve very quickly as an adaptive response to changing environments. Phenotypes that are genetically based, called polymorphisms, can be controlled by one to many genes, are heritable and may have genetic links to other traits. Multiple color morphs may also be determined by environmental conditions, such as temperature and photoperiod, or phenotypic plasticity. Here, I sought to determine the physiological mechanisms underlying the multiple color phenotypes of an abundant and widespread lepidopteran larva. *Hyles lineata* has a primary, basal coloration of yellow and green that can be observed throughout the larval body, most noticeably during the final instar. Also, this larva may have dark lateral dorsal stripes that vary in thickness during the juvenile stage. These stripes can be as minimal as a thin dotted line or so thick that the entire larval body appears black. The variation in both basal coloration and dorsal striping can be observed in the field and in the laboratory and seem to be independent of each other. I designed a series of experiments to determine which, if any, of these color patterns is genetically controlled and which, if any, is phenotypically plastic. To do so, I conducted several single-pair inheritance crosses to document the yellow to green larval color ratios expressed by their offspring. From these crosses, I was able to determine that the yellow and green coloration observed in larval *H. lineata* is, indeed, genetically based. Further, this trait is controlled by a single two-allele gene, green allele dominant, with contributions from modifier genes. A series of experiments testing variations in environments was also conducted. This included conditions of variable temperatures, photoperiod and combinations of the two. Larvae were exposed to these conditions from the second instar and the appearance of the dark dorsal patterning was noted upon the fifth instar. This patterning was found to be phenotypically plastic. While there was no variation in this trait observed under varying temperature conditions, there was an increase in stripe thickness with decreased photoperiod as well as with combinations of photoperiod and temperature. To identify and measure this variation in pigmentation, various methods were employed, from visual scoring and histological observation to chemical extraction. These methods were described and reviewed. The dark, plastic patterning observed as dorsal stripes in *H. lineata* was determined to be produced by the pigment melanin. Further, larvae that visually appeared to have greater dark patterning also had a higher percentage darkness when quantified with image analysis as well as a higher volume of extracted melanin, identified and quantified with the use of spectrophotometry. The mechanisms of the color variation observed in *H. lineata* are now better understood; this organism has a basal yellow/green coloration genetic polymorphism as well as a phenotypically plastic melanic dorsal lateral patterning. As it is known that both of these forms of phenotype variation can be linked to fitness and/or be advantageous, future research can focus on the potential adaptive consequences of these traits.

INTRODUCTION

Due to their complex relationship to fitness, animal color patterns have long been of interest in ecology and evolution (Darwin 1859; Ford 1945; Cott 1957). Coupled with behavior, the presence of color pattern greatly influences fitness via predator defense, mate choice and, in ectothermic animals, temperature regulation (Fox and Vevers 1960). For example, ways that animal color patterns have been utilized as a means to avoid predator detection include background matching, disruptive coloration, or even transparency (Ruxton et al. 2004). If detected, color patterns can also be utilized in defense as warning displays or mimicry of other warning displays (Ruxton et al. 2004).

While coloration can be beneficial, more advantageous still is the presence of multiple color phenotypes (Forsman and Aberg 2008). In populations where one species has multiple color phenotypes living together, the variation reduces their predators' visual perception (Karpestam et al. 2013). For example, the rock pocket mouse of the American southwest has a pale pelt and is commonly found on light-colored rocks; however, a population of this species which inhabits lava flow sites, is heavily melanized (Benson 1933; Dice and Blossom 1937). The strong selection pressures from the rock mice's natural, strongly visual, owl predator is likely responsible for the adaptive melanization (mutations of one gene, Nachman et al. 2003) in segregated populations (Dice and Blossom 1937), creating predator-driven color pattern variation (Karpestam et al. 2014).

Similarly, sexual selection, has been found to be the primary driver in color pattern variation in some species (Chen et al. 2012). The use of color patterns for sexual selection often include colorful, grandiose displays coupled with behavior to attract mate attention. For example, male Australian dragon lizards have not only more intense color patches than females, but also more complex color patterns (Chen et al. 2012). While this strategy is often successful in attracting a mate, the potential of attracting a predator is a high risk as well (Godin and McDonough 2003). Despite this risk, the pressures of sexual selection are greater than those of predator selection for many species.

It has long been thought that the presence of multiple phenotypes of a trait confers benefits to a species by increasing the rate at which it is able to exploit environmental resources (Dobzhansky 1951; Hedrick 1986; Leimar 2005). Further, species with multiple color phenotypes have been found to have broader habitat types and larger ranges. For example, the white-lined sphinx moth, *Hyles lineata*, is the most abundant and widespread sphinx moth in North America, with a range covering all of North America, including southern Canada and Mexico, as well as documentation in Central and South America (Powell and Opler 2009). Habitat type and elevation vary dramatically over this latitudinal gradient and include desert scrub, oak-woodland, grasslands and high-elevation meadows. The multiple phenotypes *H. lineata* expresses as a larva may contribute to this exploitation of a wide variety of resources, habitat types and extensive range.

A species' multiple color patterns can be ecologically advantageous and can contribute to its evo-

lutionary success (Hancock 1977; Forsman et al. 2008). Color patterning can be linked to fitness-related characteristics (Sinervo et al. 2001) and individuals of different phenotypic morphs may experience different costs and benefits in various ecological and morphological contexts (Meyer 1989). This phenotypic variation in coloration and color pattern may be plastic or have a genetic basis and can evolve rapidly (Majerus 1998; Reznick and Ghalambor 2001).

POLYMORPHISM

When the expression of multiple color morphs is genetically determined, it is called color polymorphism. Polymorphism is defined as two or more genetically based traits in a species, potentially from a single female, in the same habitat, in high enough frequencies to eliminate the possibility of the morph occurring due to mutation (Ford 1940). Variation in morph frequencies per population may vary spatially by habitat or geographically, but overall frequencies are stable through time in species of balanced polymorphisms. Color polymorphism is often controlled by few genes; however, these genes are often associated with fitness-related traits by shared developmental pathways (Forsman 1995; Forsman and Shine 1995; Forsman and Appelqvist 1998; Andersson 2001; Forsman et al. 2002; Forsman et al. 2008). Whether these shared pathways arise from linkage disequilibrium or trait association (Brodie 1989; Endler 1995; Forsman et al. 2008), correlational selection may not act directly on coloration at all, but on these other genetically associated traits (Brodie 1989; Andersson 2001; Forsman et al. 2008). Thus, various selection pressures on multiple traits can result in high variation of genetic recombinations that not only maintain polymorphism within a species, but over space and time, may be combined in new and potentially advantageous ways (Ford 1940; 1945; 1975). These combinations permit rapid adaptive evolutionary response to variable climatic and environmental conditions (Forsman et al. 2011) and are predicted to be the most important and relevant attribute in the face of a changing climate (Hughes et al. 2008).

Color polymorphism, in particular, has been found to increase species range, population stability, utilization of a high diversity of resources, and evolutionary potential as well as decrease vulnerability to environmental changes, extinctions, and range contractions (Forsman and Aberg 2008; Forsman et al. 2008). However, each morph may experience different fitness costs. Identifying the long-term fitness costs and benefits to each morph contributes to the understanding of the maintenance of polymorphism within a species.

PHENOTYPIC PLASTICITY

Environmental cues can create various morphs among a population; instead of differences at the genetic level, these variations, termed phenotypic plasticity, are influenced by differences in environmental conditions (Shapiro 1976). Conditions can include, but are not limited to, temperature, photoperiod, the combination of the two (i.e., seasonality), or crowding and food quality (Nijhout 1999). These cues allow an individual to better assess the state of the environment, resulting in adaptive matching of phenotype to environment (Moran 1992; Via 1993; Forsman 2015). Each of these environmental cues can elicit a response from one to many traits at a time,

including body size, survival, fecundity, development rate, diapause induction and color pattern (Caswell 1983; Hazel 2002).

The plasticity of traits that strongly influence fitness allows for greater ecological breadth (Futuyma and Moreno 1988; Sultan 2001). Thus, plastic traits are advantageous in variable environments (Via 1993) and can be favored by natural selection (Scheiner 1993). However, the ability to exhibit trait plasticity requires gene maintenance of each alternative phenotype, as well as the genes that turn on those genes to be expressed once triggered by an environmental cue (Majerus 1998; Nijhout 1999; Lee and Wilson 2006). As a consequence, the plasticity of a trait is costly to maintain (Valladares et al. 2007); and the greater the plastic variation of a trait, the higher its cost (Lind and Johansson 2009).

MELANIN

Melanism, the variation in the-deposition of melanin, is one of the most common and well-documented conspicuous phenotypes (True 2003; Liu et al. 2015; San-Jose and Roulin 2018). The pigment melanin is widespread throughout the animal and plant kingdoms (Pralea et al. 2019) where it has been observed to be heavily influenced by environmental cues (Poulton 1885). Melanin-phenotype associations have been found to have a multitude of trait associations across a wide range of taxa, resulting in various fitness advantages and disadvantages (Liu et al. 2015) and contributing to many adaptive processes in insects (Qiao et al. 2020).

Melanization in some ectothermic animals, including insects, is thought to be primarily driven by the need to thermoregulate (Wang et al. 2013; Lindgren et al. 2014). For ectotherms especially, color patterns can have large effects on thermoregulation (Kettlewell 1973; Porter 1982; Scoble 1992). Animals such as insects and reptiles rely on external heat sources (i.e., radiant, convective and conductive heat) for warmth; coupled with modified behavior (Casey 1976), this results in a more regulated body temperature (Peterson 1987). As black absorbs heat, dark or heavily melanized animals can increase their body temperature in sunlight and modify their behavior to decrease it (Kettlewell 1973). In some species with multiple color patterns, such as the pygmy grasshopper, the darker morphs not only have higher body temperatures, but prefer higher body temperatures (Forsman 2000), suggesting behavioral and physiological coevolution (Forsman et al. 2002). Other benefits of melanism include protection from ultraviolet light (Gunn 1998; Buck and Callaghan 1999; Karl et al. 2009; Hu et al. 2013), decreased juvenile development time (Hazel 2002), and an increased immune response (Lee et al. 2008; Fedorka et al. 2013); however, it can be energetically costly (Talloen et al. 2004).

Melanism has been frequently observed throughout each life stage of the Lepidopteran order (Liu et al. 2015) and noted to be influenced by several environmental cues (Poulton 1885). For example, larval melanization has been triggered by seasonality (in *Papilio polyxenes*, Hazel 2002), crowding (*Mamestra brassicae*, Goulson and Cory 1995), food quality (*Spodoptera litoralis*, Lee et al. 2008) and others (see Nijhout 1999 for a review).

While melanin is widespread and well documented in Lepidoptera, the pigment itself is difficult to work with (Pralea et al. 2019). Melanin has a negative charge, high molecular weight and is composed of polymerized phenolic and indolic compounds (Pralea et al. 2019). There are many types of melanins, derived from a wide variety of chemical precursors, found in a multitude of tissue types in animals, plants and bacteria (Pralea et al. 2019). This, coupled with its insoluble, complex and amorphous structure, can make the identification, classification, isolation and quantification of melanin difficult.

The melanin biochemical pathway has several phenol oxidative steps resulting in different colors of melanin pigments (Wittkopp et al. 2002; Yu et al. 2011; Shamim et al. 2014). As part of the inflammation response, melanin is produced by coelomocytes and participates in phagocytosis and encapsulation of the invading foreign body (Vass et al. 1993; Wittkopp et al. 2002; Yu et al. 2011; Shamim et al. 2014), thereby increasing pathogen resistance (Robb et al. 2003). Thus, it is important to understand that the melanization process strongly relates to immune response in Lepidoptera (Robb et al. 2003). If melanin production is impaired, there can be an increase in infection rate (Liu et al. 2007).

Not only may there be an increase in melanization upon the initiation of the immune response, but melanic animals may have an increased immune response (Wilson et al. 2001; Cotter et al. 2004; Liu et al. 2007; Dubovskiy et al. 2013; Fedorka et al. 2013). Thus, the increased melanization of a melanic phenotype may be an indicator of pathogen and parasitoid resistance (Vey and Götz 1986). While melanic individuals may be at an advantage if this unregulated immune response is not utilized, ultimately, life history traits are impaired (Dubovskiy et al. 2013). This critical process is energetically costly (Freitak et al. 2003) and has been found to lead to decreased body size and growth rate and increased mortality (Bascuñán-García et al. 2010). Thus, the advantages gained by the melanic phenotype may come with an important trade-off. Experiments on immunity were conducted in association with this dissertation; however, they are not presented here.

STUDY SYSTEM

Lepidopteran larvae are known to be highly diverse in their color patterning (Scoble 1992). The white-lined sphinx moth, *Hyles lineata* (Lepidoptera, Sphingidae), displays color patterning as an adult as well as a larva. Furthermore, *H. lineata* has multiple color phenotypes as larvae. While adult moths uniformly exhibit the same color pattern, brown with white stripes on the forewings and pink hindwings, the larvae vary in both color and dark patterning. In their final instar, larvae are yellow or green with or without black dorsal stripes. When stripes are present, they can vary greatly in width and intensity, so much so that occasionally the larva may appear to be solidly black. Larval color varies among individuals as well as across development. During the first instar, the larva is a transparent white, appearing green when eating plant matter, whereas it is black with white dorsal stripes in the second instar. During the third, fourth and fifth instars, variations of green, yellow and black can be observed.

