Genetic Color Polymorphism of the Whitelined Sphinx Moth larva (Lepidoptera: Sphingidae)

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Received 1 May 2020; Editorial decision 3 July 2020

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 geographical 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 with 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.

Key words: polymorphism, color variation, Mendelian gene

Animal color patterns have long been of interest in ecology and evolution due to their frequent direct and indirect links to fitness (Ford 1945, Cott 1957, Darwin 1859, Forsman 2014, Karpestam 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 McKinnon and Pierotti 2010, Wellenreuther et al. 2014 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 geographical 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 (Fig. 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

![Fig. 1. Hyles lineata in their fifth instar. Top: Wild fifth-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 fifth instar H. lineata of the green and the yellow color morph.](https://academic.oup.com/jinsectscience/article-lookup/10.1093/jinsectscience/zoz033/1958589)
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.

**Materials and Methods**

**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 10 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 marked yellow or green (see Fig. 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 ‘citroneila’ 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 larva was located, transects of ~20 m 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. Approximately 100 individuals were surveyed per site, with a total of 1,201 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. Laboratory populations were generally viable for 5–10 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, photoperiod of 16:8 (L:D) h. 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 × 30 × 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 prepupation 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 Fig. 1). Colors of laboratory-reared larvae were classified using the same Sherwin-Williams ColorSnap 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 were reared in metal trays with vented plastic lids. 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.

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 laboratory. 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 × yellow and yellow × green, were conducted with the color of all resultant first generation (F1) offspring noted (Fig. 2). 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 (P1) (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 was 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

| Outcross, P1 genotype | Expected F1 phenotype, % | Expected F1 phenotype, % |
|-----------------------|--------------------------|--------------------------|
| y × y                 | 100                      | 0                        |
| G × G                 | 0                        | 100                      |
| G × y                 | 50                       | 50                       |
| G × y                 | 25                       | 25                       |
| y × G                 | 100                      | 0                        |
| y × y                 | 50                       | 50                       |

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 F1 parental yellow color morph individuals (‘yxy’), both with the assumed ‘yy’ homozygous recessive genotype (‘yxy’ × ‘yxy’) is hypothesized to result in 100% of the offspring having a homozygous recessive genotype observed at 100% yellow phenotype, 0% green phenotype or a 1:0 yellow to green color morph ratio.
determine whether 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 subsites in which only 4% of larvae were yellow, as well as subsites 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 × 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 × yellow crosses also produced offspring color ratios not significantly different from the expected Mendelian model. Two green × 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 × green (Gy × Gy) cross. Also fitting the 1:3 ratio was the parental cross of unrelated green × green individuals (cross 14). However, green × green crosses 15 and 16, both intercrossed (F1) siblings of cross 14, differed from the expected green × 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 = 0.00001). While it is not significantly different from a 1:1 ratio (P = 0.597), this is not an expected outcome of a green × green cross in our single gene, green-dominant model.

Two other green × 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 ×

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

| Site, subite number | Surveyed larvae, n | Observed phenotype % | Observed phenotype % | Observed % “yellow/green” |
|---------------------|--------------------|----------------------|----------------------|--------------------------|
|                     |                    | yellow               | Green                |                          |
| Borrego Springs, CA, 03/2017 | 1 | 28 | 3 | 75 | 25 | – |
|                     | 2 | 28 | 3 | 50 | 50 | – |
|                     | 3 | 9 | 3 | 33 | 67 | – |
|                     | 4 | 83 | 3 | 73 | 27 | – |
|                     | 5 | 25 | 3 | 44 | 56 | – |
| Total               | 173 | 64 | 36 | – | – | – |
| Tucson, AZ, 08/2014 | 1 | 110 | 3 | 21 | 79 | – |
|                     | 2 | 143 | 3 | 97 | 3 | – |
| Oro Valley, AZ, 07/2014 | 1 | 3 | 3 | 33 | 67 | – |
|                     | 2 | 16 | 3 | 19 | 81 | – |
|                     | 3 | 19 | 3 | 16 | 84 | – |
|                     | 4 | 44 | 3 | 29 | 71 | – |
| Total               | 82 | 24 | 76 | – | – | – |
| Green Valley, AZ, 09/2013 | 1 | 80 | 3 | 12.5 | 87.5 | – |
|                     | 2 | 51 | 3 | 45 | 8 | 47 |
|                     | 3 | 241 | 3 | 4 | 57 | 39 |
|                     | 4 | 105 | 3 | 15 | 48 | 37 |
|                     | 5 | 204 | 3 | 47 | 33 | 20 |
| 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.
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 geographical 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 (Karpestam 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).

