Iridescent, Distasteful, and Blue:

Effectiveness of Short-Wavelength, Iridescent Coloration as a Warning Signal

in the Pipevine Swallowtail Butterfly (*Battus philenor*)

by

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ABSTRACT

Warning coloration deters predators from attacking prey that are defended, usually by being distasteful, toxic, or otherwise costly for predators to pursue and consume. Predators may have an innate response to warning colors or learn to associate them with a defense through trial and error. In general, predators should select for warning signals that are easy to learn and recognize. Previous research demonstrates long-wavelength colors (e.g. red and yellow) are effective because they are readily detected and learned. However, a number of defended animals display short-wavelength coloration (e.g. blue and violet), such as the pipevine swallowtail butterfly (*Battus philenor*). The role of blue coloration in warning signals had not previously been explicitly tested. My research showed in laboratory experiments that curve-billed thrashers (*Toxostoma curvirostre*) and Gambel's quail (*Callipepla gambelii*) can learn and recognize the iridescent blue of *B. philenor* as a warning signal and that it is innately avoided. I tested the attack rates of these colors in the field and blue was not as effective as orange. I concluded that blue colors may function as warning signals, but the effectiveness is likely dependent on the context and predator.

Blue colors are often iridescent in nature and the effect of iridescence on warning signal function was unknown. I reared *B. philenor* larvae under varied food deprivation treatments. Iridescent colors did not have more variation than pigment-based colors under these conditions; variation which could affect predator learning. Learning could also be affected by changes in appearance, as iridescent colors change in both hue and brightness as the angle of illuminating light and viewer change in relation to the color surface. Iridescent colors can also be much brighter than pigment-based colors and iridescent

animals can statically display different hues. I tested these potential effects on warning signal learning by domestic chickens (*Gallus gallus domesticus*) and found that variation due to the directionality of iridescence and a brighter warning signal did not influence learning. However, blue-violet was learned more readily than blue-green. These experiments revealed that the directionality of iridescent coloration does not likely negatively affect its potential effectiveness as a warning signal.

DEDICATION

This work is dedicated to my father, William Vann, whose wisdom and love of science got me to the beginning. And to my incredible husband, Ben, whose unwavering support and love got me to the end.

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PREFACE

Animals display incredible diversity in their coloration. This coloration can serve many functions, and is often naturally selected by its role in mediating interactions with other animals. Interactions with predators may lead to coloration that camouflages the prey or bright coloration that stands out against the background. Bright coloration for predator avoidance is more likely to evolve in animals that are defended in some way, making them unprofitable for predators to attack. Commonly, the prey animal is distasteful or even toxic to predators. The bright colors used to advertise these defenses are known as warning colors or aposematic colors (Poulton, 1890; Cott, 1940).

Warning colors may adaptively deter predation through several behavioral mechanisms. Warning colors may elicit innate, or unlearned, avoidance from a naïve predator (e.g. Smith, 1975; Roper, 1990). If the predator has no innate avoidance or that avoidance has been diminished due to repeated exposure, the predator may learn to associate the color with the defense by attacking the prey. Attacks not resulting in food are costly to both signaler and receiver; the prey animal may be injured or killed (Sillén-Tullberg, Wiklund, & Järvi, 1982; Wiklund & Järvi, 1982) and the predator will have wasted energy on unprofitable prey. After learning to associate bright colors with unpalatability, predators must recognize the signal and implement that association upon encountering similar aposematic prey in the future (Guildford, 1986; Speed, 2000). Therefore, a warning signal is considered more adaptive if it is innately avoided, learned with fewer taste rejections, and more quickly or accurately recognized by predators than other colors, all leading to fewer attacks on defended and warningly colored animals.

Blue and Directionally Reflecting Warning Coloration

With respect to the spectral properties of warning coloration, patterns that contain long wavelength colors, especially when combined with black contrasting patterns, are common. Presumably this is the reason that previous research on the features of warning coloration that make them effective have largely focused on orange, red, and yellow colors (e.g. Schuler & Roper, 1992; Lindström, 2001; Lindström, Alatalo, & Mappes, 1999; Aronsson & Gamberale-Stille, 2008; Théry & Gomez, 2010). However, warning colors with elements that reflect predominantly short wavelengths of light, like blue or violet, are found in many toxic or unpalatable animals, such as leaf beetles (*Oreina gloriosa*; Borer, van Noort, Rahier, & Naisbit, 2010) or blue poison dart frogs (*Dendrobates azureus*; Brodie, Jr. & Tumbarello, 1978). Moreover many of the toxic animals that display short-wavelength coloration, such as the pipevine swallowtail butterfly (*Battus philenor*; Sime, Feeny, & Haribal, 2000), pyjama nudibranch (*Chromodoris quadricolor*; Cimino & Ghiselin, 1999), or the hibiscus harlequin bug (*Tectocoris diophthalmus*; Fabricant & Smith, 2014), have blue or violet components adjacent to long-wavelength colors, suggesting that blue may be an important element of effective warning signals.

Before the experiments reported here, the potential for short-wavelength coloration to serve as a warning signal had rarely been investigated in both natural and artificial systems. Experiments have included blue prey in tests of other features of warning signals (e.g. conspicuousness or patterns; Gittleman, Harvey, & Greenwood, 1980; Gamberale-Stille & Guilford, 2003; Aronsson & Gamberale-Stille, 2008), and have found that blue coloration can be effectively associated with unpalatability. Two other

experiments explored the role of a different type of short-wavelength coloration, ultraviolet, and found that bird predators could not associate an ultraviolet coloration alone with unpalatability (Lyytinen, Alatalo, & Mappes, 2001; Lyytinen, Lindström, & Mappes, 2004). More recently, a handful of other investigations have appeared in the literature regarding short-wavelength anti-predator colors. Using visual modeling, Arenas, Troscianko, and Stevens (2014) found that red was more conspicuous than short-wavelength colors against green foliage to avian predators and was more reliably conspicuous in a variety of habitats. Fabricant, Exnerová, Ježová, and Stys (2014) demonstrated that great tits had an innate avoidance of blue and could learn to avoid blue in a warning signal context. Miller and Pawlik (2013) also found that bluehead wrasse (*Thalassoma bifasciatum)* have an innate avoidance of blue prey. These new studies added to our knowledge about short-wavelength coloration in warning signals, with split conclusions on the effectiveness of blue in warning signals, but still left many aspects of short-wavelength warning coloration uninvestigated, such as testing the role of blue warning signals in nature and how these signals are recognized.

Regardless of the hue of a warning, the mechanism by which it is produced might affect the quality of the reflection and thereby how it works in the interaction between visual predators and aposematic prey. For example, short-wavelength coloration is often produced by structural mechanisms and so can be directionally reflecting, like the iridescent blue of *B. philenor* or tarantula hawks (*Pepsis formosa*), resulting in bright, dynamic signals whose appearance changes with viewing position. While bright, conspicuous colors such as these might increase the rate at which predators learn to associate them with unpalatability (e.g. Gittleman & Harvey, 1980; Lindström et al.,

1999; Halpin, Skelhorn, & Rowe, 2008), their directionality and changes in appearance during trial and error learning could potentially slow the rate at which they are learned and recognized (e.g. Gamberale & Tullberg, 1996; Ihalainen, Lindström, Mappes, & Puolakkainen, 2008). Whether and how iridescence plays into the effectiveness of a warning signal has been recognized as an important question in the field of animal signal design, but has never been studied before (Doucet & Meadows, 2009; Théry & Gomez, 2010).

Study organism

My focal prey species was the pipevine swallowtail butterfly (*Battus philenor*). The ventral hindwings of *B. philenor* have a large field of iridescent blue within which there are seven orange spots. These wing surfaces serve as a warning signal that predators readily learn the unpalatability of this species (Brower, 1958; Codella & Lederhouse, 1990), while the dorsal surface primarily functions as a sexual signal (Rutowski & Rajyaguru, 2013). These butterflies are distasteful because the larvae sequester in their tissues aristolochic acids from their hostplants (genus *Aristolochia*; Sime, Feeny, & Haribal, 2000; Fordyce & Nice, 2008) that are also in the tissues of the adults. Not surprisingly, *B. philenor* adults have several Batesian mimics that have evolved similar orange and blue ventral hindwing coloration, including *Limenitis arthemis*, *Papilio polyxenes*, *P. troilus*, and the black form of *P. glaucus* females (Brower, 1958), each of which are avoided by predators that have had experience with *B. philenor* (Brower, 1958; Platt , Coppinger, & Brower, 1971). *B. philenor* ranges broadly from Central America northward into southern Canada and is abundant in the desert and suburban area around

Phoenix, Arizona. The larvae are relatively easy to find, collect, and rear to adulthood in the lab on field-collected or greenhouse-grown hostplant. Therefore, it is an excellent study organism for experiments on the natural context, effect of rearing conditions, and predators response to blue and orange warning coloration.

Hypotheses and Tests

The co-occurrence of blue and orange pattern elements in unpalatable species across the animal kingdom suggests an adaptive advantage to this multi-component warning signal. Additionally, many blue colors found in nature are iridescent, which could affect the effectiveness of a warning signal. These observations lead to two main hypotheses

(1) Blue coloration on many unpalatable animals functions alone as an effective warning signal in nature that elicits innate avoidance and can be easily learned and recognized by predators.

Prediction: Predators will avoid blue prey on first exposure, learn not to attack blue prey as quickly as known effective warning colors (e.g. orange), and blue prey will not be attacked by experienced predators.

(2) The property of iridescence affects warning signal effectiveness, as compared to diffusely reflecting signals.

a. Due to precise nature of the microstructures require to produce the color, structural coloration could result in more intraspecific

phenotypic variation, especially in response to environmental conditions, and variation may hinder warning signal effectiveness

Prediction: When exposed to varying environmental conditions (e.g. larval food deprivation), an iridescent color will demonstrate more phenotypic variation than a pigment-based color, and iridescent sexual signals will exhibit more variation in response to environmental conditions than iridescent warning signals

b. Because of its directionality and the resulting variation in appearance to predators, iridescent blue colors are less readily learned and recognized than diffusely reflecting colors.

Prediction: If the appearance of the unpalatable stimulus changes during learning, the rate of learning will slow.

c. Because iridescent colors are generally brighter than diffusely reflecting colors with some angles of viewing and illumination relative to the surface they will be learned more readily.

Prediction: Predators will learn a signal that is brighter than one that is less bright, faster, requiring fewer taste rejections.

d. **Iridescent animals have the potential to display different colors (hues) with the arrangement of signaler, receiver, and light source, and some hues may be more readily learned within the range of those displayed by an iridescent animal.**

Prediction: Predators will learn faster when an iridescent animal displays one color than another, within the range of the directionality of an iridescent signal.

The work described in the following chapters and appendices tests these hypotheses in several contexts. I first examined the natural context in which the colors of *B. philenor* might deter predators (Appendix A; Pegram, Han, & Rutowski, 2012). Then, because selection should favor less phenotypically variable warning signals, I investigated whether the mechanism of production (i.e. structural coloration vs. pigment-based coloration) affected the amount of phenotypic variation, specifically in response to larval food availability (Appendix B; Pegram, Nahm, & Rutowski, 2013).