This species was noted above for its abundance across a diverse array of habitat types across a vast range. Adults are generalist nectarivores and larvae feed on a wide variety of host plants, including plants from the families Portulacaceae, Nyctaginaceae, Euphorbiaceae, Onagraceae, Rosaceae and Primulaceae (Evans 2007; Powell and Opler 2009; personal observation). An increased dispersal ability can be attributed to this broad range of host plants (Beck and Kitching 2007). Additionally, *H. lineata* is a member of the subfamily Macroglossinae, known to be excellent fliers, which have migratory tendencies and are less restricted by ecological limitations (Beck et al. 2006). Macroglossinae hawkmoths are known to move pollen long distances, up to 10km (Linhart and Mendenhall 1977; Haber and Frankie 1989; Finger et al. 2014) and being able to travel thousands of kilometers to colonize new areas (Beck 2015, personal communication).

The multiple phenotypes *H. lineata* expresses as a larva may potentially aid in its ability to exploit extremely wide spatial and temporal niches. However, historically, little is understood about the morphology and physiology of larval color variation in this species. Thus, in my first chapter, I explore the potential for genetic color polymorphism in the multiple color phenotypes of this species. Specifically, I focus on the trait of the yellow and green basal coloration. I do so with a series of familial crosses: outcrosses, backcrosses and intercrosses, some over multiple generations. The yellow to green color ratios observed in the first and second generation of these crosses help us understand not only the inheritance of this trait, but how many genes may be contributing to its control. In my second chapter, I explore the potential for phenotypically plastic coloration in the larvae. Specifically, I focus on the trait of dark dorsal and lateral stripes and patterning. I do so by exposing the larvae to several controlled environments of temperature and photoperiod, throughout their juvenile development to observe any variation in coloration. Variation of the dark color patterning as a result of these treatments will indicate plasticity of this trait as well as which environmental conditions to explore further. In my third chapter, I explore the pigment that the dark patterning is comprised of. I identify what pigment is present as well as quantify volume of this pigment in various phenotypes. Further, I review common methods frequently used to quantify dark patterning in lepidopteran larvae. These methods are discussed and compared in how they vary in technical complexity and data output.

CHAPTER 1. Genetic color polymorphism of the white-lined sphinx moth larva (Lepidoptera; Sphingidae)

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APPENDIX A

For a trait to be genetically polymorphic, variants of this trait must co-occur in the wild, possibly from a single female, in high enough frequencies to eliminate the possibility of the morph occurring due to mutation. I considered each of these aspects to determine which, if any, of the *H. lineata* larval coloration aspects were genetic. Thus, field surveys in Arizona and California were conducted to confirm that both the yellow and green *H. lineata* larval color morphs co-occur. Frequencies of each color per observed population were recorded. Both yellow and green morphs were observed at each of the surveyed sites, indicating multiple populations of co-occurring color morphs. To determine if the yellow and green color morphs could possibly be related to the same maternal female, and in what ratios, single-pair crosses were conducted in the laboratory. By conducting outcrosses, intercrosses and backcrosses, I was able to determine inheritance as well as the number of genes that control this trait. Multigenerational crosses of greater than ten offspring per generation confirmed that the yellow and green basal coloration in *H. lineata* is, indeed, genetic in a Mendelian inheritance pattern complimented by modifier genes.

CHAPTER 2. Phenotypic plasticity of cuticular melanin in larval *Hyles lineata* (Lepidoptera: Sphingidae) induced by a temperature-photoperiod interaction

APPENDIX B

The influence of environmental conditions on a trait can indicate trait plasticity. I conducted a series of environmental manipulations to determine which, if any, of the color patterning observed in larval *H. lineata* were plastic. Larvae were singularly exposed to variation in individual environmental conditions of temperature and photoperiod. Larvae were further exposed to these two environmental cues simultaneously. The degree of black patterning was measured in the fifth and final instar using a visual scoring technique. Also conducted, but not presented here, were experiments on changes in dark patterning while under conditions such as crowding and ultra violet light.

The degree of black patterning was not affected by variation in temperature; however, it was affected by changes in photoperiod. This pattern variation induced by an environmental condition suggests this trait is phenotypically plastic in *H. lineata*. While there was no variation in dark patterning when exposed to various temperatures, there was significant variation when temperature and photoperiod were paired.

CHAPTER 3. Cuticular melanin determination and quantification methodology as practiced on lepidopteran larva, *H. lineata*

APPENDIX C

After exploring the physiological mechanisms by which larval *H. lineata* exhibits various color variations, I focused on determining the morphological mechanism. To determine the pigment responsible for the dark dorsal lateral stripes, I conducted and reviewed multiple melanin identification and quantification techniques. These included visual, microscopy and chemical analysis. Visual analysis included visual scoring and image analysis with the use of a digital camera and computer program, ImageJ. Three categorical scores were used to determine the degree of darkness in *H. lineata*: minimum, medium and maximum. Image analysis quantifies the degree of visual melanin as a continuous trait, resulting in percentage values of darkness per total body size. Larvae that were visually scored as having more visual dark patterning scored higher percentages. Histological cross-sections of larvae were stained and viewed with a microscope. Doing so allowed a closer look at where the pigments were located in the dermis and cuticle. The dark pigments were located in the cuticle, where melanin completes its synthesis; other possible dark pigments reside in the epidermis. I extracted melanin from larval exuviae to isolate and quantify melanin. Acid hydrolysis with DMSO was used to break down the cuticle, each exuvia underwent the extraction process five times, with the supernatant collected during each extraction. I then measured the density of suspended melanin in each supernatant sample with the use of a spectrophotometer. By including a known synthetic melanin dilution with these samples, a standard curve was used to calculate melanin quantity per exuvia. Doing so resulted in quantitative and comparative melanin volumes. Exuviae from larvae visually scored as minimum had less melanin extracted from it than exuviae from larvae scored as maximum. Melanin was determined to be the pigment responsible for dark stripes observed in *H. lineata*. Each of these methods has value for describing, determining and quantifying this pigment, however, there are trade-offs to each method.

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APPENDIX A.

Genetic color polymorphism of the whitelined sphinx moth larva, *Hyles lineata* (Lepidoptera: Sphingidae)

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ABSTRACT

For a trait to be considered polymorphic, it must fulfill both genetic and ecological criteria. Genetically, a polymorphic trait must have multiple heritable variants, potentially from the same female, in high enough frequency as to not be due to mutation. Ecologically, in a single wild population, these variants must co-occur, and be capable of interbreeding. Polymorphism is frequently considered in the context of either geographic cause or genetic consequence. However, the incorporation of both in a single study can facilitate our understanding of the role that polymorphism may play in speciation. Here, we ask if the two color morphs (green and yellow) exhibited by larvae of the whitelined sphinx moth, *Hyles lineata* (Fabricius), co-occur in wild populations, in what frequencies, and whether they are genetically determined. Upon confirmation from field surveys that the two color morphs do co-occur in wild populations, we determined heritability. We conducted a series of outcrosses, intercrosses and backcrosses using individuals that had exhibited yellow or green as laboratory-reared larvae. Ratios of yellow:green color distribution from each familial cross were then compared to ratios one would expect from a single gene, yellow-recessive model using a two-sided binomial exact test. The offspring from several crosses indicate that the yellow and green coloration is a genetic polymorphism, primarily controlled by one gene in a single-locus, two-allele Mendelian-inheritance pattern. Results further suggest that while one gene primarily controls color, there may be several modifier genes interacting with it.

Keywords: polymorphism, color variation, Mendelian gene

INTRODUCTION

Animal color patterns have long been of interest in ecology and evolution due to their frequent direct and indirect links to fitness (Darwin 1859; Ford 1945; Cott 1957; Forsman 2014; Karpes-tam et al. 2014; Janssen and Mundy 2017). The ease of visual identification and the broad range of taxa in which color variation occurs have led to many studies on the ecological impacts of predators and sexual selection, as well as changes in selection pressures across a species range (Forsman et al. 2008; McKinnon and Pierotti 2010; Wellenreuther et al. 2014). Other studies have focused on determining the genetic associations among genes that control color and other fitness-related traits (Abbott and Svensson 2005; Forsman et al. 2008). Recently, however, there has been a call to unify these ecological and genetic aspects of color polymorphism research (Wellenreuther et al. 2014). Doing so would allow the molecular effects of various selection pressures to be linked with ecological forces and applied to understanding the costs and benefits of genetic color polymorphism at the individual, population and species levels (Wellenreuther et al. 2014). Furthermore, as many color polymorphic species exhibit a geographical cline due to variation in selection pressures across a species' range, understanding the geographical and genetic context of a variable trait can ultimately lead to understanding the role that polymorphism plays in speciation (Forsman et al. 2008; McLean and Stuart-Fox 2014). Combining molecular and ecological approaches to gain insight on mechanistic control and microevolutionary processes can ultimately lead to the facilitation of identifying selection pressures and trait associations in a study system, as well as the evolutionary causes and consequences of color polymorphism (Forsman et al. 2008; McLean and Stuart-Fox 2014; Wellenreuther et al. 2014).

By definition, the genetically-based variable traits of a polymorphism in a species should co-occur in high enough frequencies to eliminate the possibility of either trait variant (or morph) being due to mutation (Ford 1940). In true (balanced) polymorphic systems, morph frequencies vary in the short term but are stable over time; conversely, transient polymorphic systems may lead to monomorphism and speciation (Suzuki and Nijhout 2006; McLean and Stuart-Fox 2014). Selection pressures that vary temporally and at local scales are thought to maintain polymorphism while broad-scale spatially variable selection can ultimately lead to population divergence (McLean and Stuart-Fox 2014). As environmental conditions and selection pressures change across a balanced polymorphic species range, morph frequencies within a population are expected to change (McLean and Stuart-Fox 2014). This is observed in many polymorphic populations, varying geographically by morph frequency, number or type (McLean and Stuart-Fox 2014).

Genetic mechanisms controlling color polymorphism are often relatively simple, involving few genes of major effect (Cain and Shepard 1954; Joron et al. 2011; Wellenreuther et al. 2014; Rankin et al. 2016; Woronik and Wheat 2017; VanKuren et al. 2019). Frequently the inheritance of these traits is explainable by Mendelian segregation (see Wellenreuther et al. 2014 for a review) and due to this simple genetic basis and high heritability, genetic polymorphism has been studied consistently with the use of Mendelian analysis (see McKinnon and Pierotti 2010). By utilizing Mendelian crosses, color polymorphism has been determined to be maintained by few loci with few alleles following Mendelian segregation in many taxonomic systems, including the

blue-tailed damselfly (*Ischnura elegans*, Sanchez-Guillen et al. 2005) and the mocker swallowtail butterfly (*Papilio dardanus*, Clarke and Sheppard 1959); see Wellenreuther et al. 2014; McKinnon and Pierotti 2010 for extensive reviews). Key to these color polymorphism studies utilizing genetic crosses, is that the species must exhibit discrete color variation among a large number of individuals (Ford 1945), which our study organism does. The white-lined sphinx moth, *Hyles lineata* (Sphingidae), has a broad geographic range, is frequently observed in large aggregations in the wild, and expresses multiple larval color morphs, as frequently noted in descriptive text in literature (Tuttle 2007; Powell and Opler 2009). However, little is currently understood about these qualities, including how the color variation is physiologically controlled or maintained across populations. Adult moths uniformly exhibit the same color pattern: brown with white stripes on the forewings and pink hindwings. However, the larvae are polymorphic for both color and pattern. While individual larvae vary in color across instars, larvae are most noticeable in their last and largest instar. At this point, they are yellow or green (Figure 1) and may or may not have two black dorsal stripes.

To understand the potential causes and consequences of multiple color morphs in this system and how color polymorphism is maintained, one must first identify co-occurrence and morph frequency variation as well as the mechanism of control behind the coloration. Here, we ask if color variants of *H. lineata* occur together in natural populations. If so, do these natural populations vary in morph frequency? Field surveys were conducted to determine co-occurrence and color morph frequency variation. Further, we ask if this color variation in *H. lineata* larvae is under genetic control. If so, are the traits of this color polymorphism controlled by few genes of Mendelian segregation, as has been shown in other taxa? Genetic crosses between individuals of various color morphs were conducted to determine the genetic basis and heritability of the color morphs.

METHODS AND MATERIALS

STUDY ORGANISM, HYLES LINEATA

Hyles lineata is the most abundant and widespread sphinx moth in North America, with documented presence in Central and South America as well (Powell and Opler 2009). Within its native range, this species experiences high variation in environmental conditions and exploits a broad array of resources. An excellent flier with migratory tendencies (Beck et al. 2006), adults have been known to move pollen up to 10 km, making them crucial pollinators of a wide diversity of plants, including several sensitive and rare species (Linhart and Mendenhall 1977; Haber and Frankie 1989; Finger et al. 2014; Skogen et al. 2016). *Hyles lineata* has been observed in habitats ranging from low elevation desert scrub, oak woodlands and grasslands to high elevation meadows and pine forests. Throughout these habitat types, the polyphagous larvae feed on a wide variety of host plants from at least ten plant families (Evans 2007; Powell and Opler 2009; personal observation). In some habitats *H. lineata* can be active most of the year, producing multiple broods.