**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 |
|--------------|------------------------|--------|------------------------|-----------------------|-----------------------------------|
| 1            | y, y                   | 346    | 100                    | 0                     | 1.0                               |
| 2            | y, y                   | 200    | 100                    | 0                     | 1.0                               |
| 3            | y, y                   | 69     | 100                    | 0                     | 1.0                               |

**G × y**

| Cross number | Outcross, P1 phenotype | F1, n= | F1 phenotype, % yellow | F1 phenotype, % green | P-value, 2-side exact binomial test |
|--------------|------------------------|--------|------------------------|-----------------------|-----------------------------------|
| 4            | G, y                   | 37     | 43                     | 57                    | 0.511                             |
| 5            | G, y                   | 22     | 45                     | 55                    | 0.831                             |
| 6            | G, y                   | 68     | 44                     | 56                    | 0.396                             |
| 7            | G, y                   | 31     | 55                     | 45                    | 0.720                             |
| 8            | G, y                   | 15     | 53                     | 47                    | 1.0                               |
| 9            | G, y                   | 112    | 59                     | 41                    | 0.72                              |
| 10           | G, y                   | 20     | 55                     | 45                    | 0.82                              |

**G × y**

| Cross number | Outcross, P1 phenotype | F1, n= | F1 phenotype, % yellow | F1 phenotype, % green | P-value, 2-side exact binomial test |
|--------------|------------------------|--------|------------------------|-----------------------|-----------------------------------|
| 11           | G, y                   | 28     | 0                      | 100                   | 1.0                               |
| 12           | G, y                   | 77     | 0                      | 100                   | 1.0                               |

**G × G**

| Cross number | Outcross, P1 phenotype | F1, n= | F1 phenotype, % yellow | F1 phenotype, % green | P-value, 2-side exact binomial test |
|--------------|------------------------|--------|------------------------|-----------------------|-----------------------------------|
| 13           | G, G                   | 48     | 31                     | 69                    | 0.319                             |
| 14           | G, G                   | 50     | 32                     | 68                    | 0.25                              |
| 15           | G, G                   | 165    | 35                     | 65                    | 0.007                             |
| 16           | G, G                   | 32     | 56                     | 44                    | <0.0001                           |

**G × G**

| Cross number | Outcross, P1 phenotype | F1, n= | F1 phenotype, % yellow | F1 phenotype, % green | P-value, 2-side exact binomial test |
|--------------|------------------------|--------|------------------------|-----------------------|-----------------------------------|
| 17           | G, G                   | 108    | 14                     | 86                    | 0.007                             |
| 18           | G, G                   | 99     | 15                     | 85                    | 0.005                             |
| 19           | G, G                   | 599    | 0                      | 100                   | 1.0                               |
| 20           | G, G                   | 25     | 0                      | 100                   | 1.0                               |
| 21           | G, G                   | 293    | 2                      | 98                    | 0.9795                            |
| 22           | G, G                   | 91     | 2                      | 98                    | 0.9795                            |
| 23           | G, G                   | 137    | 5                      | 95                    | 0.949                             |
| 24           | G, G                   | 19     | 5                      | 95                    | 0.949                             |
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 × yellow, green × yellow crosses and most of the green × 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 × green cross consist of 0:1 or 1:3 (yellow:green); Table 1. While these ratios were observed here, four of the green × green crosses 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 Mockler 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 among each color morph observed in the crosses conducted here. Similar variations in color have been attributed to modifier genes in other Lepidopteran color polymorphic species: the Mockler 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 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 Sheppard 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. 2020).

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 (Betzholz 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, traits 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 polyphenisms with trait associations and the selective pressures acting on each morph. Further studies can extend this work can 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.

Acknowledgments

We thank Judith Bronstein, Bruce Walsh, and Raymond Nagle for the comments on earlier versions of this manuscript. We also thank Ben Beal, Patty Stamper, In Lenny Park, Keaton Wilson, Chris Volansky-Wirth, John Kraft, Dawn Hendricks, Kelly Wallace, Autumn Moore, and Martin von Arx for help in the laboratory and field. This study was supported in part by National Science Foundation IOS-1053318 to Goggy Davidowitz.

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