I then did several experiments to test the effectiveness of the blue and orange wing elements in deterring predators. I used multiple predators in these experiments, with predator choice being driven by the needs of the experiment and the feasibility of working with each species. First, I measured how captive but experienced curve-billed thrashers (*Toxostoma curvirostre*), a passerine species that eats butterflies, trained not to attack *B. philenor,* recognized the ventral surface colors individually (Chapter 1; Pegram, Lillo, & Rutowski, 2013). Then, I tested the innate avoidance and learning of the blue and orange colors of *B. philenor,* which requires naïve birds and thus must be hand-raised. This is unfeasible for passerine species so I used naïve Gambel's quail, a local species whose chicks regularly consume insects (*Callipepla gambelii*; Appendix C; Pegram & Rutowski, 2014) in the lab. Finally, I measured predation rates on *B. philenor* with color alterations in the field (Chapter 2). My dissertation work concluded with an

experiment on the consequences of displaying an iridescent warning signal, measuring how variation in appearance (within the range of *B. philenor*'s angular dependence), signal brightness, and variation in hue affected learning by naïve domestic chickens (*Gallus gallus domesticus*), as they are commonly used for learning experiments and more readily available than *C. gambelii* (Chapter 3).

CHAPTER 1

IRIDESCENT BLUE AND ORANGE COMPONENTS CONTRIBUTE TO THE RECOGNITION OF A MULTICOMPONENT WARNING SIGNAL

Introduction

Warning coloration deters visual predators from attacking distasteful or toxic prey (Poulton, 1890). The decision not to attack unpalatable prey can be influenced by innate predispositions or by preferences learned through previous encounters (e.g. Gittleman & Harvey, 1980; Caldwell & Rubinoff, 1983; Turner, 1984; Schuler & Hesse, 1985; Roper & Cook, 1989). After learning in such encounters to associate bright colors with unpalatable or unprofitable prey, predators should easily recall and use that knowledge when they subsequently encounter similarly colored prey. The use of this knowledge to decide whether or not to attack an aposematic prey item is termed warning signal recognition (Guilford, 1986; Speed, 2000). Warning signals are considered effectively recognized when a predator decides not to attack a prey item based on a learned signal. This effectiveness is measured by the proportion of aposematic prey items encountered that are not attacked by experienced predators (i.e. predators that have previously learned that signal). Warning signals should be selected to facilitate predator learning and recognition, as well as elicit innate aversions (Speed, 2000) because of costs to predators and prey if unpalatable prey are not readily distinguishable (Wallace, 1889; Fisher, 1930).

Beginning with the first descriptions of warning coloration (e.g. Wallace, 1889; Poulton, 1890; Cott, 1940) and continuing in more recent reports and reviews (e.g.

Schuler & Roper, 1992; Lindström, Alatalo, & Mappes, 1999; Lindström, 2001; Aronsson & Gamberale-Stille, 2008), warning colors are either defined as long-wavelength colors (e.g. oranges, reds, and yellows) or are characterized as often reflecting long wavelengths. While there are many long-wavelength warning colors, there are also a number of unpalatable animals that display short-wavelength colorations in their warning coloration (Umbers 2013), such as the blue poison dart frog (*Dendrobates azureus*) or leaf beetles (*Oreina gloriosa*). Moreover, many of the toxic animals that display short-wavelength coloration, like the pipevine swallowtail butterfly (*Battus philenor*; Fig. 1a), strawberry poison dart frog (*D. pumilio*) or pyjama nudibranch (*Chromodoris quadricolor*) display their blue or violet coloration adjacent to long-wavelength colors. Interestingly, both color pattern elements are mimicked by palatable Batesian mimics of the Pipevine Swallowtail (e.g. *Papilio troilus, P. polyxenes, P. glaucus* and *Liminitis arthemis* (Brower, 1958; Platt, Coppinger, & Brower 1971) which leads me to suggest that the mimicked coloration is a multicomponent signal, i.e. a signal with two components in the same modality (Partan & Marler, 1999; Rowe, 1999).

There are two main hypotheses for the role of multiple components in a signal (Partan & Marler, 2005). First, blue and orange components may each be individually used by predators to recognize prey as distasteful. Thus, they would be classified as redundant signals, because they both convey the same information (e.g. Zuk, Ligon, & Thornhill, 1992; Rowe, 1999; Partan & Marler, 2005). Second, the two color components may be non-redundant, which means that only one functions as a warning color that predators learn and recognize. The other may function as an accessory signal, amplifying

or modulating the message or may be functionally irrelevant to the warning signal or defense against predators.

I tested these hypotheses using the ventral hindwing coloration of *B. philenor*, a known warning signal (Brower, 1958; Codella & Lederhouse, 1990); the main features of which are diffusely-reflecting orange spots within a field of iridescent blue (Fig. 1a). The mean hue of the ventral surface blue iridescence is around 500 nm and of the orange is about 590 nm in lab-reared *B. philenor* (Pegram, Nahm, & Rutowski 2013). Both adults and larvae of this species are unpalatable to predators due to aristolochic acids in their tissues obtained from the larval hostplant (Sime, Feeny, & Haribal, 2000; Fordyce, Marion, & Shapiro, 2005). When the ventral wing surface is presented, birds learn quickly not to attack *B. philenor*, but learn more slowly when only the dorsal surface is presented (Codella & Lederhouse, 1990). Because the dorsal surface in males only displays the iridescent blue and not the orange (Rutowski, Nahm, & Macedonia 2010), the results of Codella and Lederhouse (1990) suggest that the iridescent blue may be used as a warning signal.

In this experiment, I measured the contribution of both color elements to the warning signal of *B. philenor*. I manipulated the ventral surface signal to display only one color, both colors, or neither color and determined how this affected signal recognition by curve-billed thrashers (*Toxostoma curvirostre*) that I had trained not to attack prey with the intact coloration. If both colors function as warning signals (redundant signal), I expected birds not to attack the *B. philenor* wings when I presented wings that displayed only the blue iridescence or wings that displayed only the orange. If only one color functions as a warning signal (non-redundant signal), I expected to find significant

differences in attack rates among the wings displaying only one color. If wings displaying one color are attacked significantly more often than wings displaying the other, it would indicate that the color attacked more does not deter predators and the two colors have different signal functions.

Methods

Predators

Twenty-six adult (at least one year old), male curve-billed thrashers (*T. curvirostre*) were caught with mist nets in central Arizona, USA, between 29 March 2009 and 23 June 2009. I housed birds individually for approximately six months in 76 cm x 46 cm x 46 cm wire cages with wooden perches, water, and Mazuri Insectivore Diet (small, brown pellets; PMI Nutrition International, St. Louis, MO, USA) available at all times. After the experiment, the birds were killed for tissue analysis in an unrelated study. The capture of the birds, the housing and the experiment were approved by the Arizona State University Institutional Animal Care and Use Committee (Protocol #09-1022R). *T. curvirostre* are insectivores and butterflies can compose 13% of their diet in Arizona (Ambrose, Jr., 1963).

Prey

I presented *B. philenor* wings with freeze-killed mealworms that were either palatable (soaked in water) or rendered unpalatable by soaking them in a solution of 4% quinine hydrochloride and 2% mustard powder for 30 minutes (Forsman & Merilaita, 1999). I used *B. philenor* wings cut from lab-reared animals, and then glued together

using non-toxic glue (Elmer's Glue-All®, Westerville, OH, USA) with only the ventral surface exposed in a posture like that of perched individuals (Pegram, Han, & Rutowski, 2012). I created four different wing treatments using black, non-toxic permanent markers (Sharpie®, Oak Brook, IL, USA). For 'black' wings, the entire ventral wing surface was blackened. For 'only blue' wings, I blackened the orange spots. For 'only orange' wings, I blackened the iridescent patch. The color on the 'unaltered' wings was left intact. Wings were not treated on the dorsal surface because it was not visible at any time in the presentations. Because *B. philenor* wings contain aristolochic acids (Sime et al., 2000), they may have an unpleasant taste. However, birds usually only moved the wings to get to the mealworm, not consuming or mouthing the wings, and did not display any reaction (e.g. bill wiping, head shaking) in response to contact with or ingesting the wings as they did for the unpalatable mealworms.

I presented each prey item in a 55 mm diameter Petri dish with white printer paper on the bottom to provide a consistent background. In addition, I placed a wooden stick (length 4.5 cm, diameter 3 mm) on the bottom of the Petri dish under the paper about 1 cm from the dish's center. I placed the wings on the stick with the line from the wing base to the tail parallel to the stick and the distal part of the wing resting on the stick (Fig. 2). This consistent positioning of the wing reduced potential variation in apparent reflectance due to the iridescent nature of the blue coloration, which changes in appearance with the angle of the viewer and the light source (Rutowski et al., 2010). The stick allowed the wing to be at the same angle whether or not a mealworm was present underneath the wing.

Experiment conditions

Birds remained in the same room with each individual in its cage and visually isolated from birds in adjacent cages throughout training and testing. The room was lit by overhead fluorescent lighting. As expected, the irradiance of the room light had large peaks around 445 nm, 550 nm, and 620 nm. However, all experimental procedures, including training, occurred under the same lighting. Both prior to and during the experimental procedures, there was no food restriction (pellet food was available at all times). I did not exceed 20 presentations (training or recognition tests) per bird in a day and the maximum number of days a bird received training or tests was 23 (mean = 8.4). The birds readily ate mealworms throughout the trials.

Training

An experiment began with a series of training presentations. The subjects may have had experience with sympatric *B. philenor* prior to capture but the training presentations ensured that all birds had recent experience with and had learned not to attack the intact color pattern of *B. philenor*. First, I trained birds to associate 'black' wings with a palatable mealworm as a control for two reasons: (1) as a control for effects of the size and shape of the butterfly wings (Forsman & Merilaita, 1999) and (2) for comparing the attack responses for colored prey to prey that is not warningly colored.

I began the training procedure by presenting a mealworm on top of the black wings. Once the bird ate the mealworm, I did another presentation in which all but 5 mm of the mealworm was hidden by the black wings. If the bird ate the mealworm partially hidden by the wings, I did another presentation in which the mealworm was entirely

hidden by the black wings so that the bird had to move the wings to reveal the mealworm. Each presentation lasted five minutes. If the bird did not eat the mealworm within five minutes when the mealworm was partially or completely hidden by the wings, the next presentation moved back a step (e.g. if the bird did not eat a mealworm partially hidden by the wings, the mealworm would be on top of the wing in the next presentation). Once a bird consistently retrieved mealworms hidden underneath black wings, I considered it trained. The average number or presentations required for training was 8.9 (range = 4 - 27) and 26 out of 28 birds were successfully trained. I then moved on to training them to not attack an unaltered wing.

I trained the birds not to attack the ventral color pattern of *B. philenor* by pairing an intact *B. philenor* wing pattern with an unpalatable mealworm hidden underneath. I scored an attack if the bird touched the wing with its beak. Because observers were absent from the room during all training and testing presentations, any wings that had moved and mealworms that were gone were recorded by observers upon re-entry but attacks were confirmed by reviewing digital video recordings of the birds made of each presentation. I repeatedly presented each bird with the unaltered wings and unpalatable mealworms underneath until the bird did not attack two unaltered wings in succession. All birds either ate or picked up the unpalatable mealworm at least once. After two unaltered wings were not attacked, I ran a series of five alternating presentations of black and unaltered wings. If birds did not attack all of the unaltered wings and attacked all of the black wings, I considered the bird successfully trained. This stringent requirement meant that only 42% of the birds (11 out of the 26) showed clear evidence that they consistently recognized intact *B. philenor* as distasteful and required an average of 36.5

presentations (range = 5-106). This requirement also ensured that the birds had learned not to attack *B. philenor* based on the ventral surface warning coloration and that I were testing warning signal recognition. The eleven birds that met the requirement were then immediately tested to determine which of the available color cues were being used by them to recognize *B. philenor*.