FIELD-OBSERVED LARVAL COLOR CLASSIFICATION

The color of larval *H. lineata* varies across instars as well as within an instar at various body parts of an individual. During the first instar, the larva is transparent white, whereas it is black with white dorsal stripes in the second instar. During the third and fourth instars, variations of green, yellow and black can be observed. While the amount of black is usually reduced from that of the fourth instar, black dorsal stripes may occur in the fifth instar. When stripes are present, they can vary greatly in width and intensity, so much so that occasionally the larva may appear to be solidly black. However, such density of black striping rarely occurs in the laboratory. Most often, in the fifth and final instar, larvae are markedly yellow or green (see Figure 1). While we classify larvae as either ‘yellow’ or ‘green’, a gradient between the two can be observed. Furthermore, this color varies slightly across the larval body: it is more intense on the dorsal side, whereas the ventral side is quite pale and the areas of thicker chitin, the anal plate and head capsule, can be shades darker than the body. Therefore, color was determined during the fifth instar, at four points along the body: 1) head capsule, 2) anal plate, 3) central dorsal abdomen and 4) central lateral abdomen. Using the Sherwin-Williams ColorSnap Visualizer iPhone application (The Sherwin-Williams Company, Cleveland, OH), under ambient lighting conditions, larvae that matched color #6915 ‘citronella’ or were more intensely yellow than this standard were identified as yellow, whereas #6705 ‘high strung’ and colors with greater green intensity were identified as green. However, prior to this classification system, field data were collected using three color categories: yellow, yellow/green and green. Surveyed larvae appearing bright yellow with minimal green tint and brownish head capsules were classified as ‘yellow’. Green larvae with minimal yellow and green head capsules were classified as ‘green’, and larvae that were an even combination of the two were classified as ‘yellow/green’. The development of color standards and the use of the color application provided a more narrow color point to identify yellow larvae, eliminating a blended ‘yellow/green’ designation.

WILD POPULATION COLOR RATIOS AND FIELD SURVEYS

To verify that *H. lineata* color morphs co-occur and are not geographically separated, field surveys were conducted in Pima and Cochise Counties, southeast Arizona as well as San Bernardino County, southern California, during the 2013, 2014 and 2017 summer seasons. Based on variation noted in many species descriptions (Evans 2007; Powell and Opler 2009) and in previous literature (Casey 1976; Mock and Ohlenbusch 1981), we hypothesized that the larval color morphs of *H. lineata* occurred sympatrically in wild populations.

Once a dense population of wandering (highly mobile) fifth instar larvae was located, transects of approximately 20 meters were walked and the color morph of each individual encountered recorded. Transects (hereafter referred to ‘sub-sets’) were repeated, when possible, several meters away from previous transects to avoid repeat recordings of individuals. Survey sites consisted of Anza Borrego Desert State Park, Borrego Springs, CA; Continental Road near Madera Canyon, Green Valley, AZ; Oro Valley, AZ; Portal Road, Portal, AZ; Yetman Trail, Tucson Mountain Park, Tucson, AZ and San Pedro River Valley Reserve, Sierra Vista, AZ. Approximate-

ly 100 individuals were surveyed per site, with a total of 1201 individual larval color morph observations recorded.

COLONY AND EXPERIMENTAL ANIMAL CARE

Individuals used for crosses came from multiple generations of a laboratory colony of *H. lineata* that were initially collected from populations in southeastern Arizona. Lab populations were generally viable for five to ten generations, with at least 200 adults per generation, with wild-caught adults continuously added to the main colony to avoid inbreeding. Larvae, pupae and adults were kept at 27°C, 16L:8D photoperiod. The different morphs were kept clearly labeled and separate from each other, resulting in several breeding populations. Within each breeding population, larvae were reared in metal trays with vented plastic lids. Trays were lined with paper towels to absorb excess moisture and contained a raised hardware cloth stage to provide adequate surface area for molting and to separate larvae and food from frass. Larvae were fed *ad libitum* a fresh wheat-germ based artificial diet (100% diet; see Davidowitz et al., 2003). Overcrowding was avoided and trays were cleaned daily. Once a fifth instar larva concluded feeding and began to clear its gut in preparation for pupation (noted by very loose frass and highly active wandering), coloration was noted and then it was moved into a labeled wooden pupation block. Adults of each stock population were maintained separately in large plexiglass mating chambers with continuous access to a 20% sucrose sugar solution and live *Oenothera sp.* (Onagraceae) plants for oviposition. Eggs were used as breeding stock to maintain large enough populations to provide experimental animals, as well as to maintain population genetic diversity. Labeled individuals of each population were selected at random for experimental single-pair matings while in the pupal stage. Adults were allowed to eclose and were then paired with another virgin adult of known larval color in a 30 x 30 x 30 cm plastic and mesh mating chamber with access to sucrose and oviposition plant. In these experimental crossings, eggs were collected from plants daily. The offspring of each pairing were reared in low-density trays as above, and kept completely isolated from other families and the stock colony throughout their entire lifecycle. Upon entering pre-pupation at the end of the fifth instar, coloration of all experimental single-pair mating offspring was noted.

LABORATORY-REARED LARVAL COLOR CLASSIFICATION

Because animals reared in the laboratory were fed artificial diet (see above) that lacks plant carotenoids, the yellow and green hues differed slightly from those found in the wild (see Figure 1). Colors of laboratory-reared larvae were classified using the same Sherwin-Williams Color-Snap Visualizer iPhone application, but with slightly different colors defined. Yellow was classified as #6409 ‘edgy gold’ for the head capsule and anal plate and #9030 ‘limon fresco’ for the abdomen. Larvae with more intense or darker shades of yellow or brown hues than these colors were recorded as yellow. Green larvae observed in the laboratory were defined by #6417 ‘tupelo tree’ (for head capsule) and #6710 ‘melange green’ (abdomen). Those with deeper, darker or more intense green or blue hues were also recorded as green.

INHERITANCE AND GENETIC CROSSES

To determine if the color variation observed in larval *H. lineata* is genetically determined, we conducted a series of outcrosses, intercrosses and backcrosses using adults of larvae that had exhibited yellow or green larval coloration (subsequently referred to as ‘yellow’ and ‘green’, respectively) from the color-based populations maintained in the lab. Based on pilot crosses and observations made while maintaining an *H. lineata* colony, we hypothesized that coloration was genetically determined. Further, based on pilot studies, we hypothesized that the larval color polymorphism is a single-locus trait with the yellow allele recessive (‘y’) and the green allele dominant (‘G’).

Single-pair outcrosses of green by green (cross denoted by ‘x’), yellow x yellow and yellow x green, were conducted with the color of all resultant first generation (F1) offspring noted (Figure 1). Some of these F1 offspring were then mated with a sibling (intercross) or with an unrelated adult of the same larval coloration as the parental generation (P) (backcross), noting the larval coloration of all second generation (F2) offspring. While the offspring of the F1 generation display the dominant phenotype, the F2 generation of a backcross can reveal the number of loci controlling the genotype by the distribution of the phenotype. The trait for larval coloration is on a single locus when offspring have equal, discontinuous phenotypic classes mirroring those of the parent generation, or is polygenic if intermediate phenotypes are present (Silver 1995) Our expectations for phenotype frequencies were based on the Mendelian model of a single gene with two alleles, where the allele for green larval coloration is dominant, are outlined in Table 1. In this model, some crosses might have multiple yellow/green larval coloration ratio possibilities. Thus, the resultant F1 and F2 color ratios of each familial cross were tested against all possible model outcomes for that cross. There is no data on differential mortality between color morphs in *H. lineata* larvae reared in the laboratory; thus, we assume equal mortality between morphs. However, to ensure that our crosses provided conclusive data, we conducted a power analysis to determine family size needed to infer inheritance pattern. A power analysis with a significance level of 0.05 indicated that a sample size of 10 offspring per familial cross would allow for these different ratios to be declared significant 80% of the time. Families with fewer than 10 offspring were excluded from data analysis. The yellow to green larval color ratios from the resultant offspring were analyzed using a two-sided binomial exact test to determine if these ratios were significantly different from that of the expected model (all statistical analyses were done in R ver. 3.5.1.; www.R-project.org).

RESULTS

FIELD SURVEYS

Both green and yellow fifth-instar *H. lineata* larvae were present at most of the field sites surveyed (Table 2). Some sites, such as Portal, Arizona, during the 2013 wet summer monsoon season, had sub-sites in which only 4% of larvae were yellow, as well as sub-sites where 100% were yellow.

GENETIC CROSSES

Larval color ratios from the single-pair mating crosses indicated that the yellow and green coloration observed in *H. lineata* is a genetic polymorphism, primarily controlled by one gene in a single-locus, two-allele Mendelian-inheritance pattern. All single-pair yellow x yellow outcrosses produced F1 offspring that were 100% yellow (see Table 3 for details of crosses). A binomial exact test indicated that this ratio was not significantly different from that predicted by the model. All green x yellow crosses also produced offspring color ratios not significantly different from the expected Mendelian model. Two green x yellow crosses (crosses 11 and cross 12) resulted in an F1 that did not vary from the expected model of 0% yellow:100% green. Both of these families became the parental generation of back/intercrosses (multigenerational families denoted by ‘*’ in Table 3; see Table 4 for complete multigenerational family lineage). One of the 100% green offspring from cross 11 was backcrossed with an unrelated yellow individual, producing an F2 fitting the 1:1 yellow/green Mendelian model (cross 10). This 1:1 phenotypic distribution in an F2 backcross indicates that the genotype of the yellow and green larval coloration is controlled by a single gene (Silver 1995). Two of the 100% green F1 offspring from cross 12 were intercrossed (cross 13), resulting in an F2 fitting the 1:3 color ratio expected from a green x green (Gy x Gy) cross. Also fitting the 1:3 ratio was the parental cross of unrelated green x green individuals (cross 14). However, green x green crosses 15 and 16, both intercrossed (F1) siblings of cross 14, differed from the expected green x green, yellow-recessive Mendelian ratio (Table 4). From the cross 14 F1 results, we can infer that both of the two green individuals crossed were heterozygous dominant. In our model, intercrossing these offspring would be expected to result in an F2 with yellow:green phenotypes ratios of 0:1 or 1:3. However, when two green F1 siblings of this family were intercrossed (cross 15), the resulted F2 expressed phenotypes of 35% yellow:65% green color ratio, a 1:2 ratio not expected from single-locus inheritance. This ratio is significantly different from both the 1:3 model ($p=0.007$) and the 100% green model ($p<0.0001$). Similarly, another pair of green color morph F1 siblings from cross 14 were intercrossed (cross 16), expressing an F2 ratio of 56% yellow:44% green offspring. This ratio also differs significantly from both models (1:3 $p<0.0001$, 0:1 $p<0.0001$). While it is not significantly different from a 1:1 ratio ($p=0.597$), this is not an expected outcome of a green x green cross in our single gene, green dominant model.

Two other green x green crosses, 17 and 18, also differed from the expected 0:1 or 1:3 ratios. Both of these families resulted in approximately 15% yellow:85% green offspring, a 1:6 color ratio (cross 17, significantly different from 0:1 model- $p=0.007$, $p=0$ 1:3 model; cross 18- 0:1 model- $p=0.005$, 1:3 model- $p=0$). The remaining crosses, approximately half of all single-pair green x green outcrosses conducted, yielded > 95% green offspring in the F1 (crosses 19-24), a result not significantly different from the expected 100% green Mendelian model.

DISCUSSION

Field surveys confirmed that the various color morphs of *H. lineata* occur together in the same habitat in multiple populations. Because each site was only surveyed once, it is impossible to determine whether these polymorphic populations are balanced or transient and how each population varies over time, but we can confirm that the polymorphic color variation is not allopatric or associated with a geographic cline. While the surveys are consistent with our co-occurrence hypothesis, it must also be noted that these surveys were performed over a limited portion of *H. lineata*'s exceptionally broad geographical range. Survey results may vary throughout the range. At the landscape scale, variants of each morph within a population may be exposed to similar spatial and temporal environmental conditions (Ford 1945; McLean and Stuart-Fox 2014). However, while color morphs may occur together and be using a habitat similarly, they may be exploiting different microhabitats, resulting in various phenotypes present in an assortment of microhabitats (Karpeštam et al. 2012). Relative fitness of each morph may be dependent on microhabitat selection (Ahnesjo and Forsman 2006) where a generation of one color morph may be favored, resulting in maladapted individuals of the other morph (Roulin 2004). Furthermore, a polymorphic trait may be evolutionarily maintained in a species in continually changing selective environments, in a changing climate or across a species range, leading to differential fitness among color morphs (Jaworski and Lattanzio 2017). With this, different color morphs may employ different evolutionary and/or ecological strategies (Forsman et al. 2002; Ahnesjo and Forsman 2006; Suzuki and Nijhout 2006). For example, color morphs of the pygmy grasshopper, *Tetrix undulata*, vary in substrate selection, one color morph selecting substrate to aid in thermoregulation, another selecting color-matching substrate in predator avoidance (Ahnesjo and Forsman 2006). With the understanding that the various color morphs of *H. lineata* co-occur in the multiple populations surveyed here, we can now begin to investigate how each morph is being selected for or against within a population. Understanding how selection pressures and thus morph frequencies vary throughout a species range can clarify the role that polymorphism plays in speciation (Suzuki and Nijhout 2006; McLean and Stuart-Fox 2014).

The results of our genetic crosses indicate that the yellow and green coloration observed in *H. lineata* larvae is a genetic polymorphism, primarily controlled by one gene in a single-locus, two-allele Mendelian inheritance pattern. Through single-pair matings, we derived both yellow and green offspring, in ratios not significantly different from the expected Mendelian models, indicating simple genetic control of coloration. All of the yellow x yellow, green x yellow crosses and most of the green x green crosses conducted here resulted in offspring ratios that suggest that the allele for green larval coloration is dominant.

Though the majority of our data point towards a single-locus, two-allele inheritance pattern, there were some crosses that produced unexpected results. Expected ratios of a green x green cross consist of 0:1 or 1:3 (yellow:green); Table 1. While these ratios were observed here, four of the green x green crossed families resulted in ratios of 1:6, 1:2 and 1:1, not fitting either of these expected Mendelian models. These crosses suggest that while green may be the dominant color morph, incomplete penetrance is occurring when the genotype of the trait is not expressed phenotypically (Silver, 1995). Thus, the families that expressed green phenotypes in a 1:6 ratio, or in 85% of the F1 offspring, have a green genotype with 85% penetrance. The offspring that do not

express this phenotype, the 15% yellow offspring, carry the dominant green genotype, but this genotype is not translating or incompletely penetrates into the phenotype. However, the understanding of the genetic inheritance of this trait is only one piece of the puzzle. To fully understand these inconsistencies in the Mendelian inheritance pattern observed here, we may need to incorporate multiple levels of organismal information and potential environmental interactions (Gawne et al. 2018).

For a trait to be exclusively controlled by one gene, the trait variation must be discontinuous (Silver 1995). Yet, the coloration among each color morph of *H. lineata* expressed considerable variation. This variation suggests that yellow and green coloration is controlled by more than one gene. While the primary coloration may be Mendelian and fall on a single locus, there may be modifier genes interacting with this gene, potentially affecting its expression. These modifier genes may explain the discrepancies from the expected Mendelian inheritance patterns and the variation of color among each morph observed in the crosses conducted here. Similar variations in color have been attributed to modifier genes in other Lepidopteran color polymorphic species: the Mocker swallowtail (*Papilio dardanus*; Clarke and Sheppard 1959) and the Diadem butterfly (*Hypolimnas mysippus*; Gordon and Smith 1998). Both of these butterfly species were found to have heritable coloration on genes that segregate in a Mendelian pattern as well as variation among each color morph (Clarke and Sheppard 1959; Gordon and Smith 1998). The few genes controlling color polymorphism coupled with modifier genes, variable penetrance of these genes, and linkages among genes, may form a supergene complex (Gordon and Smith 1998; Joron et al. 2011; Rankin et al. 2016). These supergenes have the potential to be coupled with life history-related traits (Cain and Shepard 1954), resulting in significant variation of fitness between color morphs (Abbott and Svensson 2005). Recent genomic work has revealed such supergenes associated with coloration during various life stages in several species, including those of the *Heliconus* and *Papilio* genera (Joron et al. 2011; Kunte et al. 2014; Wellenreuther et al. 2014; Saenko et al. 2019; Yoda et al. 2020). Furthermore, the different supergene complexes that have been found to control coloration at different life stages have also been found to be decoupled from each other (Medina et al. 2019).

Selection for coloration, or the genetically associated traits, can result in various expression combinations over space and time to be combined in new, potentially advantageous, ways (Ford 1940; 1945; 1975). For example, Suzuki and Nijhout (2006) show in another hawkmoth species (*Manduca sexta*) that discrete color polyphenisms can arise through genetic accommodation of a mutation in the pathway of the developmental hormone JH (juvenile hormone) in response to environmental stress. The mechanisms that regulate developmental hormones can act as evolutionary capacitors introducing novel phenotypes into a population (Suzuki and Nijhout 2006). Consequently, color polymorphism can aid in the utilization of a high diversity of resources (Dobzhansky 1941; 1951), as well as increased efficiency of resource exploitation (Betzholtz et al. 2017), and genetic diversity contributes to the species' ability to take advantage of a broad niche (Forsman and Aberg 2008; Forsmen et al. 2008) and various microhabitats (Pizzatto and Dubey 2012). As *H. lineata* exploits a wide variety of resources, occupying a very broad niche, the presence of multiple juvenile color morphs may aid *H. lineata* in this ability. Confirming that

the color morphs co-occur and that they are, indeed, genetic polymorphisms, was the first step in determining if color variation aids in these benefits observed in *H. lineata*. The variation in potential selection pressures on each color morph, trait associations and genetic recombinations, make understanding the maintenance of color polymorphism, and the costs and benefits associated with it so complicated, yet critical to understanding a polymorphic species population persistence (Forsman 2016). Determining some of these potential variation in selection pressures and trait associations between color morphs in the *H. lineata* larvae will further our understanding of the costs and benefits of color polymorphism in this system. As the presence of color polymorphism in a species may increase range potential and population stability and decrease vulnerability to environmental changes, range contractions, and extinction (Forsman and Aberg 2008; Forsman et al. 2008), insight into the differentiation of these ecological traits in a species with reduced extinction risk may help identify predictors of vulnerability in threatened species (Kotiaho et al. 2005; Betzholtz et al. 2017).

By unifying ecological and genetic work in this study, we can begin the process of linking ecological and evolutionary forces with molecular effects and genetic trait associations in this species. The crosses conducted here explain the genetic of coloration of the larval *H. lineata*. We have shown that yellow and green coloration are controlled by a single gene, with yellow being the recessive allele, potentially affected by several modifier genes. By understanding that part of the color patterning is genetically derived, we can begin to connect color polymorphisms with trait associations and the selective pressures acting on each morph. Further studies can extend this work to understanding the cost and benefits of each morph as well as broader ecological questions, including those focused on the evolutionary forces that maintain and shape population-level phenotypic differences.

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Figure 1. *Hyles lineata* in their 5th instar.

Top- Wild 5th instar *H. lineata* larvae, of both the yellow and the green color morphs, located together during the Portal 2013 field survey.
Bottom- Laboratory-reared/artificial- diet fed 5th instar *H. lineata* of the green and the yellow color morph.

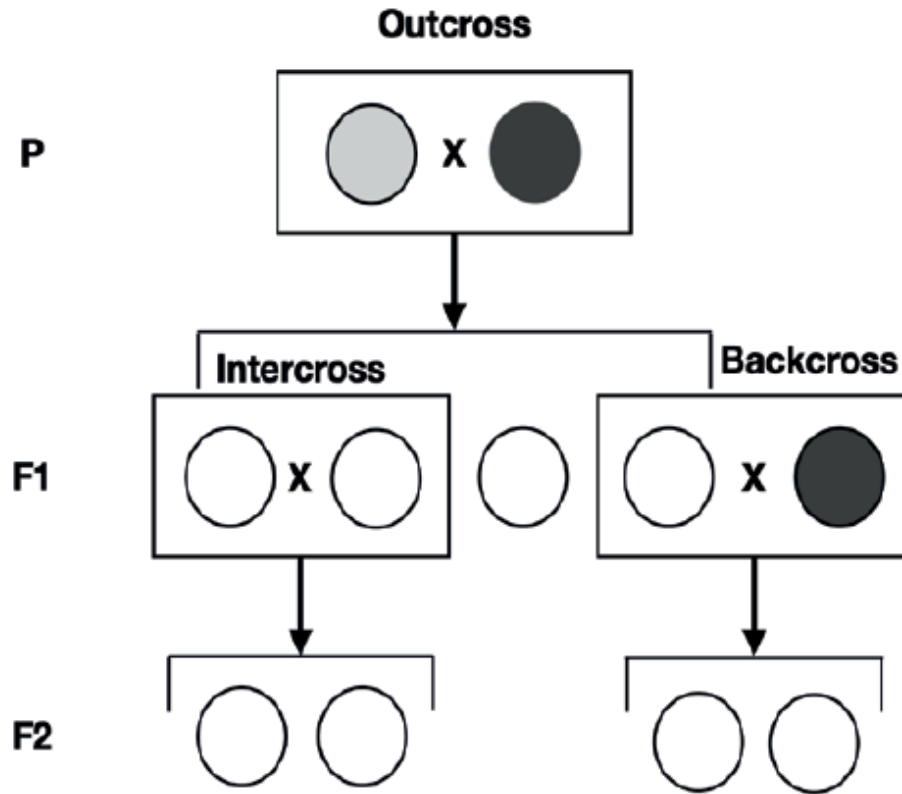


Fig. 2. Single-pair mating systems. Parental generation (P) outcrosses producing first generation filial offspring (F1). These offspring may have been further mated with a sibling (intercross) or an individual of the same larval coloration as one of the P individuals (backcross), resulting in second generation filial offspring (F2). Here, combinations of adult *H. lineata* of yellow and green larval coloration (represented by black and gray) were paired. The subsequent generations were reared and larval coloration (represented by white in diagram) noted during the final larval instar.

Table 1. Inheritance and genetic crosses-model expectations of a single, two-allele locus gene of Mendelian inheritance

Outcross, P1 phenotype	Outcross, P1 genotype	Expected F1 genotype, % 'yy'	Expected F1 genotype, % 'Gy'	Expected F1 genotype, % 'GG'	Expected F1 phenotype, % yellow	Expected F1 phenotype, % Green	Expected F1 phenotype color ratio, yellow:Green
y × y	yy × yy	100	0	0	100	0	1:0
G × G	GG × GG	0	0	100	0	100	0:1
G × G	GG × Gy	0	50	50	0	100	0:1
G × G	Gy × Gy	25	50	25	25	75	1:3
G × y	GG × yy	0	100	0	0	100	0:1
G × y	Gy × yy	50	50	0	50	50	1:1

Based on the hypothesis that the green color morph is genetically dominant and the yellow color morph is genetically recessive. Phenotypes are noted by 'G' (dominant green) and 'y' (recessive yellow). Genotypes include 'yy' (homozygous recessive), 'GG' (homozygous dominant), and 'Gy' (heterozygous). Crossed phenotypes (denoted by 'x') are paired with their possible genotype as well as the percent (%) offspring per family expected of each genotype and phenotype class. For example, crossing two P1 parental yellow color morph individuals ('yy'), both with the assumed 'yy' homozygous recessive genotype ('yy × yy') is hypothesized to result in 100% of the offspring having a homozygous recessive genotype observed as 100% yellow phenotype, 0% green phenotype or a 1:0 yellow to green color morph ratio

Table 2. Wild population color ratios as observed during field surveys

Field surveys		Site, subsite number	Surveyed larvae, #	Observed phenotype % yellow	Observed phenotype % Green	Observed % "yellow/green"
Borrego Springs, CA, 03/2017		1	28	75	25	-
		2	28	50	50	-
		3	9	33	67	-
		4	83	73	27	-
		5	25	44	56	-
		Total	173	64	36	-
Tucson, AZ, 08/2014		1	110	21	79	-
Oro Valley, AZ, 07/2014		1	143	97	3	-
Green Valley, AZ, 09/2013		1	3	33	67	-
		2	16	19	81	-
		3	19	16	84	-
		4	44	29	71	-
		Total	82	24	76	-
Sierra Vista, AZ, 08/2013		1	80	12.5	87.5	-
Portal, AZ, 08/2013		1	51	45	8	47
		2	241	4	57	39
		3	105	15	48	37
		4	204	47	33	20
		5	9	100	0	0
		Total	610	25	42.5	32.5

Conducted at six sites, with up to five subsites per site in San Bernardino County, CA; Pima and Cochise Counties, AZ. Early surveys were conducted with three color categories: 'yellow', 'green', and 'yellow/green' and later changed to either 'yellow' or 'green'. At each site/subsite, the total number of *H. lineata* larvae surveyed and the percent of larvae of each color morph observed was recorded.

Table 3. Inheritance and genetic crosses-single-generation outcrosses

Cross number	Outcross, P1 phenotype	F1, n=	F1 phenotype, % yellow	F1 phenotype, % green	P-value, 2-side exact binomial test
<i>y</i> × <i>y</i>		Expected ratio: 100:0			
1	<i>y, y</i>	346	100	0	1.0
2	<i>y, y</i>	200	100	0	1.0
3	<i>y, y</i>	69	100	0	1.0
<i>G</i> × <i>y</i>		Expected ratio: 50:50			
4	<i>G, y</i>	37	43	57	0.511
5	<i>G, y</i>	22	45	55	0.831
6	<i>G, y</i>	68	44	56	0.396
7	<i>G, y</i>	31	55	45	0.720
8	<i>G, y</i>	15	53	47	1.0
9	<i>G, y</i>	112	59	41	0.72
10	<i>G, y</i> *	20	55	45	0.82
<i>G</i> × <i>y</i>		Expected ratio: 0:100			
11	<i>G, y</i> *	28	0	100	1.0
12	<i>G, y</i> *	77	0	100	1.0
<i>G</i> × <i>G</i>		Expected ratio: 25:75			
13	<i>G, G</i> *	48	31	69	0.319
14	<i>G, G</i> *	50	32	68	0.25
15	<i>G, G</i> *	165	35	65	0.007
16	<i>G, G</i> *	32	56	44	<0.0001
<i>G</i> × <i>G</i>		Expected ratio: 0:100			
17	<i>G, G</i>	108	14	86	0.007
18	<i>G, G</i>	99	15	85	0.005
19	<i>G, G</i>	599	0	100	1.0
20	<i>G, G</i>	25	0	100	1.0
21	<i>G, G</i>	293	2	98	0.9795
22	<i>G, G</i>	91	2	98	0.9795
23	<i>G, G</i>	137	5	95	0.949
24	<i>G, G</i>	19	5	95	0.949

Individuals of yellow ('y') and green ('G') color morphs were crossed in either yellow × yellow, yellow × Green or Green × Green combinations. Number of offspring (F1) resulting from each parental cross (P1), as well as the percentage of each color morph observed in the fifth instar per family. Families are numbered and grouped by the best fit two-allele, single-gene expected model which they were compared to using a two-sided exact binomial test. A *P*-value of > 0.05 indicates that the observed ratio is not significantly different from a ratio one would expect from a single, two-allele gene. Families that were one generation of a multigenerational cross are noted by an '*'; complete multigenerational family lineage can be seen in Table 4.