Recognition Test

A recognition test consisted of four presentations with no mealworm offered, in randomized order, with one each of the four different wing color alterations (black, only blue, only orange, and unaltered). Wings were left in the cage for five minutes with one minute in between and wings used in a given recognition test were not used in other recognition tests. Again, video recordings were reviewed to determine whether or not the wing was attacked. The person reviewing the video did not know the wing color presented. Additionally, another author reviewed approximately 25% of the recognition test videos and confirmed that attacks or the lack thereof had been accurately scored by the initial reviewer.

Due to the relatively low number of birds that met the stringent requirements, I subjected some birds to multiple recognition tests. I was also interested in whether birds would make consistent decisions on multiple encounters with these prey items, as may occur in nature. Nine out of the 11 birds completed more than one test, for an average of 3.6 tests (range = 1-10) per bird. After the first test, a bird had to make correct decisions on an unaltered wing and a black wing before starting another test. This requirement

resulted in birds completing different numbers of tests as some did not continue to make the correct decisions and I instead focused the effort on other birds.

Statistical analysis

I used generalized linear mixed models with binomial distributions in R v. 2.15.2 (R Foundation for Statistical Computing). I first analyzed only the first recognition test completed by each bird ($N = 11$). In this model, the dependent variable was whether or not the wing was attacked during a presentation, wing color alteration was entered as a fixed factor, and bird identity entered as a random factor (since birds responded to four different wings).

In the analysis of the repeated tests, wing color alteration and recognition test number were included as fixed factors and bird identity was entered as a random factor with slopes (but not intercepts) allowed to differ between individuals (Schielzeth & Forstmeier, 2009), and whether or not the wing was attacked as the dependent variable. This inclusion of the random factor with random slopes allows the model to account for non-independence. I used the general linear hypothesis testing function for my three *a priori* comparisons and Bonferroni corrected the level of significance to 0.016 to account for multiple comparisons. Otherwise, inferences for both models were made at the 0.05 level of significance.

Results

Eleven birds met the training criteria and completed at least one recognition test, with four wing treatments, for a total of 44 responses (Fig. 3). If, after initial training,

blue iridescent coloration and orange coloration were recognized as warning signals, I expected that the number of attacks on wings displaying each of these colors individually to be significantly less than the number of attacks on wings displaying no coloration. With all black wings considered the reference (intercept) for fixed effects in the model, wings displaying only the blue iridescent coloration (estimate =-2.60, z=-2.136, p=0.033), wings displaying only the orange spots (estimate =-2.60, z=-2.136, p=0.033) and unaltered wings (estimate =-2.60, z=-2.136, p=0.033) were attacked significantly less than black wings. If blue iridescent coloration and orange coloration are redundant warning signals, I also expected to find no difference in the number of attacks for wings displaying only orange and wings displaying only blue coloration. This was the case as they each had the same number of attacks. Unaltered wings also had the same number of attacks as only orange wings and only iridescent wings. The variance explained by bird identity was 3.312 (standard deviation = 1.82).

When including repeated tests, I completed a total of 40 recognition tests to yield 160 responses (Fig. 4). The pattern of response to the colors followed that for the first tests. Only blue (estimate =-3.98, z=-3.665, p<0.001), only orange (estimate =-3.66, z=-2.927, p=0.003), and the unaltered wings (estimate =-5.04, z=-3.936, p<0.001) I all attacked significantly less than all black wings. The attack rate for only blue and only orange wings was not significantly different (p=0.588). The attack rate on unaltered wings was not significantly different from only orange wings (p=0.033[α=0.016]) or only iridescent wings (p=0.150). The variance explained by bird identity for each level of the fixed effects is reported in Table 1.

However, the birds were not very consistent in their responses across multiple tests. Recognition test number was a significant predictor of whether or not a wing was attacked (estimate =-0.55, z=-2.181, p=0.029). This effect of test number appears to be a result of birds changing their responses to stimuli as they went from one test to the next. Interestingly, the likelihood of a change in response appears to be greatest for the only blue wings and the only orange wings, that is, those stimuli displaying only one of the two main color components. As a measure of this I compared whether the response of a bird to a stimulus was the same or different from its response in the previous test. For stimuli with only one color component a change in response occurred in 27 out of 58 comparisons (46.5%). In contrast, there were 6 changes (20.7%) in response for the wings with intact coloration and 4 changes (13.8%) for the all black wings in 29 comparisons each. The rate of changes in response from one test to the next for those stimuli with one component displayed was significantly higher than for stimuli with both components (χ^2=5.48, df=1, p=0.019) or neither component (χ^2=8.15, df=1, p=0.004).

Discussion

Overall my results suggest that, under the conditions of this study, curve-billed thrashers that did not attack the intact coloration of *B. philenor* used both blue and orange coloration to recognize this butterfly as distasteful. First, attacks were significantly less frequent when the colors (i.e. orange, iridescent blue) were present compared to when the wings had been fully blackened. Second, I find no significant difference in frequency of attacks between the two colors. Finally, the rates of attack on the wings displaying only blue and only orange were not significantly different from the unaltered wings. From

these results I conclude that orange and blue are likely redundant components during recognition of this multicomponent warning signal, equally effective at deterring predator attacks.

Redundant signals may be advantageous for several reasons. First, redundant visual signals may be more effective in a greater diversity of light environments, against more diverse backgrounds, or with different predators. The orange and blue color components differ in hue, and therefore may differ in conspicuousness when the spectral composition of ambient light changes throughout the day (Endler, 1993). In addition, the two components vary in the percent of incident light reflected. The iridescent blue reflects a larger proportion of incident light than the diffusely reflecting orange (mean brightness = 14.5% reflectance for orange and 46.8% for iridescent blue), which could allow for a more conspicuous reflection under low light conditions, such as when they are perching (Pegram et al., 2012). Second, signals with multiple rather than single components may also increase detection, discriminability, and associative learning, which are all likely to reduce attack rates by predators on aposematic animals (Rowe, 1999). Blue and orange presented together may make a more effective signal, classified as an enhanced redundant signal (Partan & Marler, 2005). This experiment showed no significant difference between attacks on the unaltered wings and wings with the single components. However, I did find that in the multiple attacks, birds were more likely to change their response between tests to wings only displaying one component. In nature, this could translate to increased attacks on warningly colored animals with only one component, indicating an advantage to displaying a redundant signal.

The role of blue coloration in warning signals has received very little attention (Umbers, 2013). Some studies have demonstrated unpalatability in animals with blue coloration or blue patches (e.g. Brodie, Jr. & Tumbarello, 1978; Karuso & Scheuer, 2002; Saporito et al., 2007; Borer, Van Noort, Rahier, & Naisbit, 2010; Williams, 2010) but research has not parsed the role of blue versus other color pattern elements in the warning displays of these animals (Umbers, 2013). My experiment demonstrates that predators can use blue coloration to recognize that a prey is distasteful. However, because the experiences of the birds prior to capture was not controlled, I could not test what pattern elements the birds are learning. Therefore, I cannot be sure if the birds have learned during my training or in the field to associate blue coloration with unpalatability and are recalling this knowledge in the recognition test or if they are generalizing from associations with stimuli formed prior to capture that now deters them from attacking prey with blue coloration. Nonetheless, these colors were able to effectively deter predation. I do not know if orange and blue are equally effective as warning colors during different stages of warning signal use, such as learning or innate aversion, or what features of these colors make them more effective (Stevens & Ruxton, 2012), but this is under investigation. The conclusion that blue is an effective warning color may be strengthened by follow-on studies of the role of blue components in predator learning and innate avoidance.

Short-wavelength coloration is often structurally produced and directionally reflecting, as it is in *B. philenor*. The structures that produce directionally reflecting colors focus the reflected light like a mirror or shiny surface as opposed to diffusely reflecting colors which scatter the light they reflect in many different directions (Prum,

2006). The light reflected from directionally reflecting blue coloration has the potential to produce a much brighter and more conspicuous signal. Bright, conspicuous signals increase warning signal effectiveness (review in Ruxton, Sherratt, & Speed, 2004). However, the nature of many structural mechanisms that produce iridescent colors is such that the bright appearance is restricted to a small set of viewing angles with almost no light reflected from other viewing angles (Rutowski, Macedonia, Kemp, & Taylor-Taft, 2007; Rutowski et al., 2010). In this experiment, I controlled the angle of the wings during presentations in an effort to reduce some of the potential variability, but neither the angle at which the birds first viewed the iridescent blue prey nor the angle of predator approach were controlled. Therefore, the appearance of the iridescent blue wings may have been variable or could have been brighter for some individual birds or in some trials than in others. This same type of variation may also be experienced in nature which may select against using iridescent coloration as part of a warning signal. The nature and magnitude of these consequences is currently under investigation in my lab.

I am confident that the important roles of the iridescent blue and orange hindwing areas in warning signal recognition revealed by my experiments are also at play in nature. The ambient illumination in the indoor setting of my experiments was not natural, but the peaks in the spectral output of the fluorescent light illumination were not far from the peak reflectances of the *B. philenor* iridescent blue areas (mean = 491 nm) and the orange spots (mean = 589 nm). Therefore, the fluorescent lighting provided substantial light energy in the dominant wavelengths of the hindwing reflectance, as does solar radiation. This suggests that differences in the appearance of the hindwing to predators between experimental and field settings would be relatively small and that the features used for

recognition would be similar in both settings. Additionally, my use of wild-caught predators means that the birds had prior experiences with prey that could have shaped their decisions about whether or not to attack prey. However, most predators encountering warningly colored prey in nature will also have had prior experience and, again, I am confident that my results reflect what happens in nature.

My conclusions demonstrate that the characterization of warning colors should not be limited to long-wavelength colors and that multicomponent visual warning signals should be considered in studies of visual antipredator signals.

Table 1. Results for random effects in the generalized linear mixed model on all tests

Random Grouping Factor	Fixed Effect	Variance	Standard Deviation
Bird Identity	Intercept	1.53×10^{-11}	3.92×10^{-6}
Bird Identity	All Black Wings	16.47	4.06
	Only Iridescent Wings	4.07	2.02
	Only Orange Wings	1.64	1.28
	Unaltered Wings	3.78	1.94
Bird Identity	Test Number	0.20	0.49

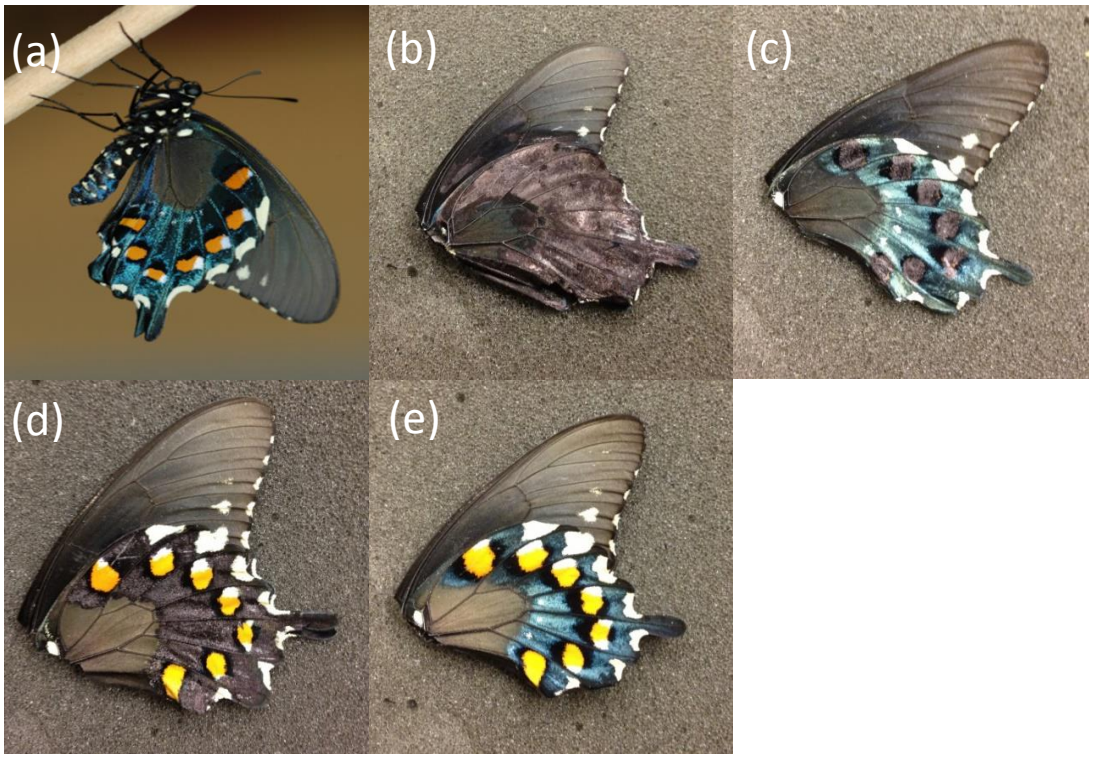

Figure 1. (a) *Battus philenor*, with the ventral surface visible; (b) 'black' wings with ventral surface coloration blackened; (c) 'only blue' wings with orange coloration removed; (d) 'only orange' wings with blue iridescent coloration removed; (e) 'unaltered' wings.