Table 4. Inheritance and genetic crosses-multiple-generation outcrosses

Cross number	Cross type	Outcross P1 phenotype	F1, n	F1, expected y:G phenotype ratio	F1 phenotype % yellow	F1 phenotype % green	P-val, 2-side exact binomial test	F1, cross phenotype type	Cross number	F2, n	F2, expected phenotype y:G ratio	F2 phenotype % yellow	F2 phenotype % green	P-val, 2-side exact binomial test
11	Backcross	G, y	28	0:100%	0	100	1	G, y backcross	10	20	50:50%	55	45	0.824
12	Intercross	G, y	77	0:100%	0	100	1	G, G intercross	13	48	2.5:7.5%	31	69	0.319
14	Intercross	G, G	50	2.5:7.5%	32	68	0.25	G, G intercross	15	165	2.5:7.5%	35	65	0.007
								G, G intercross	16	32	2.5:7.5%	56	44	<0.0001

Crosses in which a backcross or intercross were conducted with the F1 offspring of a P1 parental cross. For each family, number of offspring as well as the observed yellow:green color ratio, the expected color ratio and the P-value yielded by a two-sided exact binomial test for both F1 and F2 provided. Two pairs of F1 family 14 offspring were successfully mated resulting in two F2 generations within family 14.

APPENDIX B.

Phenotypic plasticity of cuticular melanin in larval, *Hyles lineata* (Lepidoptera: Sphingidae) induced by a temperature-photoperiod interaction

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ABSTRACT

Plasticity of fitness-related traits is advantageous in changing and novel environments. Many organisms with trait plasticity can interpret current environmental cues to forecast future environmental conditions to match a phenotype that best suits a particular environment. Because of this, adaptive phenotypic plasticity may be a critical mechanism for organisms in the face of climate change. However, as changes in our climate occur, the environmental cues and the current evolutionary adaptive responses they prompt are becoming less reliable. Furthermore, the phenological decoupling of interactions has been observed. As environmental cues and drivers of selection decouple, it is critical to determine the environmental cues, the selective pressures, the relationship between them and the potential adaptive traits to predict possible responses to climate change. However, to understand the value and limitations of trait plasticity, we must first identify the environmental information being used and understand the cue-trait interaction. Here, we seek to understand the plastic trait of cuticular melanization in a lepidopteran larva, *Hyles lineata* (Sphingidae), and identify which environmental cue influences this trait. The change in the environmental cues of temperature, photoperiod and the two together were replicated while larval cuticular melanization was measured. We found that cuticular melanin in *H. lineata* is primarily controlled by photoperiod with integration of the secondary information of temperature. This result suggests that *H. lineata* integrates information from multiple cues simultaneously. This ability to integrate information may be imperative for the organism as the environment continues to change and cues such as photoperiod and temperature decouple.

INTRODUCTION

The response of a trait to environmental cues, or phenotypic plasticity, occurs when one genotype can produce multiple phenotypes (Mayr 1970; Shapiro 1976; Scheiner 1993). Cues such as temperature and photoperiod (Nijhout 1999) can elicit responses from one to many traits at a time, including body size, fecundity, development rate, survival, diapause induction and color pattern (Caswell 1983; Hazel 2002). A single trait can respond to multiple simultaneous environmental cues (Davidowitz et al. 2005). Furthermore, it allows for greater ecological breadth (Futuyma and Moreno 1988; Sultan 2001) and is hypothesized to reduce the risk of extinction in the face of climate change (Valladares et al. 2007; Forsman et al. 2008; Bell and Gonzalez 2009; Chevin et al. 2010; Forsman et al. 2012; Valladares 2017).

Currently, climate change is altering abiotic conditions, including temperature and precipitation in disproportionate ways across environments (IPCC 2014, Walker et al. 2019). Additionally, environments characterized by high seasonal variation are predicted to be dramatically affected (Williams et al. 2017) as seasonal cues of temperature and precipitation become disjoined from photoperiod. Consequences may include a mismatch between species and their environmental interactions, potentially resulting in increased rates of extinction (Williams et al. 2017). Extensive research has been conducted to predict how species will respond to the decoupling of seasonal variables, such as temperature and precipitation (see Hughes 2000 for review), yet few studies have focused on temperature and photoperiod (Walker et al. 2019). The continuation of the consistency of photoperiod, coupled with the shift in temperature, has the potential to have detrimental effects to the individual while profoundly impacting species' fitness and survival (Walker et al. 2019). The species that currently utilize this information are forecasted to experience greater decoupling of phenological interactions (Hughes 2000), especially among taxa such as plants and their pollinators and/or herbivores.

Ectothermic animals rely on external heat sources (i.e., radiant, convection and conduction) for warmth; coupled with modified behavior, this can result in a more regulated body temperature (Kettlewell 1973; Casey 1976; Peterson 1987; Nielsen et al. 2018). It is hypothesized that darker individuals have an advantage over lighter individuals in environments of low temperature due to the increased relative absorption of solar radiation and resultant warming (Watt 1968; Kettlewell 1973; Peterson 1987; Bennett et al. 1992; Forsman 2000; Clusella Trullas et al. 2007; Sibilía et al. 2018). Melanization, in some ectothermic animals, is thought to be primarily driven by the need to thermoregulate (Lindgren et al. 2014). When cuticular melanization deposition is plastic, a decrease in temperature can induce greater pigmentation in an attempt to alter thermoregulation potential (Kettlewell 1973; Porter 1982; Scoble 1992). This response has been considered an adaptation to fluctuating thermal conditions (Solensky and Larkin 2003) and has been observed in the larvae of various lepidopteran species including *Danaus plexippus* (Solensky and Larkin 2003), *Papilio polyxenes* (Hazel 2002) and *Battus philenor* (Nice and Fordyce 2006). Warmer body temperatures can lead to increased feeding rates (Porter 1983; Fielding and DeFoliart 2005), decreased development time (Goulson 1994; Hazel 2002; Clusella Trullas et al. 2007;

Soarers et al. 2015), reduced mortality (Barker and Enz 1993) and ultimately higher fitness (Bennett et al. 1992) in ectothermic animals.

While temperature has a direct effect on melanization, it can vary considerably in the environment. Photoperiod is considered to be a more predictable indicator of seasonal timing (Han and Gatehouse 1991). Photoperiod changes in small, gradual increments (in contrast to highly variable fluctuations in temperature), which make it a reliable cue of future environmental conditions (Bradshaw and Holzapfel 2007). Thus, similar to the plastic response to cold temperatures, shortening photoperiod may result in increased cuticular melanin. While this has been observed in larvae of several lepidopteran species (Hazel 2002; Musolin and Numata 2003), photoperiod and its relationship to thermal melanin is still poorly understood (Sibilia et al. 2018). Photoperiod cues are predictable enough to forecast upcoming conditions and initiate the hormonal cascade of fitness-related trait responses such as diapause, migration and developmental switches associated with seasonal polyphenism (Bradshaw and Holzapfel 2007; Nijhout and Davidowitz 2009).

Temperature and photoperiod may each elicit plastic trait responses. However, they are often coupled in temperate environments (Shintani et al. 2015; Stoehr and Wojan 2016). As photoperiod is consistent and precise it is frequently used by plants and animals as a primary cue to predict future conditions, while temperature is utilized as a secondary cue, providing current environmental information to modify response timing (Bradshaw and Holzapfel 2007). The coupling of these two cues creates a more accurate indication of seasonal transitions and associated response times (Williams et al. 2017; Walker et al. 2019). Along with the precise timing of major life history events that are often initiated by photoperiod, the interaction of photoperiod and temperature has been found to vary in the degrees of darkness in the stink bug (*Nezara viridula*, Musolin and Numata 2003), as well as varied degrees of cuticular melanin in crickets (*Allonemobius socius*, Fedorka et al. 2013) and in lepidopteran larvae (*Papilio polyxenes*, Hazel 2002).

Adaptive phenotypic plasticity of certain traits may be an important mechanism in adapting to climate change (Williams et al. 2017). However, the adaptive potential that phenotypic plasticity provides has limits and, ultimately, may not be enough for some species as climate change progresses (Visser 2008). To understand the value of plasticity and these limits, it is critical to identify the environmental variables that act as cues, which traits they affect and in what ways they affect them (Williams et al. 2017).

Here, we focus on the environmental cues of temperature and photoperiod and how the information provided by both is integrated into a plastic response of cuticular melanin. Specifically, we observed the plastic response of the black dorsal patterning on the larvae of a common lepidopteran species, the white-lined sphinx moth, *Hyles lineata* (Lepidoptera, Sphingidae). This hawkmoth expresses a broad range of larval colors, as frequently noted in descriptions of the species (Hodges 1971; Tuttle 2007; Powell and Opler 2009). However, little is currently understood about this species' morphological mechanics of variation in color patterning. We examine the primary larval body colors of yellow and green in Francois and Davidowitz (2020), and focus on the black dorsal stripe patterning and its environmental cues here. We seek to determine if the

observed black pigmentation on the last (fifth) and largest instar of *H. lineata* is a plastic response to the environmental conditions of temperature, photoperiod, and/or the interaction of the two. We predict the degree of melanization will increase: 1) at lower temperatures, 2) in a shorter photoperiod and 3) the cuticular response will vary from that of these cues independently when temperature and photoperiod are paired. We manipulated photoperiod and temperature in the laboratory and measured melanin by visual estimation to test these predictions. Understanding how *H. lineata* integrates environmental information into an adaptive response such as cuticular melanization may help us predict responses of other organisms with changing climate and identify those that may be in danger with no potential response at all.

METHODS AND MATERIALS

STUDY ORGANISM, HYLES LINEATA

Hyles lineata is most widespread and abundant sphinx moth in North America (Powell and Opler 2009), with its highest densities occurring in the southwest desert (USA). Within its extensive native range, spanning from southern Canada to South America (Powell and Opler 2009), individual larvae and adults have been observed in habitats ranging from low-elevation desert scrub, oak-woodlands and grasslands, to high-elevation meadows and pine forests. Throughout these habitat types, *H. lineata* can be active most of the year, potentially experiencing high variations in environmental conditions. Multiple broods of larvae per year are frequently observed en-masse in the wild feeding on a wide variety of host plants. Such an expansive spacial and temporal range suggests that *H. lineata* has the capability to exploit a broad array of resources.

Adult moths exhibit one color pattern: brown with white stripes on the forewings and pink hindwings. In contrast, the larvae are polymorphic for both basal coloration and black cuticular patterning. These variations in coloration have been observed within a population as well as within a family (Francois and Davidowitz 2020). Individual larvae also vary in color across instars (unpublished data). During the first instar, the individual larva is transparent white. In the second instar, they are black with white dorsal stripes. During the third and fourth instars, variations of green, yellow and black can be observed. Most often, in the fifth and final instar, larvae background coloration is markedly either yellow or green. While the amount of cuticular black patterning is usually reduced from that of the fourth instar, black dorsal stripes may occur in the fifth instar. These stripes can vary greatly in width and intensity when present (Figure 1), so much so that occasionally the larva may appear to be solidly black.

COLONY AND EXPERIMENTAL ANIMAL CARE

Individuals in these experiments came from a laboratory colony of *H. lineata* that was initially generated from wild populations in southeastern Arizona. Laboratory populations were continuously bred for five to ten generations, with at least 200 adults per generation and with wild-caught adults continuously added to the colony to avoid inbreeding. Adults were maintained in large plexiglass mating chambers with continuous access to a 20% sucrose sugar solution and

live *Oenothera sp.* (Onagraceae) plants for oviposition. Eggs were used as breeding stock to maintain populations large enough to generate individuals for experiments and to maintain genetic diversity. Colony larvae were reared in metal trays with vented plastic lids. Trays were lined with paper towels to absorb excess moisture and contained a raised hardware cloth stage to provide adequate surface area for molting and to separate larvae and food from frass. Larvae were fed *ad libitum* a fresh wheat-germ-based artificial diet (Davidowitz et al., 2003). Overcrowding was avoided and trays were cleaned daily. Colony larvae, pupae and adults were kept at 27°C, 16L:8D photoperiod.

Experimental larvae were individually separated into 1-ounce plastic cups (Solo) with vented lids containing fresh diet. Upon entering the second instar, they were randomly selected for control or treatment. At the fifth instar, individuals were transferred to clear 9-ounce plastic cups (Solo) with vented lids to accommodate growth. Diet was changed daily and cups were cleaned or replaced every other day. Once a fifth instar larva concluded feeding and began to clear its gut in preparation for pupation (noted by very loose frass and highly active wandering), the degree of pigmentation was recorded (see below). Environmental conditions were simulated by use of Percival incubators (136VLC8) with control conditions mimicking those of the colony (27°C, 16-hour light photoperiod) and experimental conditions, as noted below.

MELANIN QUANTIFICATION

Black pigmentation was verified as melanin by cellular and chemical analysis (unpublished) and quantified here by visual scoring. We created a discontinuous visual-estimation scale for scoring cuticular melanization in fifth instar *Hyles lineata*, which includes ‘minimal’, ‘medium’ and ‘maximal’ categorical levels of degree of melanic pigmentation (Figure 1). Larvae were individually scored using this scale for each treatment below.

TEMPERATURE

Larval melanin response was tested in various temperatures, including extremes of 35°C (‘hot’) and 20°C (‘cold’). All incubators (Percival, 136VLC8), including the 27°C control, were programmed to a 16-hour light photoperiod and 5°C cooler night temperature (not ramped). From the early second instar, larvae were kept in individual cups and maintained in one treatment temperature: cold, control or hot, throughout development (N= 91, 216, 189, respectively). Artificial diet dried out very quickly under hot conditions, so food in this treatment was changed frequently (up to twice a day) and individual cups were replaced daily to discourage mold growth.

PHOTOPERIOD

To observe the effects of photoperiod on larval coloration, the control incubator was set to standard rearing conditions, mimicking colony conditions- 16 hour light long-day length (‘long’; 16L:8D; N=36), while short-day length photoperiod treatment was set to 8 hours light, 16 hours dark (‘short’; 8L:16D; N=114); both at 27°C.

TEMPERATURE BY PHOTOPERIOD INTERACTION

The interaction between temperature and photoperiod was tested by coupling previously tested temperature conditions with a short photoperiod. As standard rearing conditions included a long photoperiod and all of the temperature experiments were conducted under a long photoperiod, long photoperiod by temperature combinations were not repeated. Thus, all incubator conditions were set to short photoperiod (8L:16D); cold conditions set to 20°C for 8 hours of light, 16 hours of dark ('cold/short'; n= 192), 27°C/8L:16D control conditions ('control/short'; n= 213), and 35°C/8L:16D in hot conditions ('hot/short'; n= 283).

STATISTICAL ANALYSIS

To determine if the tested variables are independent, data were analyzed with a goodness-of-fit test. Our test variables, temperature, photoperiod and their interaction, as well as our response variable (cuticular melanin visual scores), were all categorical units and sample sizes were all relatively large; thus, a Pearson's chi-square (χ^2) test was performed in JMP (Version 14. SAS Institute Inc., Cary NC 1989-2019). The melanin score values from each test temperature were compared to those of the control temperature to determine if there was variation in cuticular melanin with different temperatures. Photoperiod and temperature by photoperiod were also analyzed this way.

RESULTS

TEMPERATURE

As it is so common in arthropods, we predicted that a decrease in temperature would result in increased cuticular melanization. However, this hypothesis was not supported here. The degree of cuticular melanization of experimental larvae did not significantly differ from the control during the temperature experiment (χ^2 (df = 4, N = 496) = 4.787, p = 0.3098; Figure 2.A.).

PHOTOPERIOD

As predicted, exposure to the short photoperiod treatment had a significant effect on the amount of cuticular melanization (χ^2 (df = 2, N = 150) = 43.725, p <.0001; see Figure 2.B.). While only minimally melanized animals were observed under control/long-day conditions, a full range of melanization (approximately 20-30% of each minimum, medium and maximum) was observed under short photoperiod treatment (Figure 2.B.).

TEMPERATURE BY PHOTOPERIOD INTERACTION

A melanic response was observed when photoperiod and temperature were combined. As observed during the photoperiod experiment, when larvae were exposed to a short photoperiod, cu-

cuticular melanization increased. When exposed to a short photoperiod *and* various temperatures, melanization varied from that of photoperiod alone (x^2 (df = 4, N = 688) = 362.820, $p < .0001$; Figure 2.C.). As melanization was uniformly minimal with temperature alone, the observed melanization with temperature and photoperiod was not additive. Instead, the degree of cuticular melanization observed with these cues together suggests an interaction between the cues.

DISCUSSION

Many ectothermic animals, including larvae of many Lepidopteran species, exhibit increased dark patterning in response to decreased temperature to aid in thermoregulation (Kettlewell 1973). However, we show that cuticular melanin plasticity in *Hyles lineata* is *not* affected by temperature variation. In contrast, variation in photoperiod did have an effect with an increase in melanization with decreased day-length. This response aligns with our predictions. Similar results are seen in other Lepidopteran species (Nylin et al. 1989). More importantly, we observed a maximal response in cuticular melanization to the interaction of temperature and photoperiod.

The use of multiple environmental cues provides more reliable information about future conditions than single cues, making the integration of more information ideal for accurate forecasting by facilitating an optimally timed, better-adapted response to the expected conditions (Bradshaw et al. 2000; Stoehr and Wojan 2016; Williams et al. 2017; Walker et al. 2019). This is particularly interesting in the case of seasonality, specifically regarding photoperiod and temperature, as only one of these cues (temperature) applies selective pressure (Williams et al. 2017; Walker et al. 2019). Despite no direct effect on fitness, photoperiod provides predictable indications of future conditions of the selective pressure, temperature (Walker et al. 2019). Photoperiod's consistent and gradual cue is considered to be the most accurate indicator of time (Bradshaw and Holzapfel 2007) and one that many organisms rely on to indicate change in season (Bradshaw et al. 2000). Thus, it is no surprise that the more-reliable environmental cue of photoperiod acts as a primary cue, while a secondary cue, such as temperature, provides information to fine-tune the timing of such critical responses (Bradshaw and Holzapfel 2010), as observed here. Photoperiod acted as a primary cue when it was the only information provided as well as when paired with temperature. However, temperature acted as a secondary cue only when paired with photoperiod. On its own, temperature does not induce a plastic response in cuticular melanization in *H. lineata*.

The adaptive responses to temperature, photoperiod and the interaction between the two have long been of interest to ecologists (Shapiro 1976); however, our understanding of it is still incomplete (see Williams et al. 2017). Photoperiod in the context of climate change has not yet been examined (Walker et al. 2019). This may be due to the predictable and consistent nature of photoperiod, despite changing abiotic variables such as temperature (Walker et al. 2019). However, as a component of seasonality, the photoperiod cue and the selective pressure of temperature have begun to decouple as a consequence of climate change (Hughes 2000; Walker et al. 2019). This has begun to result in a misalignment between a species and their ecological interactions as well as between a species and the other species they interact with (Donnelly et al. 2011; Walker et al. 2019).

Identifying the phenotypic traits that have the potential to limit population persistence (Chevin et al. 2010), as well as understanding the adaptive response of these traits to environmental cues is critical and more urgent than ever in the face of climate change (Walker et al. 2017; Williams et al. 2019). Here, we tested the plasticity of the cuticular melanic response to multiple environmental cues in *H. lineata*. It was determined that *H. lineata* uses photoperiod as a primary source of information to cue the increase of cuticular melanin and can incorporate information provided by temperature into its response as needed. While photoperiod is likely not the driver of selection for this trait, it is used as a cue. Thus, future work should focus on determining the selection pressure and its relationship with photoperiod, perhaps starting with temperature. Once established, this will help determine potential species' response to the decoupling factors of seasonality, specifically with regards to photoperiod and temperature.

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Figure 1. Visual estimate melanin pigmentation scoring scale in laboratory reared fifth instar *H. lineata*
Discontinuous scoring scale consisting of 'minimal', 'medium' and 'maximal' categorical levels of dark pigmentation.

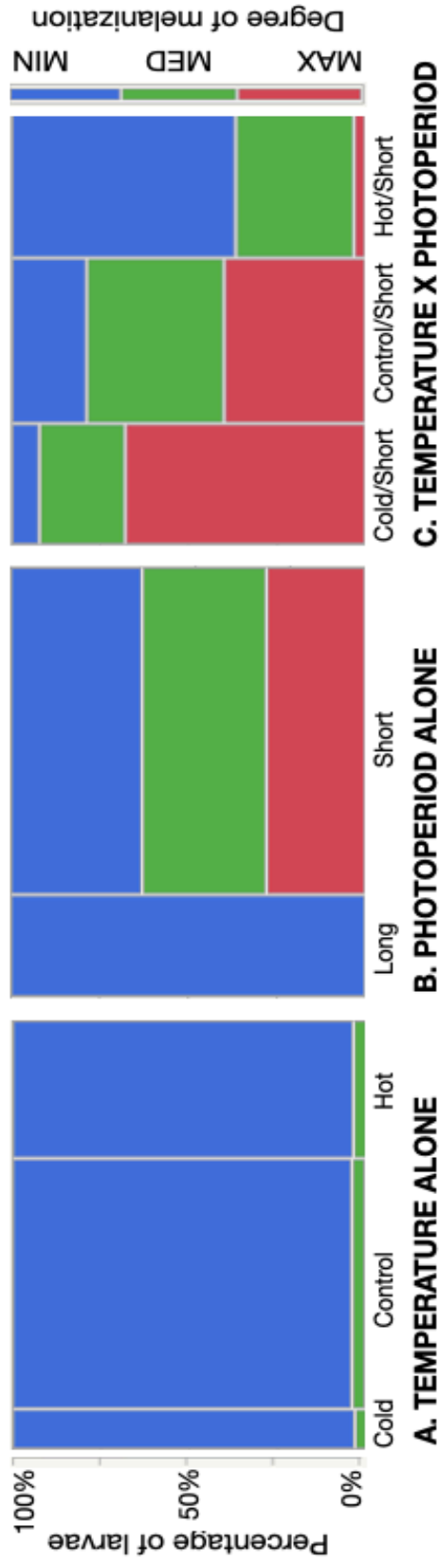


FIGURE 2. Mosaic plots depicting variation in melanin among larva from each treatment- percentage of larvae per treatment per color category. Pearson's chi-squared test applied to determine statistical significance in each treatment.

A. Temperature treatment alone- no statistical difference in melanization observed among *H. lineata* larvae reared under 'hot', control and 'cold' conditions. B. Photoperiod treatment alone- no variation in melanization among larvae in long photoperiod treatment; however, statistically significant variation in melanization among those in short photoperiod treatment. C. Temperature by photoperiod- short photoperiod coupled with various temperature treatments resulting in statistically significant differences in larval melanization indicating an interaction between the two environmental cues.

APPENDIX C.

Cuticular melanin determination and quantification methodologies as practiced on the Lepidopteran larva, *H. lineata*

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Target journals-

Insect Physiology, submit as a methods paper;

Arthropod Structure & Development

ABSTRACT

Melanin is an important contributor to color patterns throughout the animal, plant and fungi kingdoms. For this reason, research involving melanin is widespread in many disciplines, including Entomology. While some publications simply assume the coloration in question is melanin or refer to it as ‘dark patterning’, others include published methods used to identify and quantify melanin. These methods vary greatly in time/effort intensity, cost, statistical power and data output. In part, this may be due to how complicated melanin is to work with. This pigment can be derived from multiple sources, is present in various chemical structures, resides in a very broad range of tissue types and is hydrophobic and insoluble by nature. Thus, consideration of melanin identification and/or quantification methods can be overwhelming. Here, we review several of the most commonly used methods applied to arthropods. Our goal is to identify the ‘dark patterning’ observed in larval *H. lineata*. Additionally, we would like to quantify how the pigment volume varies among *H. lineata* phenotypes. Thus, we review multiple methods and discuss our use of each. These methods include visual analysis- visual scoring and image analysis, cellular analysis- viewing the cells with the help of microscopy and chemical analysis- spectrophotometer and HPLC and the wide array of chemical extraction techniques used in sample preparation. The ‘dark patterning’ observed in *H. lineata* was identified as the pigment melanin with the help of cellular and chemical analysis. The amount of melanin was quantified by visual and chemical analysis. The larvae that appeared to have greater ‘dark patterning’ or were visually scored as ‘maximal’ darkness had more melanin volume per exuvia (final molt) than those visually scored as ‘minimal’ darkness.

INTRODUCTION

Form, function and variation of animal color patterns have long been of interest in ecology and evolution (Darwin 1859; Ford 1945; Cott 1957; Lee and Wilson 2006; Wittkopp and Beldade 2009). Work has focused on coloration because patterns can be influenced by a wide range of ecological pressures as well as having the potential to have a considerable influence on fitness (via predator defense, sexual selection, thermoregulation), ultimately, affording great ecological and physiological advantages (Fox and Vevers 1960; Via 1993; Wittkopp and Beldade 2009; Forsman 2015). Coloration is highly variable, and can be expressed in a plethora of colors and patterns and can vary dramatically among species, population and individuals, as well as across life stages and body parts of an individual (Wittkopp and Beldade 2009). Such variability has been observed and studied extensively in insects, particularly that of lepidopteran larvae (Shapiro 1976; Lee and Wilson 2006; Nice and Fordyce 2006), which are known to have an extensive diversity of color patterns (Scoble 1992).

The pigment melanin is a main contributor to the high diversity of coloration and patterning (Poulton 1885; True 2003; Lee and Wilson 2006). This is punctuated by melanin's widespread presence across the animal kingdom as well as throughout plant and fungi/bacteria taxa (Pralea et al. 2019). In addition to taxonomic diversity, melanin can also be found in many different tissue types (Pralea et al. 2019). Furthermore, melanin can be derived from multiple sources (various phenolic or indolic monomers melanins including tyrosine and L-Dopa) resulting in a wide variety of melanin types and colors (see d'Ischia et al. 2013; Pralea et al. 2019 for reviews). Melanin has been found to have many biological functions, from protection against ultra violet radiation to a role in immune responses (Nappi and Vass 1993; see San-Jose and Roulin 2018 for a review).

Despite the extensive research conducted on animal color patterns and the contribution that melanin makes to these color patterns, little is actually known about the molecular structure and mechanics of melanin (Wittkopp et al. 2002; True 2003; Adachi et al. 2005; Futahashi and Fujiwara 2005; Shirataki et al. 2010; Shamim et al. 2014). Historically, this general lack of understanding has led to the frequent use of terms such as 'darkness', 'dark patterning' or just the assumption of melanin without supporting data (Kayser-Wegmann 1976 ; d'Ischia et al. 2013). This is likely due to the term 'melanin' being so broadly applied and yet so complicated to chemically define (d'Ischia et al. 2013). The chemical structure of melanin is heterogenous in nature due to the many sources that it can be derived from and the extensive spectrum of tissue types it can be found in (see Pralea et al. 2019 and/or d'Ischia et al. 2013 for a review of melanin types). Generally, melanin is described as a polymeric compound that is frequently tightly bonded with other cellular components, is hydrophobic and insoluble in organic solvents making it typically resistant to chemical degradation (d'Ischia et al. 2013; Pralea et al. 2019; Shamim et al. 2014). These variables make melanin difficult to isolate and adds to the complexity of identification and quantification (Wigglesworth 1972; Kayser-Wegmann 1976; Shamim et al. 2014).