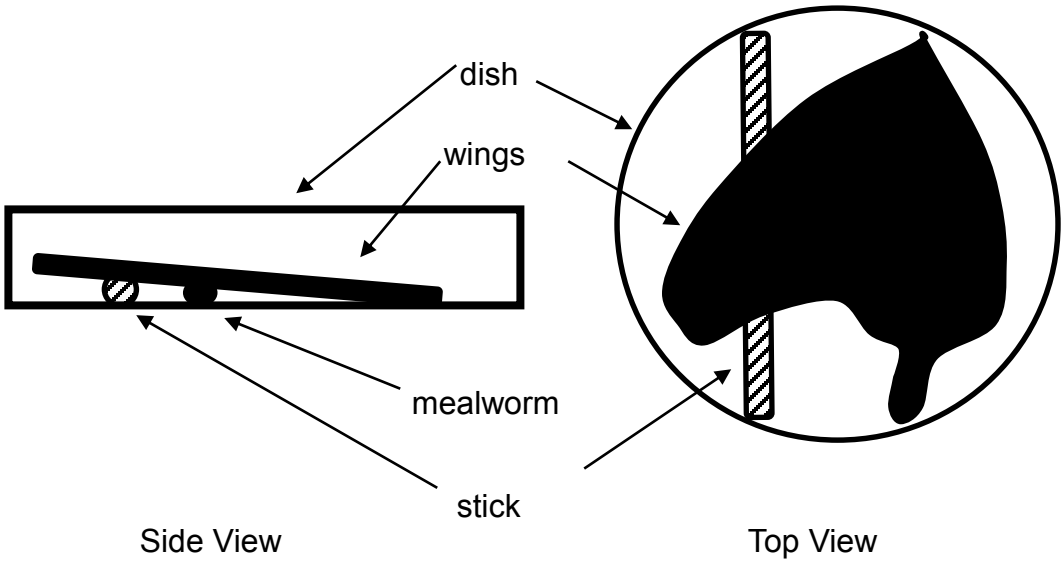

Figure 2. Diagram of prey presentation to demonstrate placement of dowel and wings in a Petri dish. White paper was placed between the dowel and the wings to provide a consistent background.

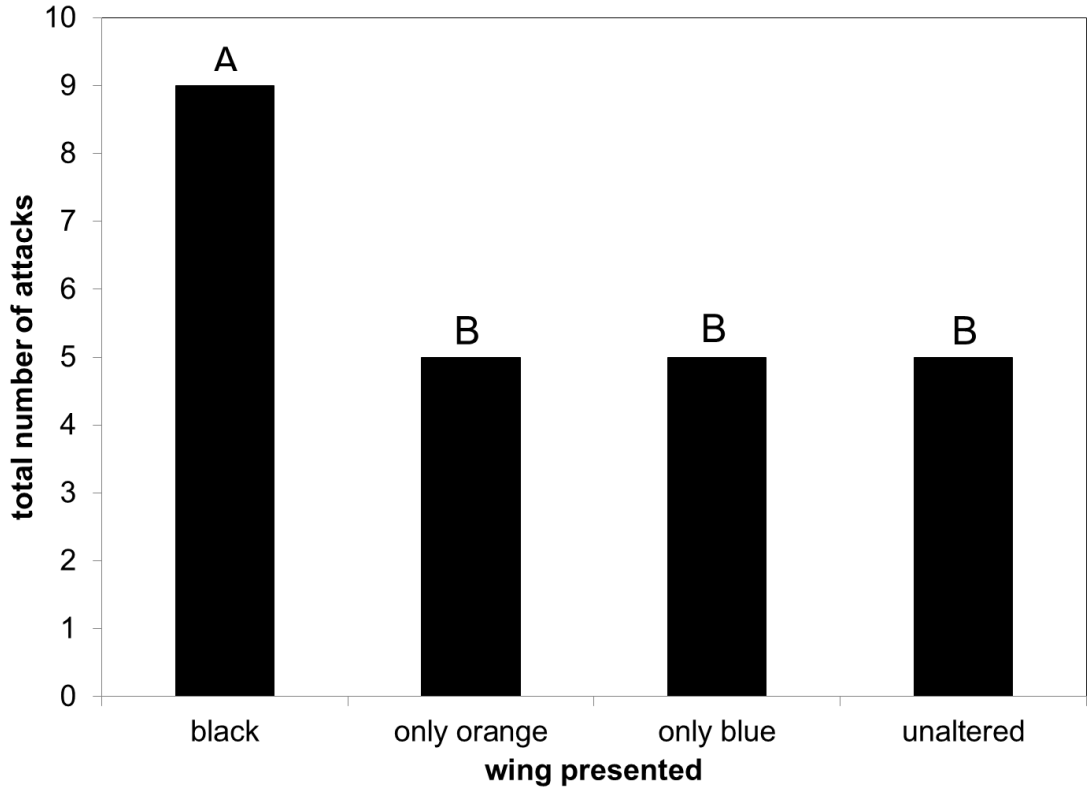

Figure 3. Results of only first recognition test on each bird (*N* = 11). Wings displaying only blue, only orange, and unaltered wings were all attacked at the same rate and less than the black control wings. Different letters represent significantly different number of attacks, while shared letters indicate that the number of attacks were statistically the same.

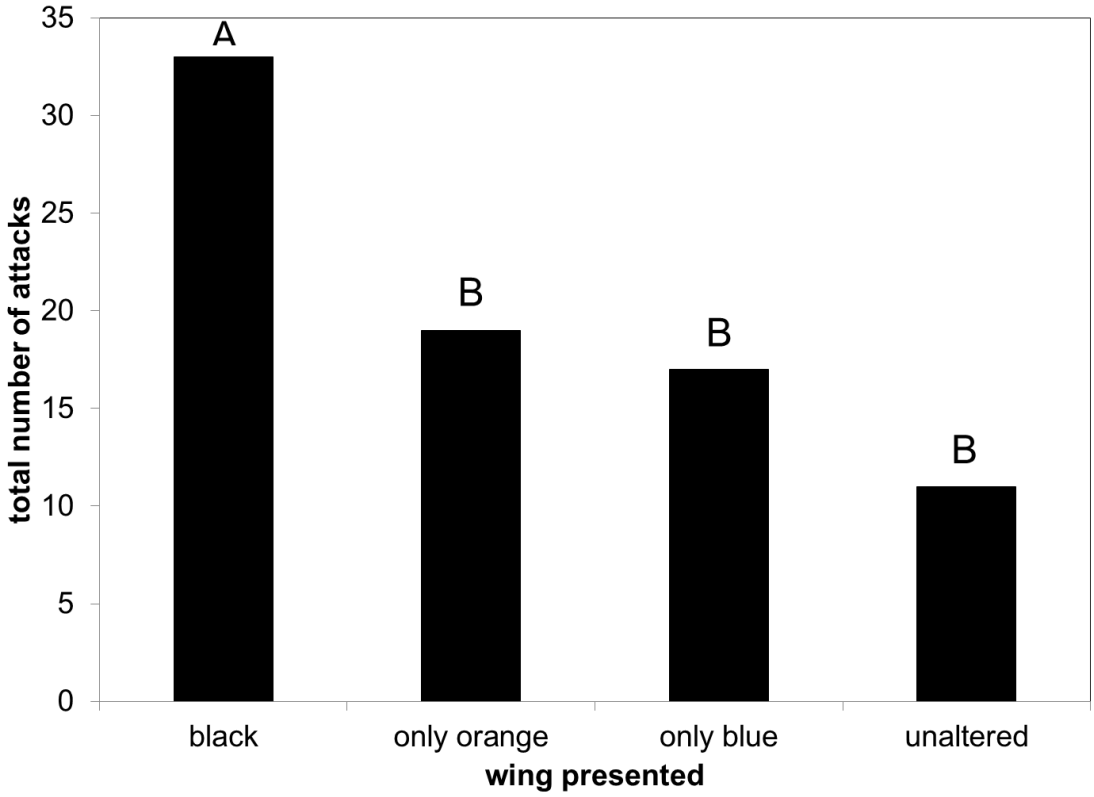

Figure 4. Results of all recognition tests (N = 40). Wings displaying only blue or only orange were attacked less than the black control wing. Wings displaying both colors were attacked the least. Different letters represent significantly different number of attacks, while shared letters indicate that the number of attacks were statistically the same. Wings displaying only orange and wings displaying both colors were not significantly different after Bonferroni correction.

CHAPTER 2

WARNING SIGNAL EFFICACY IN NATURE: EFFECT OF COLOR, IRIDESCENCE AND TIME OF DAY

Introduction

Unpalatable or toxic animals often display bright, conspicuous coloration that advertises their chemical defense and deters predation (Poulton, 1890; Ruxton, Sherratt, & Speed, 2004). These warning signals are understood to have two elements: the 'strategic element', i.e. the part of the signal that contains the information, and the 'tactical design' or 'signal effectiveness' element, i.e. the features and characteristics of the signal that determine how well the information is transmitted (Guilford & Dawkins, 1991; Endler 1993a). A warning signal's effectiveness is measured by how it affects predator psychology, specifically, the stimulation of innate aversions to the signal or the rapidity with which a predator learns to associate the color with unpalatability (reviewed in Rowe & Skelhorn, 2004; Stevens & Ruxton, 2012). Both will lead to reduced attacks on prey bearing an evolved warning coloration. Specific design features of warning signals, such as color or pattern, may increase the effectiveness of the signal. I investigated two visual warning signal features found in natural warning signals that may increase effectiveness: color and iridescence.

Investigations of warning colors to date have largely focused on long-wavelength, diffusely-reflected colors (e.g. oranges, reds, and yellows; Umbers, 2013). Stevens and Ruxton (2012) propose that long-wavelength colors may be more effective warning signals than short-wavelength colors (e.g. ultraviolet, blue and green) because they (1)

are more conspicuous when viewed against green foliage, (2) are more reliably seen across a diversity of habitats, (3) permit distant-dependent camouflage and (4) are distinctive from colors displayed by profitable species. The first two of these hypotheses have been supported by results from visual modeling (Arenas, Troscianko, & Stevens, 2014). Moreover, empirical studies suggest that, in some animals, coloration rich in short wavelengths is not an effective warning signal (Lyytinen, Alatalo, & Mappes, 2001; Lyytinen, Lindström, & Mappes, 2004; Hegna, Saporito, & Donnelly, 2013; Cibulková, Veselý, & Fuchs, 2014). Nonetheless, patches of short-wavelength colors are prominent components of the coloration of a number of toxic or unpalatable animals (e.g. Schultz, 2001; Borer, van Noort, Rahier, & Naisbit 2010) and often adjacent to long-wavelength color patches such as in pipevine swallowtail butterflies (*Battus philenor*) and strawberry poison dart frogs (*Oophaga pumilio*).

Predators are able to learn to associate short-wavelength color features with unapalatability. In the process of testing the effects of conspicuousness and patterns, previous research has shown that domestic chickens are able to learn to avoid blue items (Gittleman, Harvey, & Greenwood, 1980; Gamberale-Stille & Guilford, 2003; Aronsson & Gamberale-Stille, 2008) and two experiments have specifically demonstrated that avian predators can learn to avoid blue prey in a warning signal context (Fabricant, Exnerová, Ježová, & Stys, 2014; Pegram & Rutowski, 2014). Also, fish (Miller & Pawlik, 2013) and great tits (*Parus major*; Fabricant et al., 2014) have an innate avoidance of blue prey and both the short-wavelength and long-wavelength components of the *B. philenor* ventral wing surface equally deter predation by experienced captive

avian predators (Pegram, Lillo, et al., 2013). Therefore, blue coloration can effectively serve as a warning signal under some conditions.

Short-wavelength colors in animal displays are often produced by structural mechanisms that, in turn, produce directionally reflecting, very bright, and dynamic signals that change in appearance with the angle of illumination and angle of viewing. Iridescent coloration is a type of directionally reflecting color that can change in both brightness and hue (e.g. Rutowski, 1977; Loyau et al., 2007; Doucet & Meadows, 2009). Under the right spatial arrangement of reflecting surface, light source and receiver, iridescent colors are brighter and more chromatic (saturated) than diffusely reflected signals but are not perceptible under other arrangements (Rutowski et al., 2007; Doucet & Meadows, 2009; Rutowski, Nahm, & Macedonia, 2010). Therefore, iridescent colors have the potential as warning signals to be very conspicuous (and therefore likely more effective) but less reliably visible than diffusely reflecting colors. Whether or not iridescence makes a warning signal overall more or less effective than diffusely reflecting signals is unknown (Doucet & Meadows, 2009; Théry & Gomez, 2010).

In addition to specific tactical design features of the signal, time of day may also significantly influence the effectiveness of a warning color and selection on the signal. As time of day changes, so does the quality and quantity of ambient light available and, potentially, the suite of predators that attack insect prey and their visual capabilities. The appearance of a color signal to a predator (or any animal receiver) is a function of the ambient light available, the light reflected from a color signal, the medium through which the light is transmitted, and the visual capabilities of the predator (Endler, 1990).

The amount and wavelengths of light available vary greatly with the time of day and habitat (e.g. sun and shade; Endler, 1993b), and therefore the appearance and conspicuousness of colors may vary throughout the day (e.g. Schultz, Anderson, & Symes, 2008; Théry, Pincebourde, & Feer, 2008; Arenas et al., 2014) and variation in light environments may influence predation on defended animals (Rojas, Rautiala, & Mappes, 2014). Little light is available at dawn and dusk and tends to be rich in short wavelengths so that diffusely-reflected long-wavelength visual signals may be difficult to see. In contrast, the structures that produce directionally reflecting short-wavelength signals, like iridescent coloration, reflect the light in one direction and therefore may provide a brighter and more effective signal during dawn and dusk (Olofsson, Vallin, Jakobsson, & Wiklund, 2010; Pegram, Han, & Rutowski, 2012). Additionally, the predator community structure may change throughout the day and different types of predators are likely to vary in their visual capabilities and tendency to attack aposematic prey (e.g. Endler & Mappes, 2004; Kelber & Roth, 2006; Ratcliff & Nydam, 2008; Mochida, 2011; Valkonen et al. 2012; Nokelainen, Valkonen, Lindstedt, & Mappes, 2014). The visual capabilities of predators can also change throughout the day (e.g. Kacelnik, 1979) resulting in differences in attack decisions.

To test the role of short-wavelength coloration, iridescent coloration, and time of day on warning signal effectiveness, I measured predation rates on *B. philenor*, the pipevine swallowtail butterfly, in the field. *B. philenor* is extremely distasteful to insectivorous birds (Brower, 1958; Codella & Lederhouse, 1990) due to the sequestration of aristolochic acids in their tissues during the larval stage (Sime, Feeny, & Haribal, 2000; Fordyce, Marion, & Shapiro, 2005). The ventral hindwing warning signal is

composed of orange spots in a field of iridescent blue (Codella & Lederhouse, 1990; Rutowski et al., 2010). Previous experiments with *B. philenor* have found that captive avian predators can use both the ventral iridescent blue and the orange to recognize this species as distasteful (Pegram, Lillo, et al., 2013) or the male dorsal surface alone (Codella & Lederhouse, 1990), which displays iridescent blue. Additionally, both orange and blue elements are found in the coloration of Batesian mimics of *B. philenor*: *Papilio polyxenes*, *P. troilus*, female black form of *P. glaucus* and *Limenitis arthemis* (Brower, 1958; Platt, Coppinger, & Brower, 1971). Despite many investigations into the behavior and ecology of *B. philenor* (e.g. Hazel & West, 1979; Rausher, 1980; Papaj, 1986; Rutowski, Alcock, & Carey, 1989; Weiss, 1997), I know very little about predation on adults and the suite of natural predators. Invertebrates and lizards have been observed preying on adult *B. philenor* (Rausher, 1980; Odendaal, Rausher, & Benrey, 1987), but insectivorous birds are also likely predators. I also do not know what time of day *B. philenor* adults are most susceptible to predation but I do know they form nocturnal aggregations that likely increase the effectiveness of the warning signal (Pegram et al. 2012), among other factors.

I tested these ideas in nature using procedures liked those used in other studies studying the effect of color and pattern on predation (e.g. Stevens, Graham, Winney, & Cantor, 2008; Rowland et al., 2008; Lindstedt et al. 2011; Finkbeiner, Briscoe, & Reed, 2012). I placed various types of models in the field in central Arizona and noted the rates at which they were attacked. I manipulated the coloration of *B. philenor* wings to produce models that were all-black, only-iridescent-blue, only-orange, iridescent-blue-and-orange (intact signal), or matte-blue-and-orange (iridescent blue painted over with

diffusely reflecting blue paint). I hypothesized that displaying both colors makes a more effective warning signal and, based on laboratory results, that long-wavelength and short-wavelength coloration are equally effective at deterring predation. To test this hypothesis, I compared attack rates on the iridescent-blue-and-orange (intact signal), only-iridescent-blue, only-orange and all-black models. If short-wavelength coloration functions as an effective warning signal in nature, the only-iridescent-blue models will be attacked less than models with only black coloration, which provides a control for size and shape of the butterfly. If long-wavelength colors and short-wavelength colors are equally effective at deterring predators, rate of attacks on only-iridescent-blue models and only-orange models will be the same. My second hypothesis was that iridescence per se will have an impact on warning signal effectiveness. To test this hypothesis, I compared the attack rates on the iridescent-blue-and-orange model (intact signal) and the matte-blue-and-orange model, because the only difference between the two models is in the iridescent nature of the blue coloration. If iridescence affects warning signal effectiveness (either positively or negatively), there will be a difference in attack rates between the two models. Lastly, I hypothesized that warning signal effectiveness may vary with time of day. If time of day affects signal effectiveness, I will find that time of day in which a model is attacked is not independent of the model type, suggesting that a color is more effective at one time of day than another. These results will inform my understanding of the specific tactical design features that affect warning signal effectiveness in nature with a suite of predators.

Methods

I performed the experiment on the grounds of the Desert Botanical Garden (DBG) in Phoenix, Arizona, USA (N 33 27.589 W 111 56.959), a 59 hectare desert preserve. DBG is within the range of *B. philenor* and I and other observers have seen the adult butterflies flying at this location and nearby neighborhoods. I ran 14 replicates of the experiment, four from 17-May-2010 to 21-Jun-2010 and ten from 16-Apr-2011 to 16-May-2011. Each replicate was run in a different 3000-5000 m^2 area within DBG in areas not open to the public to minimize human disturbance.

During each replicate I placed 25 model butterflies out in the field. The models consisted of freeze-killed *B. philenor* with the wings folded over the back as they are when the animals are perched (Pegram et al. 2012). I altered the ventral color patterns with non-toxic tempera paint, glued the wings together, and inserted a pin in the thorax for field placement. The models were equally divided among five different wing pattern treatments: iridescent-blue-and-orange (intact signal), all-black, only-iridescent-blue, only-orange, and matte-blue-and-orange (Table 2). On the iridescent-blue-and-orange butterfly model I did not alter the ventral hindwing pattern (Fig. 5a) but I did paint a portion of the black forewing with black paint as a control. For the all-black model I painted over the entire hindwing pattern except for the white margin spots with black paint (Fig. 5b). The only-iridescent-blue model displayed iridescent blue and black; I painted over the orange spots with black paint (Fig. 5c). The only-orange model displayed the orange spots and black (Fig. 5d); I painted over the iridescent blue patch with black paint. For the matte-blue-and-orange model, I painted over the iridescent blue with a diffusely reflecting blue paint that matches the hue of the natural blue coloration

(see further description below) and did not alter the orange spots (Fig. 5e). For all model types, I left uncovered the white spots around the margin of the hindwing and covered the iridescence on the butterfly abdomens with black paint. The specimens were lab-reared from field-collected larvae and eggs; larvae were fed ad libitum in the lab (for details on the rearing conditions and field site see Rutowski et al., 2010).

The matte blue paint was chosen to match the average hue of the *B. philenor* ventral iridescence at the angle of peak iridescent reflectance (Fig. 6). I used spectrophotometry to choose the color and decided to match the hue and not chroma, brightness, or avian perception because iridescent colors are often much brighter and chromatic (at the peak reflectance) than diffusely reflecting colors (Vukusic, Sambles, Lawrence, & Wootton, 1999; Osorio & Ham, 2002; Doucet & Meadows, 2009). Therefore, (1) the increased brightness and chroma of the iridescent signal could contribute to an increased effectiveness of iridescent colors and (2) iridescent signals will likely look significantly different from diffusely reflecting signals in the eyes of a predator. The hue of field-collected *B. philenor* ventral iridescence at peak reflectance ranges from 461-560 nm, with an average of 504 nm for males and 514 nm for females (Rutowski et al., 2010). I took reflectance spectra on the paint after application to the model. The wavelength at peak reflectance for the paint was 506 nm. In addition, I used AVICOL v.6 (Gomez 2006) and a visual model based on Vorobyev and Osorio (1998) to determine the magnitude of the perceived differences in the wings painted blue and the natural iridescent blue in the eyes of avian predators. Using the spectral sensitivities and cone proportions for Blue Tits (*Cyanistes caeruleus*, Hart, Partridge, Cuthill, & Bennett, 2000), a species commonly used to represent passerine bird predators, I found that the

average Just Noticeable Difference (JND) between the diffusely reflecting blue paint and the *B. philenor* ventral iridescence was 3.8. The average JND between the black paint and the black of the *B. philenor* forewing was 0.6. A JND value larger than 1 is considered to be discriminable by bird predators (Vorobyev, Osorio, Bennett, Marshall, & Cuthill, 1998). These results were as expected, because the black paint should not be discriminable from the black wing and the diffusely reflecting blue, due to differences in brightness and chroma, looks different than the ventral iridescence.