This complex chemical nature of melanin, coupled with its far reaching effects in evolution, ecology and animal physiology, has created a great need to verify and quantify melanin (Davis et al. 2004). However, methods in purification, extraction and sample preparation vary dramatically throughout current literature, resulting in data that is frequently inconsistent and incomparable (Pralea et al. 2019). While there is a demand for standardization of methodologies (d’Ischia et al. 2013), there are many melanin quantification methods making it a vast and overwhelming task. Main factors that researchers consider when choosing a methodology include equipment accessibility, cost, complexity and skill set requirements. Here, we briefly review the primary methods frequently employed to identify and quantify melanin variation in arthropods. These methods include visual, cellular and chemical analysis, applied here to identify and quantify dark pigmentation in the lepidopteran larvae of the whitelined sphinx moth, *Hyles lineata* (Fab).

As mentioned previously, lepidopteran larvae are known to be highly diverse in their color patterning (Scoble 1992) and *H. lineata* (Lepidoptera, Sphingidae) is no exception to this. While adult moths uniformly exhibit the same color pattern: brown with white stripes on the forewings and pink hindwings, the larvae are polymorphic for both color and dark patterning. In their final instar, larvae are yellow or green with or without black dorsal stripes (Figure 1). When stripes are present, they can vary greatly in width and intensity, so much so that occasionally the larva may appear to be solidly black. Some of the mechanisms of color generation have been explored in previous work by our group. The black dorsal stripes and patterning have been found to be plastic, varying with environmental cues such as photoperiod (Francois and Davidowitz, unpublished data) and crowding (unpublished data). The yellow and green coloration has been found to be genetically controlled, primarily by a single allele (Francois and Davidowitz 2020).

Here, we focus on the black dorsal stripes and patterning, which, prior to the work presented here, we presumed were generated by melanin. The main aims of this study were to: 1) identify whether the dark pigment and coloration patterns in *H. lineata* were generated by melanin, 2) quantify variation in melanin and patterning in our laboratory colony of *H. lineata* larvae, and 3) to compare and contrast a variety of techniques that are currently used to identify and quantify melanin content. We view this last aim as particularly important, given the spectrum of methods used by researchers, and the wide array of results from different studies in the field that make comparisons and generalizations about melanic coloration challenging. Because a large portion of the work presented here is aimed at comparing and contrasting these methods, we use portions of the methods section below to further describe the history and nuance of each approach, as well as outlining the methodology we used for comparisons. Ultimately, the goal with this work is to review melanin determination methods most frequently utilized in arthropod research to simplify methodology selection and review the effort required for the use of each method and compare the data generated from each.

METHODS

STUDY ORGANISM

The whitelined sphinx moth, *H. lineata*, is the most abundant and widespread sphinx moth in North America with a range that extends as far north as Canada and as far south as Mexico, as well as documentation in Central and South America (Powell and Opler 2009). Habitat type and elevation range vary dramatically over this latitudinal gradient and include desert scrub, oak-woodland, grasslands and high elevation meadows and pine forests. Females may have multiple broods per year and adults and larvae are active during much of the year in parts of its range (specifically the southwest, personal observation). Adults are generalist nectivores and larvae feed on a wide variety of host plants, including plants from the families Portulacaceae, Nyctaginaceae, Euphorbiaceae, Onagraceae, Rosaceae, Primulaceae (Evans 2007; Powell and Opler 2009; personal observation). Larval color varies both across individuals (described above) but also across development. During the first instar, the larva is a transparent white appearing green when eating plant matter, whereas it is black with white dorsal stripes in the second instar. During the third, fourth and fifth instars, variations of green, yellow and black patterning can be observed.

COLONY AND ANIMAL CARE

Individuals used in melanin evaluation methods came from a laboratory colony of *H. lineata* that was initially generated from wild populations in southeastern Arizona. Laboratory populations were sustained for five to ten generations, of at least 200 adults per generation, with wild-caught adults continuously added to the colony to avoid inbreeding. Adults were maintained in large plexiglass mating chambers with continuous access to a 20% sucrose sugar solution and live *Oenothera sp.* (Onagraceae) plants for oviposition. Eggs were used as breeding stock to maintain populations large enough to maintain population genetic diversity. Colony larvae were reared in metal trays with vented plastic lids. Trays were lined with paper towels to absorb excess moisture and contained a raised hardware cloth stage to provide adequate surface area for molting and to separate larvae and food from frass. Larvae were fed *ad libitum* a fresh wheat-germ based artificial diet (Davidowitz et al. 2003). Overcrowding was avoided and trays were cleaned daily. Colony larvae, pupae and adults were kept at 27°C, 16L:8D photoperiod.

At the end of the fifth instar, as a larva concluded feeding and began to clear its gut in preparation for pupation (noted by very loose frass and highly active wandering), the degree of pigmentation was recorded and photographs taken (methods described below). If being used in the chemical extraction technique, larvae were allowed to pupate and fifth instar exuviae was removed shortly after molting. Larvae used for histological purposes were not allowed to pupate but set aside for sample preparation mid-fifth instar instead.

VISUAL ANALYSIS

The simplest, and oldest, color variation estimation method is visual scoring (Garman 1920). This method can vary greatly in complexity. For example, scoring scales can be as straightforward as a presence/absence score (e.g. Valimaki et al. scoring *Chiasmia clathrata* melanistic morphs (2015)) or a simple numerical ranking (e.g. Goulson used a 1-4 ranking mechanism to

score melanization of *Mamestra brassicae* (1994)). Alternatively, visual scoring can be as complex as assigning values to characteristics, color and textures. For example, Kazimirova (1992) used seven categories considering lateral bands, dorsal side coloration and ranking from lack of melanin and ‘velvety’ melanin in describing coloration patterns in *M. brassicae*. Similarly, Sandre et al. (2013) included dorsal/lateral coloration and presence/absence of spots while working with the highly variable geometrid *Ematurga atomaria* and Gunn (1998) considered head capsule color, longitudinal lines and percentage of several different colors in *Spodoptera exempta*.

To visually score the black pigmentation in fifth instar *H. lineata* larva, we created a discontinuous scoring scale which included ‘minimal’, ‘medium’ and ‘maximal’ dark color patterning categorical delineations (Figure 1). To minimize the subjective nature of this methodology, there were pictorial definitions of each category (e.g. there were photographed reference larvae that served as the ‘platonic ideal’ for each category), subjects were photographed, and the same team member consistently scored larvae.

Conceptually similar to the visual scoring approach but integrating current technology, digital photography and image analysis software, image analysis can be used to process, quantify and analyze images using algorithmic models (Stevens et al. 2007). Frequently used in historic studies of animal coloration, photography is accessible to a wide range of researchers and adds preservable data for future examination and replication (Stevens et al. 2007). Programs such as Photoshop (Reindeer Graphics, Inc.), GIMP (Sun Microsystems, Inc.) or Image J software (U.S. National Institutes of Health, Bethesda, Maryland) add standardization, precision and consistency to this method (Stevens et al. 2007).

Here, we use a typical procedure outlined in (Stevens et al. 2007), where a sample image is first converted to greyscale after which dark patterning can be measured as a percentage of the photographed two-dimensional total larval body area. By dividing the area of black by the area of the total body, the resultant percentage is a quantifiable and standardized value of dark pigmentation. This technique is dependent on careful lighting standardization when photographing the subject, as is the determination of a black standard and consistent subject positioning to avoid shadowing. To quantify dark patterning in *H. lineata*, larvae were placed in a linear position parallel to a photo card in a white photo box with a fixed light. A digital camera Canon PowerShot SX40 H was fixed on a tripod inside light box, raised approximately 30cm above staging area. Once the image was digitized, the entire two-dimensional larval body was outlined, converted to grey-scale and percent darkness calculated with the help of Image J software. Photographs of 224 individual larvae were processed. Variation in percent darkness was compared among groups of visually-scored larvae with a oneway analysis of variance (ANOVA) and a Tukey-Kramer HSD (JPM, Version 14 SAS Institute Inc., Cary, NC, 1989-2020).

CELLULAR ANALYSIS

Microscopy is a common and useful method for discerning morphological characterization and understanding the cellular structure of melanin (Pralea et al. 2019). This technique entails mak-

ing histological cross sections of the sample epidermis and cuticle to view under high powered microscopes such as a transmission electron microscope or compound light microscope. Early observations of dark coloration in insects note that dark pigments seem to be spatially separated from other colors by occurring in the cuticle and most other pigments present in the epidermis (Wigglesworth 1972; Lhonoré et al. 1980; Maisch and Bückmann 1987; Bear et al. 2010; Nijhout 2010; Shamim et al. 2014). Based on the more complete molecular understanding of the melanin synthesis pathway, it is now well understood that cuticular pigments are indeed melanin (Kayser-Wegmann 1976; Hobkins and Kramer 1992; Futahashi and Fujiwara 2005; Futahashi et al. 2010; Shamim et al. 2014; Fukutomi et al. 2017). Unlike other pigments, melanin occurs in the cuticle due to the presence of the required oxidation proteins and enzymes (Futahashi et al. 2010). While the melanin precursors are produced in the epidermis, they are individually transferred from the epidermal cells to the cuticular layer during the molting process (Futahashi et al. 2010). There, dopamine is enzymatically converted to melanin and deposited into the exocuticle (Hobkins and Kramer 1992; Ashida and Brey 1995; Futahashi and Fujiwara 2005; Futahashi et al. 2010; Shamim et al. 2014; Nie et al. 2014; Fukutomi et al. 2017).

Depending on the polymer it is derived from (DOPA or Dopamine), melanin can appear as several colors. DOPA melanin appears grey or black. However, Dopamine-derived melanin can appear not only as black or grey, but also as tan, brown, yellow, red or reddish-brown when synthesized from NBAD (Futahashi et al. 2010; Yu et al. 2011; Shamim et al. 2014). These pigments have been observed in lepidopteran larvae including *Papilio xuthus* (Futahashi and Fujiwara 2005) and *Bombyx mori* (Yu et al. 2011). All of these pigments, derived from DOPA or Dopamine, finish synthesis when it is met by the conversion enzyme (DCE) in the cuticle (Futahashi and Fujiwara 2005; Futahashi et al. 2010; Shamim et al. 2014). However, black or brown Dopamine-melanin is the primary melanin product in insects (Futahashi and Fujiwara 2005; Futahashi et al. 2010). Of course, pigments found in insects include anthraquinones, pterins and carotenoids and can be present in a wide variety of colors, including black (Shamim et al. 2014). Epidermal ommochrome pigments, similarly to melanin, can be black and produced by the insect (independent of plant matter). However, this pigment would be present in the epidermis (Shamim et al. 2014).

Ultimately, this means that melanin may be several colors, but occur in the cuticle, and several non-melanin pigments are black and brown, but will not occur in the cuticle, but in the epidermis, haemolymph and fat bodies (Shamim et al. 2014). Thus, for simple confirmation of melanin, histological cuts can be informative based simply on pigment location. Here we looked at histological pigment location to confirm the presence of melanin in fifth instar *H. lineata* larvae. After being photographed, fifth instar larvae were fixed by injection of hot Bouin's solution (Sigma-Aldrich). Five mm sagittal sections of dorsal integument were dehydrated in graded ETOHs, embedded in paraffin and sectioned at 5-6 microns. De-paraffinized sections were stained with H&E (hematoxylin/eosin). Stained slides were viewed in an Olympus compound microscope (BX50 258 L98-029) with QCapture Pro cellSens image-capture computer software (Media Cybernetics, Inc. Rockville, Maryland) and photographed using a DP72 digital camera.

CHEMICAL ANALYSIS

In the methods covered thus far, dark patterning (assumed to be melanin) is measured as a percentage of body coloration (visual analysis) or the presence of an unknown volume of melanin is confirmed by pigment location in the cuticle (cellular analysis). Yet, there are methods that can provide more quantitative data, including spectrometry and chromatography.

Spectrometry detects amounts of melanin in a sample by measuring absorbance at a specific wavelength that is unique to that compound. To do so, the density of the sample is measured by light transmittance. By including a known amount of melanin in a dilution series with a sample, researchers can observe the absorbency of the known volumes and use the known values of the standard curve to calculate the volume of the unknown samples. Similarly, high-performance liquid chromatography (HPLC), measures *adsorbance* /desorbance of the sample components through a porous filter. Each component of the sample travels through the filtration substrate at different rates. How each substrate is absorbed by the filter over time, compared to known amounts of melanin in a calibration curve, can identify and quantify components of the sample. See Ito and Wakamatsu (2003) for specific HPLC method details as well as variations. Both of these methods are used frequently to measure melanin in various tissue types, including insect cuticle (Barak et al. 2017), fruits and seeds (Kannan and Ganjewala 2009), mollusk scallop shell (Sun et al. 2017) and human/mammal tissues (Watts et al. 1981). However, spectrophotometry and HPLC both require chemical extraction of pigment from the test tissue to characterization, identify and quantify potential melanin.