In the field, each model was placed on a tree or shrub using a set-up that provided a surface that would securely hold the model, protect it from ants, and attach it securely to the vegetation. This set-up consisted of a gray PVC pipe 45.5 cm in length, a cylindrical piece of black foam made to insulate PVC pipes, and Fluon® (Fig. 7). The black foam, cut to approximately 5 cm wide, was slipped onto and centered on the PVC pipe. Above and below the foam I painted a ring of Fluon on the pipe to prevent scavenging from ants, the results of which could be mistaken for bird predation. The model butterfly was pinned to the foam and the pipe was slipped over a branch on the vegetation between 0.4 and 2 m above the ground (mean=1.1 m). These pipes and the attached models were set-up so that they were at least 2 m from one another (but usually farther apart). The model placed on each set-up was a random selection from the five model types and was placed at a haphazard compass orientation and therefore was not placed in any particular orientation in relation to the sun.

Each replicate lasted 72 h and started between 1100 and 1300. I checked for attacks on models at three times each day: in the hour before sunset, in the hour after sunrise, and at midday (1100-1300). During these checks, all models were examined for

signs of damage. I considered a model to have been attacked if there was damage to the model, such as missing body parts or pieces of wing, or if no longer present. Twenty-four models were clearly hit and damaged by nearby branches moved by wind and so were not included as attacks. Previous studies of field predation vary in whether they consider models no longer present to have been attacked (e.g. considered attacked: Hossie & Sherratt, 2013; Carroll & Sherratt, 2013; not considered attacked: Cuthill et al., 2005; Stevens et al., 2008). I considered models that were no longer present to have been attacked because my video data (see results and discussion) demonstrated that some predators carried away the entire model during an attack and missing models can indicate complete removal of a prey item that should be considered an attack. Furthermore, my models were not composed of inedible components as in other studies, such as clay or paper wings.

I gathered data to identify the predators that attacked the models in the field. In the first spring (2010), I used two continuous-recording, digital, standard definition video cameras (Panasonic SDR-S7; Panasonic Corporation, Secaucus, NJ). Each was set on a tripod 1-5 m away from a model turned on to record during the day between the dawn and the dusk checks. The cameras were powered by external battery packs (Duracell Powersource Mobile 100, Duracell, Bethel CT). In the second spring (2011), I used the same two standard definition cameras as above, and added three continuous-recording, high definition video cameras (JVC Everio GZ-HM300; JVC Kenwood Corporation, Wayne, NJ) and two motion-activated cameras capable of recording at night (Bushnell Trophy Cam, Models #119415 and #119435C; Bushnell Outdoor Technology, Overland Park, KS). All continuous-recording cameras were covered by a box built of cardboard

and foam. The motion- activated cameras were sometimes mounted on a tripod, but more often strapped to a nearby branch or tree, and set to record a one minute video when triggered with a medium sensitivity setting. The motion activated cameras were left throughout the trial (including overnight), with the memory cards and batteries changed regularly. I reviewed the video recordings when a model was attacked to identify the attacker.

I analyzed the results using survival analysis. Survival analysis can account for censored values, i.e. those models that survived through the 72 hours or had damage not consistent with an attack. I used Kaplan-Meier to estimate the survival functions and a Mantel-Cox log-rank test statistic, adjusted for trial, to compare survival curves. To compare amongst treatments, I used planned post hoc tests. The post hoc test consisted of the calculation of odds ratios (OR), which compare the odds an attack on one model type to the risk on another model type, and a chi-square test to determine significance. Based on the hypotheses presented in the introduction, I had seven planned comparisons. Because the comparisons were planned a priori, they do not require the correction of post-hoc p-values (Ruxton & Beauchamp, 2008). I used a chi-square contingency table to determine whether the time of day in which a model was attacked was dependent or independent of the model type. All analyses were performed with a 0.05 significance level.

Results

Attacks on models

I completed 14 72-hr trials over two years, collecting data on a total of 350 models, 70 models of each type. Overall, 139 models (40%) were attacked. There was a significant effect of model type ($\chi^2 = 13.696$, $df = 4$, $p = 0.007$; Fig. 8). Contrary to expectations, there was no significant difference in the attack rates for all-black models, with the orange and blue ventral color elements replaced with black, and iridescent-blue-and-orange models (intact coloration; OR = 1.89, $\chi^2 = 3.492$, $df = 1$, $p = 0.062$), although the difference was close to significant and in the expected direction. The remaining planned comparisons are presented here and summarized in Table 2.

Models with attack rates significantly lower than all-black models are considered to have coloration that deters predation. Like the iridescent-blue-and-orange models, I compared the attack rates of black models to the only-iridescent-blue models and only-orange models. Black models were attacked significantly more than only-orange models (OR = 2.8, $\chi^2 = 8.552$, $df = 1$, $p = 0.003$), but not significantly more than only-iridescent-blue models (OR = 1.19, $\chi^2 = 0.256$, $df = 1$, $p = 0.613$). Only-iridescent-blue models were not attacked more often than iridescent-blue-and-orange models (intact coloration; OR = 1.60, $\chi^2 = 1.166$, $df = 1$, $p = 0.280$). Similarly, there was no significant difference in attack rate between the iridescent-blue-and-orange model and the only-orange models (OR = 1.48, $\chi^2 = 1.165$, $df = 1$, $p = 0.280$).

When I replaced the iridescent blue coloration with a matte blue coloration, I found that this did not change the effectiveness of the warning signal. The attack rate on matte-blue-and-orange models was not significantly less than the attack rate on

iridescent-blue-and-orange models (OR = 0.94, χ^2 = 0.029, *df* = 1, *p*=0.065). Long wavelength colors may be more effective than short wavelength colors because the attack rate on only-orange models was significantly lower than the attack rate on only-iridescent-blue models (OR = 0.425, χ^2 = 5.908, *df* = 1, *p* = 0.015).

I found that the time of day in which the model was attacked was independent of model color (χ^2 = 10.483, *df* = 8, *p* = 0.232; Fig. 9), indicating that a model type was not more effective at deterring predators during one time of day than another time of day.

Video recordings

Over the 14 trials, I gathered approximately 1590 h of video recordings from the continuous recording cameras and had the motion-activated cameras trained on models for approximately 1000 h. I video recorded 69 models, each for at least one measurement period (6 h) and, of those, 22 were damaged or went missing while being recorded. Eleven were clearly attacked by predators and seven were blown away by wind. The cause of damage or disappearance of the remaining is unknown because the motion-activated camera trained on each did not activate at the time the model was damaged or went missing.

All of the predators recorded attacking models were insectivorous, passerine birds. I identified three Abert's towhees (*Pipilo aberti*), one brown-crested flycatcher (*Myiarchus tyrannulus*), five cactus wrens (*Campylorhynchus brunneicapillus*), one curve-billed thrasher (*Toxostoma curvirostre*), and one Northern mockingbird (*Mimus polyglottos*). The birds attacked by either removing parts of the model, such as the head or abdomen, or departing with the entire butterfly. From the videos, I could see the birds

attacking the models but could not resolve whether they ate either the model or piece of model that was grabbed.

Discussion

Role of short-wavelength color in warning signal effectiveness

My results indicate significant differences in the rates at which the various models were attacked although some of these differences were as expected and some not. Especially surprising was that there was no significant difference between the attack rates on all-black models and those on the intact iridescent-blue-and-orange model. I expected to be different based on the results of my previous captive predator studies (Pegram, Lillo, et al., 2013) and others (Brower, 1958; Codella & Lederhouse, 1990). Similarly, short-wavelength blue coloration of the *B. philenor* ventral surface did not effectively deter predators, which is not consistent with my previous study with captive but experienced insectivorous birds (Pegram, Lillo, et al., 2013). In both cases given that the difference reported here was in the direction expected from previous studies and that the difference was close to significant, I conclude the lack of difference in the present study was an artefact of uncontrolled variability inherent in field studies such as the seasonal variation in predator experience (Mappes, Kokko, Ojala, & Lindström, 2014).

Several studies have suggested that orange and red may make more effective warning signals (Stevens & Ruxton, 2012; Hegna et al., 2013; Arenas et al., 2014). I found some support for this hypothesis, because only-orange models were attacked significantly less than only-iridescent-blue models. My results did not support the hypothesis that two adjacent short-wavelength and long-wavelength colors make a more

effective warning signal, as the models with both colors were attacked as often as models with only one color.

In this experiment, I kept the natural patterning of *B. philenor* (i.e. when a model displayed a color, it was where that color was found naturally) because I were expecting attacks by natural predators that had likely had previous experiences with *B. philenor*. Therefore, there were significant differences in the area of the warning signal between only-iridescent-blue models and only-orange models. The total area of orange spots combined is smaller than the size of the iridescent patch (Pegram, unpublished data), and larger signal sizes are expected to be more effective (Gamberale & Tullberg, 1996; Forsman & Merilaita, 1999; Lindstedt, Lindström, & Mappes, 2008). However, I still found that the models with only orange coloration were attacked less often. The natural coloration of *B. philenor* also has significant continuous intraspecific variation (Rutowski et al., 2010; Pegram, Nahm, & Rutowski, 2013), so there was variation in the natural colors. However, color variation may have few consequences for warning signal effectiveness in natural environments (Lindstedt et al., 2011). Altering the color and iridescence of my models likely changed some characteristics of the prey and their warning signals, such as the conspicuousness or the distinctiveness. For example, only-orange models may have been more conspicuous than the only-iridescent-blue models. However, being more conspicuous in nature is one reason orange signals may be more effective (Stevens & Ruxton, 2012; Arenas et al., 2014; but see Fabricant & Herberstein, 2014), so I did not control these characteristics.

Role of iridescent coloration

I hypothesized that iridescent colors are more effective warning signals than diffusely reflecting colors because iridescent coloration has the potential to be a much brighter and conspicuous signal. I also hypothesized that iridescent signals are more effective than diffusely reflecting colors at times of day when little light is available because the structures that produce iridescence do not scatter the light in as many directions (Olofsson et al., 2010). These hypotheses were not supported by my data because there was no significant difference in attack rates between iridescent-blue-and-orange models and matte-blue-and-orange models. There was also no relationship between model type and time of day to suggest that the blue iridescent coloration was more effective around dawn and dusk.

Iridescent coloration and other directionally reflecting colors can be much brighter than diffusely reflecting colors (reviewed in Doucet & Meadows, 2009), a feature that may improve warning signal effectiveness (Prudic, Skemp, & Papaj, 2007), or may even create effective flashing signals that startle or draw the attention of a predator or obscure the prey's position (Hinton, 1973). However, iridescent colors are only bright from a limited range of angles and may have little or no apparent reflectance from other angles (Rutowski et al., 2007, 2010; Perez i de Lanuza & Font, 2014), which may make for a less reliable signal. Additionally, iridescent coloration may shift in hue when viewed from different angles (Rutowski et al., 2010). This variation in appearance of iridescent coloration could slow or hinder learning and recognition processes or could simply make the signal ineffective if predators approached from directions relative to the wing surfaces where there is no apparent reflectance. This experiment was not designed

to test these ideas individually, only the overall role of iridescence per se as a tactical design element as compared to diffusely reflecting colors (not including directionally reflecting but not iridescent colors). Therefore, these potential positive and negative consequences of iridescence could have both been influencing the effectiveness of the iridescent coloration to the point where no effect was discernable in my experiment.

The two models compared here to measure the effect of iridescence were both static and both had the diffusely reflecting orange spots still present. Iridescent warning signals or warning signals in general may function differently in nature when prey are able to move (Paluh, Hantak, & Saporito, 2014), unlike my static models, and the orange spots could have influenced the attack rate on the two models. However, the goal of the experiment was to measure the role of iridescent coloration in its natural context, with the orange spots. Iridescent coloration can be costly to produce (e.g. Kemp & Rutowski, 2007) and the ventral surface iridescence does not likely function in the intra-sexual signals of *B. philenor* (Rutowski & Rajyaguru, 2013), therefore the possible adaptive advantages of displaying iridescent blue coloration for *B. philenor* and potentially other animals that combine orange and iridescent blue are unknown. This adaptive advantage was not revealed by this study and highlights the need for further studies on the potential consequences and benefits of iridescent warning signals.

Role of time of day

I hypothesized that model types would vary in effectiveness through a 24 hour period. I found that this was not the case as the time of day in which a model was attacked was independent of model type. Changes in color appearance due to changes in

ambient light may be mediated by predator generalization of warning signals (Ham, Ihalainen, Lindström, & Mappes, 2006; Ruxton, Franks, Balogh, & Leimar, 2008; Svádová et al., 2009) or changes in detectability (Rojas et al., 2014). Also, warning signals, especially multicomponent signals, may evolve to be effective across a broader range of light environments and predator perception (Partan & Marler, 2005). Two components that differ in hue might provide insurance, increasing the chance that in any given light environment at least one component of the signal will be conspicuous when viewed by a predator. However, as with the different colors, I saw no indication that iridescent-blue-and-orange models were more effective at one time of day than another. This could be a good area for further research in more controlled conditions or through modeling of predator visual perception under a range of light conditions.

Conditions of warning signal use and video analysis

My digital recordings of attacks in the field provide new information on the potential receivers of the warning signal of *B. philenor*. I recorded 11 attacks on models - all by insectivorous birds. There are reports of invertebrate predators (e.g. dragonflies and spiders) and lizards in the field (Rausher, 1980; Odendaal et al. 1987) predating on *B. philenor*, but I saw no evidence of this in my videos. Perhaps I did not catch them on video or dragonflies and spiders focus on capturing moving prey. The dragonfly in Rausher's (1980) observation caught the *B. philenor* adult mid-flight. Swallowtail butterflies are expected to be under higher predation pressure when perching (Rawlins & Lederhouse, 1978; Lederhouse, Codella, & Cowell, 1987) and I have found that *B. philenor* in an enclosure may disappear when perching at night, likely due to predation

(Pegram et al. 2012). While I had hoped to shed some light on nocturnal or crepuscular predation through the use of motion-activated cameras running in the dusk through dawn period, no attacks were recorded in this period.

The video recordings also allow me to assess the causes of model disappearance. I had 22 models disappear or incur damage while being recorded, but the videos only showed clear attacks for 11 of those models. The models that were not attacked on video were blown away by the wind or hit by nearby wind-blown branches. While these abiotic factors add some noise to my overall attack data, they should act on models without respect to model type, and I still found significant effects of model type. To better understand both the biotic and abiotic factors influencing data beyond treatment, I suggest that future field studies incorporate the use of cameras. I found that among the cameras I used, my relatively-inexpensive continuously-recording cameras provided the most complete and reliable records (but see Willink, García-Rodríguez, Bolaños, & Pröhl, 2014).

Applicability of conclusions to other populations and prey

Like other field experiments on warning coloration, my study was done only in a restricted geographic region with a blocking design to minimize the potential for one predator to attack multiple models (e.g. Borer et al., 2010; Lindstedt et al., 2011; Finkbeiner et al., 2012; Valkonen et al., 2012). Other parts of the range of *B. philenor* will have different local predators that potentially have different learning abilities and innate aversions (Merilaita & Kaitala, 2002; Mappes, Marples, & Endler, 2005; Valkonen et al., 2012) or variation in local prey communities could lead to variation in how

predators respond to an unpalatable prey such as *B. philenor* (Merilaita & Kaitala, 2002; Sherratt, 2003; Mappes et al., 2005; Ihalainen, Rowland, Speed, Ruxton, & Mappes, 2012). However, field guides report little variation in *B. philenor* coloration (Scott, 1986) which suggests that the responses of predators to color pattern elements that I have observed both in the field and lab (Pegram, Lillo, et al., 2013) and consequent selection pressures on coloration are similar from one region to the next. Also, the basic patterns of predator avoidance of this species in response to coloration were found in other experiments in other regions (Brower, 1958; Codella & Lederhouse, 1990). While I am confident that my results will apply across the geographic range of *B. philenor*, it will be of interest to see if the functional relationship between orange and blue components in warning colorations suggested by my studies apply in other similarly colored taxa.

Conclusion

Despite the long history of interest in warning coloration, many questions remain unanswered about the tactical design features of warning signals that contribute to their effectiveness. I have provided a start at answering these questions, especially those highlighted in Stevens and Ruxton (2012) and Théry and Gomez (2010). I found that long-wavelength colors are more effective in natural situations of predator recognition and that short-wavelength colors provided little deterrence in these circumstances but this could be dependent on predator experience. I also found that the iridescent reflectance of *B. philenor*'s blue coloration did not affect predation rate in the field as compared to matte blue coloration. Time of day also did not seem to affect the effectiveness of warning coloration. Studies of warning color effectiveness in the laboratory provide

important data, but may not be directly applicable to nature. In a natural setting, there is a complex array of light environments, backgrounds, alternative food sources, and predators that can all influence predation and the selection on animals that display warning colors. This experiment is a first step in exploring some of the untested ideas about warning signal effectiveness in nature, and I suggest that these ideas, including why some colors are more effective than others, be further pursued in controlled settings.

Table 2. Summary of model types, alterations performed, percent attacked and results of comparisons. Higher than or lower than under significantly different model types refer to attack rates.

			results of comparisons		
Model type	Alteration	Percent attacked	Attacked more often than	Attacked less often than	Attacked same rate as types
all black	black paint covers ventral surface warning coloration, white margin spots left in place	72%	only orange		iridescent blue and orange, only iridescent blue
only iridescent blue	orange spots covered by black paint	62%	only orange, iridescent blue and orange		all black
iridescent blue and orange	intact warning coloration, black painted on forewings	52%			only orange, only iridescent blue, black, matte blue and orange
matte blue and orange	iridescent blue painted over with diffusely reflecting blue paint	48%			iridescent blue and orange
only orange	iridescent blue covered by black paint	46%		all black, only iridescent blue	iridescent blue and orange

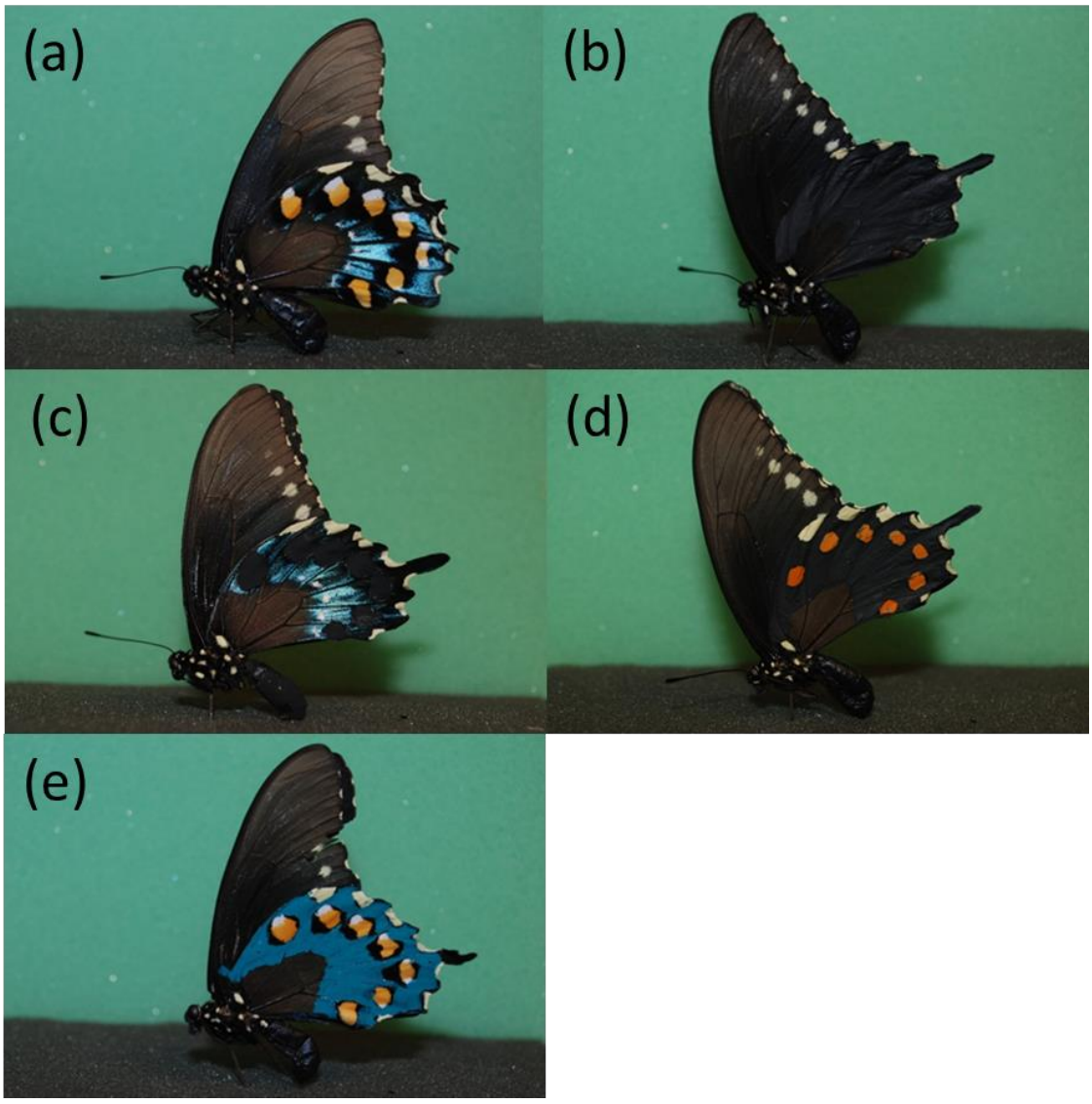

Figure 5. *B. philenor* model types placed at the field site. (a) Iridescent-blue-and-orange model (intact warning coloration), (b) All-black model, (c) Only-iridescent-blue model, (d) Only-orange model, and (e) Matte-blue-and-orange model