As melanin is hydrophobic, insoluble and frequently tightly bonded with other cellular components (d'Ischia et al. 2013; Shamim et al. 2014; Pralea et al. 2019) isolation and extraction from tissues is challenging (Wigglesworth 1972; Kayser-Wegmann 1976; Wakamatsu and Ito 2002; True 2003; Adachi et al. 2005; d'Ischia et al. 2013; Shamim et al. 2014; Pralea et al. 2019). Further, melanin is resistant to chemical degradation (Pralea et al. 2019), yet many extraction methods have been rejected due to decarboxylation and the break down of the pigment granule or skeleton (d'Ischia et al. 2013). Methods can vary dramatically and must take tissue type and melanin characterization type into consideration (Pralea et al. 2019). There are many melanin isolation, purification and extraction methods, frequently including an acid hydrolysis to purify (Sun; wang; Pralea et al. 2019) and an alkaline extraction such as alkaline hydrogen peroxide oxidation (d'Ischia et al. 2013; Barak et al. 2017), hydrochloric acid (Sun et al. 2017) or dimethyl sulfoxide (DMSO) (Zhou et al. 2012; Debecker et al. 2015; Chung et al. 2019). Methods can vary dramatically and must take tissue type and melanin characterization type into consideration (Pralea et al. 2019). For in-depth detail about various chemical extraction methodologies, see d'Ischia et al. 2013, Pralea et al. 2019 and/or Wakamatsu and Ito 2002.

Here, we used a melanin extraction protocol based on work by Zhou et al. (2012) as modified by Debecker et al. (2015). Individual fifth instar exoskeleton molts, exuviae, were collected after larvae molted into pupal stage. The exuviae from 15 visually scored 'minimal' larvae and 15 visually scored 'maximum' larvae were analyzed. Exuviae were independently weighed to the

nearest 0.01mg on a microbalance (Mettler Toledo XS3CU, Columbus, OH) then finely ground with a plastic pestle in the eppendorf tube. A subsample of these grounds was then combined with 1 M NaOH/ 10% DMSO and the volume of solution was adjusted per subsample weight (extraction volume in $\mu\text{L} = 200 \times$ subsample mass, in mg). Samples were then incubated in an 80°C water bath (VWR 5L Avantor) for two hours and centrifuged at 12000g (VWR Galaxy 16D Avantor) for 10 minutes. Supernate was siphoned and harvested while 1 M NaOH/ 10% DMSO was added to the precipitate to repeat the extraction process again, for five extractions total. This was done as a precautionary measure as this larval exuviae seemed very thick and samples remained dark after first extraction. Aliquots (45 μL) from each extraction for every sample was plated (96 well microtiter plate) in triplicate along with a synthetic melanin (Sigma-Aldrich) serial dilution from 1000 μg melanin/0.5 mL DMSO to 15.63 μg /0.5mL DMSO, also plated in triplicate. Absorbance at 470 nm, 25°C, was analyzed using a spectrophotometer (ThermoFisher Scientific Multiskan GO 1.00.40 spectrophotometer, Thermo Scientific, Waltham, MA). Concentration of melanin per sample (melanin μg per exuviae weight mg) was calculated using a standard curve from the melanin dilution series. Variation in melanin extracted from exuviae was compared among larvae with a oneway analysis of variance (ANOVA)(JPM, Version 14 SAS Institute Inc., Cary, NC, 1989-2020). [KW to add a sentence on how the values from each round of extraction were compared to the total extraction values per exuvia, see Figures 3 and 4]

RESULTS AND DISCUSSION

VISUAL ANALYSIS

Visual scoring scales can be as simple or as complex as needed; here we created a relatively simplistic scale with three, categorical delineations of minimum, medium and maximum levels of dark pigmentation. For evaluating dark pattern intensity in *H. lineata*, this seemed to work well. However, points of concern have been raised with visual scoring. While this method is inexpensive and unburdened by equipment, it is subjective and potentially inconsistent or lacking in detail. Visual scoring scales can be more complex and detailed to avoid missing morph nuances, however, the data yielded is categorical, reducing its statistical power (Davis et al. 2004; Byers 2006; Lee and Wilson 2006). Further, it did not identify the pigment responsible for dark patterning in *H. lineata*, and only categorically quantify it.

By using a more objective method of visual scoring, image analysis, we were able to assign numerical values to the degree of melanization by quantify visual dark patterning into percent black over total body. Thus, individuals with greater dark patterning scored a higher percentage of black- larvae visually scored as minimum averaged approximately 14% black, medium 43% black and maximum 75% black. Means were significantly different from each other (one-way ANOVA Tukey-Kramer, $n=224$, $p<.0001$). While image analysis did not identify the pigment responsible for the dark patterning observed in *H. lineata*, it did quantify it as a percentage dark patterning, meeting one of our two aims. This method is numerically quantifiable and more standardized than visual scoring it can be expensive and time consuming. Camera and computer software were, obviously, required making the method a bit more expensive and equipment

heavy. This method also took more time in that the lighting and positioning should be standardized as well as double checking each data point in the software program for miscalculations due to light reflection, glare or shadows.

CELLULAR ANALYSIS

Microscopy and histological sections of *H. lineata* integument show dark pigmentation restricted to the epi- and exo- cuticle (See Figure 2). The presence of the pigmentation being in the cuticle suggests that this pigment is melanin. Further, the epidermal cells under the non-melanized cuticle are much larger than the those under the melanized cuticle, possibly due to their containing urate granules and/or granules of other pigments (Hu et al. 2013). With this methods, we were able to can confirm that the dark pigmentation observed is melanin, however, this data does not quantify said melanin, only meeting one of our two melanin aims. While this method is most likely used as a tool to deduce cellular pigment location, researchers have also attempted to derive quantifiable data from it. Miot et al. (2012) have used digital image analysis on human skin sections to quantify dark area as a computational morphometric evaluation of melanin. Similar to dark patterning quantification of the entire larval body, they quantified percent of black granules in the cuticle. Unfortunately, this method can be expensive to execute and timely. Making the histological slides requires specialized equipment that may or may not be possible to do in-house. Viewing the slides also requires moderately expensive microscope equipment and software.

CHEMICAL ANALYSIS

To confirm the presence of and quantify melanin in the cuticle of *H. lineata*, we started by extracting and isolating melanin using DMSO. Once extracted and isolated, we measured sample absorbance and calculated volume allowing us to quantify amount of melanin per exuviae. Exuviae obtained from larvae visually scored as ‘minimum’ averaged 59.1 μg of melanin per 1mg of exuvia, ranging from samples with 34.4 μg melanin/1mg exuvia to 74.8 μg melanin/1mg exuvia. Larvae scored ‘maximum’ had an average of 67.6 μg melanin/1 mg exuvia with a range from 54.5 μg /1mg exuvia to 93.6 μg melanin/1 mg exuvia. Calculated values of melanin from the ‘minimal’ samples were compared to those from the ‘maximal’ and were found to be statistically significantly different from each other (ANOVA, $n=31$, $p=.0247$).

While the spectrophotometer method can provide data to determine melanin volume per sample will great resolution, the data collected is only as good as the sample is prepared. The physiology of a tissue sample, as well as the biochemistry a potential melanin type and classification need to be factors in understanding the biochemistry of how to isolate melanin and thus, choose a chemical extraction method. Here we chose to grind the exuviae samples and extract with the aid of DMSO. However, to do so successfully, we needed to modify this method to include multiple rounds of extraction. After five rounds of extraction, the values per exuvia were added together to result in total melanin per exuvia. Once graphed it is clear that the value from one extraction of *H. lineata* exuviae in DMSO significantly under represents total potential melanin (Figures 3

and 4). However, the total after three rounds of extraction is much closer to the total value after five rounds of extraction.

Not doing so would have altered our results considerably. These biochemical considerations are critical when further preparing your samples for HPLC and choosing your solvent for the mobile and the filtration material of the stationary phase. Things to consider with spectrophotometry include potential background absorbance (Wakamatsu and Ito 2002) or the different light wavelength absorbance of different melanin types (see Pralea et al. 2019 for a review).

Overall, spectrophotometry did work considerably well. It not only identified our pigment as melanin, but also quantified the variation we were observing in the samples, meeting both of our first two aims. Unfortunately, however, this method is labor and time intensive and requires a moderate amount of bench skill. As mentioned, it also requires an understanding of the study system and thoughtful consideration of the approach.

CONCLUSION

Working with melanin can be complicated and overwhelming. Here we explored some of the more common methods used to identify and quantify cuticular melanin in entomological literature. These methods each met at least one of our aims to identify or quantify melanin in *H. lineata*, and one met both. These methods vary dramatically in required skill level, equipment needs and time to execute. To identify melanin in *H. lineata* and to categorically quantify melanin, visual scoring (and histology in conjunction with image analysis as in Miot et al. 2012) were sufficient. Visual scoring can be sufficient if designed to the detail needed and is still the preferred method for some (Sandri et al. 2013). However, more information and statistical power is gained by the identification and precise quantification of melanin provided gained with chemical analysis. Perhaps the compromise is visual scoring with categorical measurement units based on the analysis of a subsample with more complex method, like chemical analysis. It is important to consider melanin type and tissue type of your sample when evaluating methods (Wakamatsu and Ito 2002) as well as the potential for cross-study comparisons. Not all methods provide equivalent, comparable data (Pralea et al. 2019) and the relative costs and benefits of each method needs to be weighed in advance of any study.

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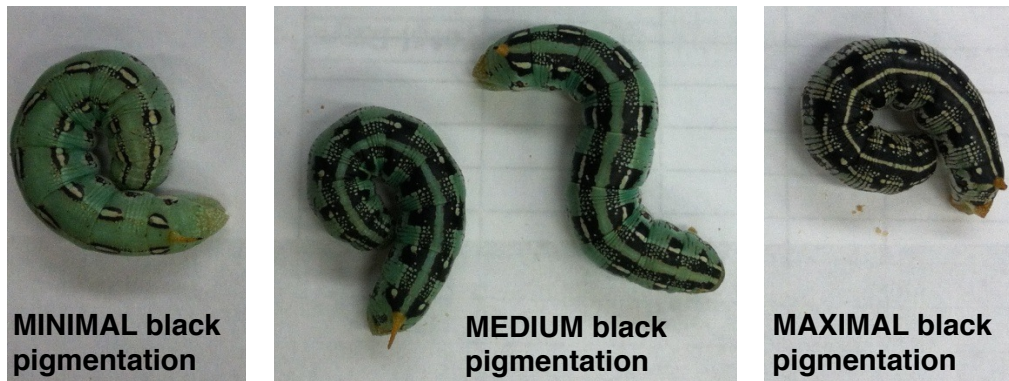


Figure 1. Color pattern divided into three groups, minimum, medium and maximum degree of black pigmentation. Larvae were scored using this scale while in their fifth instar, upon wandering, pre-pupation.

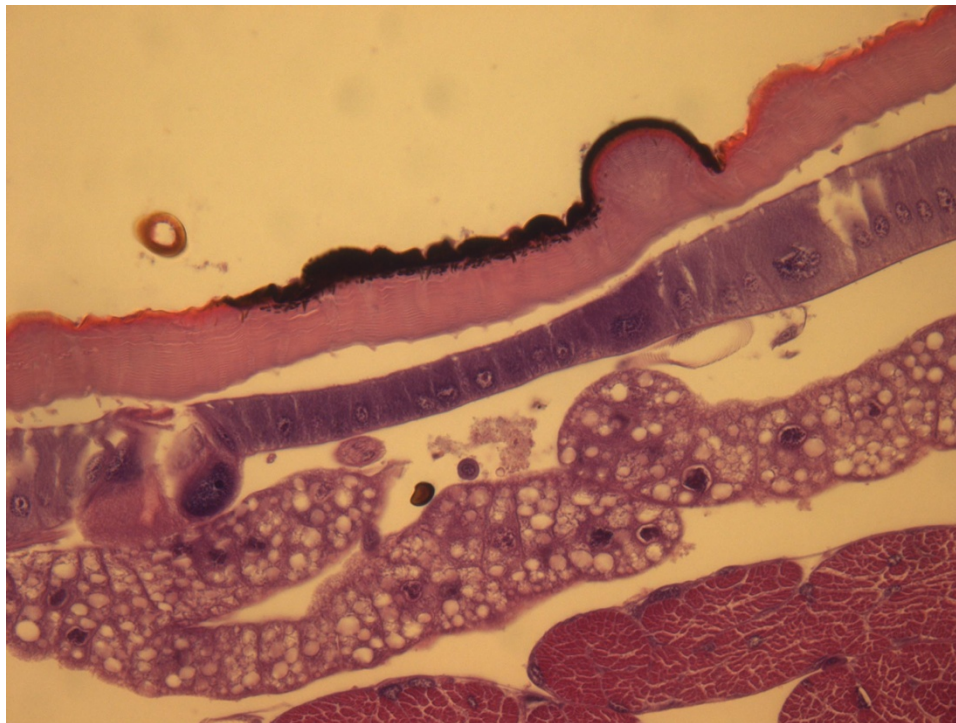


Figure 2. Histological cross section of *H. lineata* integument. The dark pigmentation is limited to the exocuticle providing confirmation that the pigment is, indeed, melanin. Furthermore, the epidermal cells under the non-melanized cuticle are larger than those under the melanin cuticle, possible due to the presence of other pigments and/or uric acid.

Figures 3 and 4 double panel (see next page)

Increasing the number of melanin chemical extractions gets you closer to the total melanin value, but with diminishing returns. Percent of total melanin extraction value by the accumulated volume after each of five rounds.