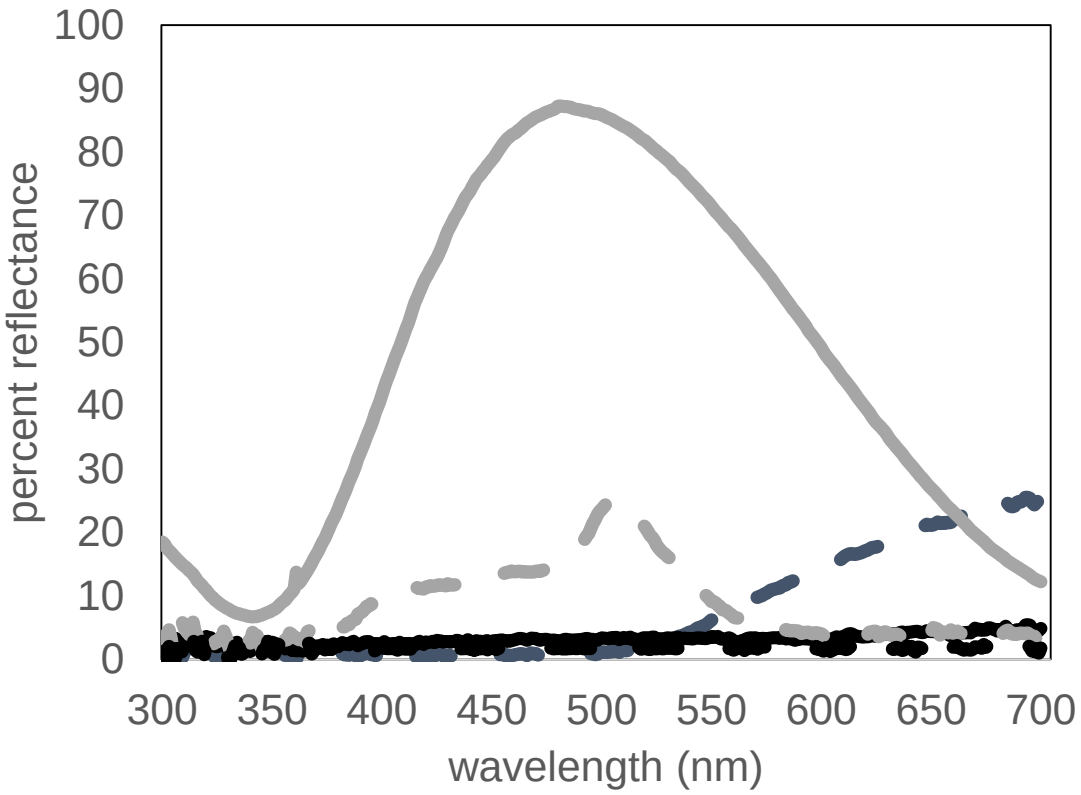

Figure 6. Reflectance spectra taken from models. The iridescent blue (light grey solid line), orange spots (dark grey solid line), and black (black solid line) data are averaged from four lab-reared specimens. The light grey line is the averaged spectra of the matte-blue paint and the dashed black line is the averaged spectra of the black paints. Reflectance spectra were taken as described in Pegram, Nahm, & Rutowski (2013).

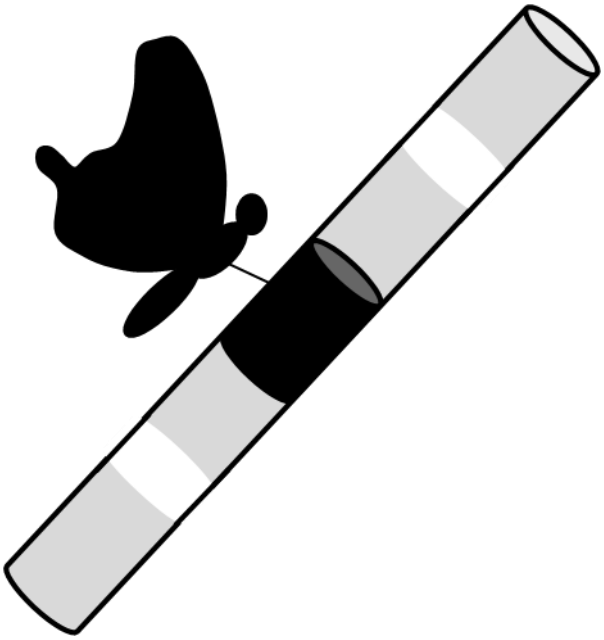

Figure 7. Diagram of field set-up that held butterfly models. Gray PVC pipe had foam in the middle with Fluon painted on either side. Model was pinned to foam and then the pipe was slipped over branch on natural vegetation.

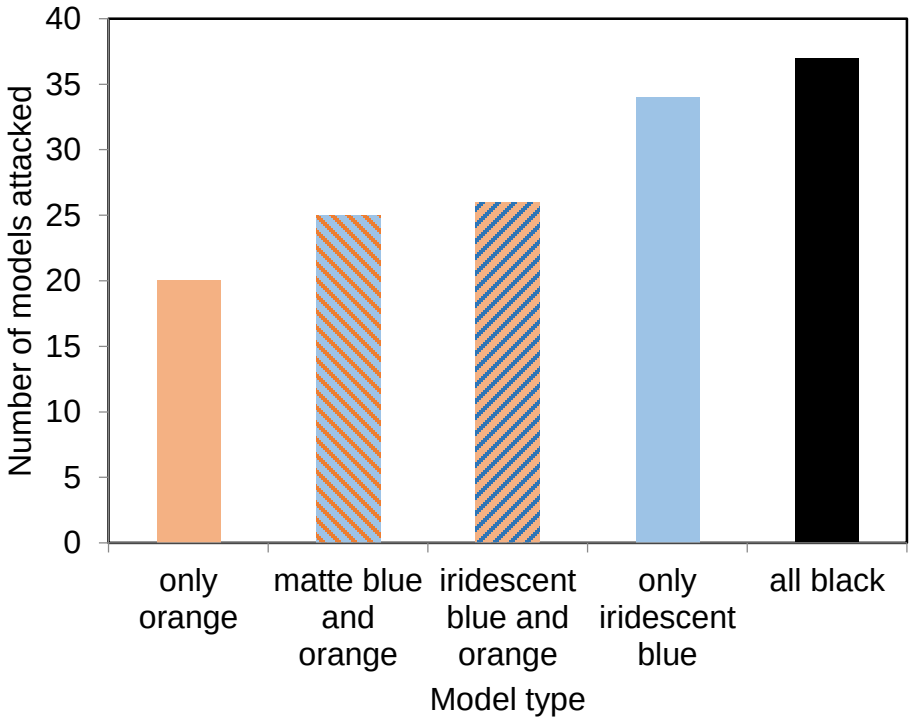

Figure 8. Total number of models attacked across all trials based on model type. Model type significantly influenced attack rate (χ^2 = 13.696, *df* = 4, *p* =0.007). Different letters represent significantly different results. Sample size = 350 models, 70 of each type.

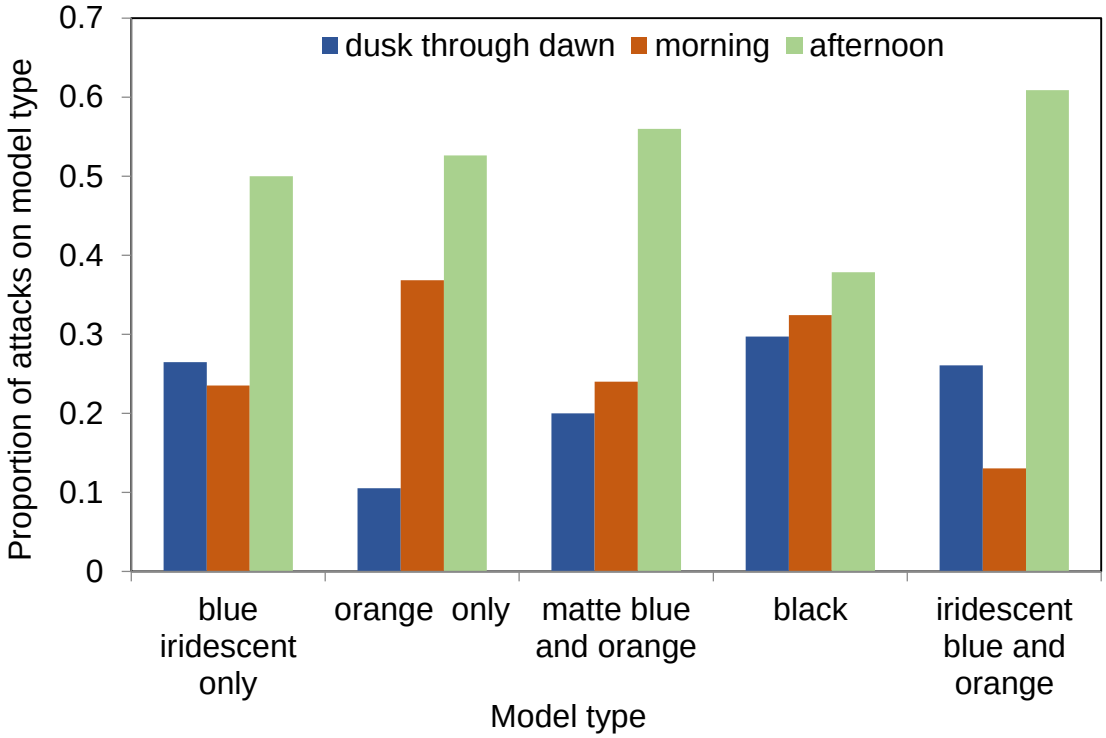

Figure 9. Proportion of models attacked by time of day in which attack occurred. The time of day in which a model was attacked was independent of that model's type.

CHAPTER 3

IRIDESCENT WARNING SIGNALS: THE EFFECT OF DIRECTIONALITY, SIGNAL INTENSITY, AND SHORT-WAVELENGTH HUE ON LEARNING

Introduction

Some of the flashiest colors in nature, such as those of the gorgets of hummingbirds or the dorsal blue of *Morpho* butterflies, are produced by structural features of the reflecting surface (Hailman, 1977; Bradbury & Vehrencamp, 2011). Such features often produce iridescent coloration which is unique in that the surface reflection changes both in hue (i.e. the color or wavelengths reflected) and brightness (i.e. the amount of light reflected) as the position of the viewer and light source change relative to the surface (Land, 1972; Vukusic, Sambles, Lawrence, & Wooton, 2001; Doucet & Meadows 2009). As a result, compared to matte pigment-based colors, iridescent reflections are much more directional and, thus, brighter and more conspicuous signals (Kemp 2002; White, Zeil, & Kemp, 2015). However, directionality also restricts the bright appearance to certain angles of viewing and approach with little or no light visible from other angles (Rutowski et al., 2007), which could also affect the effectiveness of an iridescent signal. Trade-offs in signal directionality and effectiveness for iridescent colors have received relatively little attention (Doucet & Meadows, 2009), and the limited work to date in this area has focused on sexual signals (e.g. Loyau et al., 2007; Dakin & Montgomerie, 2009; Schultz & Finke, 2009; Sicsú, Manica, Maia, & Macedo 2013; White et al., 2015). Testing the role of iridescence in natural systems presents challenges because the effect of the iridescence (i.e. directionality in brightness and hue) must be

separated from the effects of the color. Here I address this gap in understanding via studies of an iridescent warning signal.

Warning colors, or aposematic colors, are displayed by toxic or unpalatable animals to deter predation (Poulton, 1890; Cott, 1940). Predators can have innate reactions to these colors (e.g. Smith, 1975; Roper, 1990) or learn to associate them with prey defenses through trial-and-error learning (e.g. Gittleman & Harvey, 1980; Roper & Wistow, 1986). Several possible examples of iridescent warning signals include the tropical butterflies *Heliconius cydno*, *H. sapho*, and *Parides sesostris*, and the blue-ringed octopus, *Hapalochlaena lunulata*. All of these have iridescent colors and are unpalatable to predators or are defended (Ghiradella, 1985; Pinheiro, 1996; Mäthger et al., 2012), but the connection between their color and distatefulness has not been shown. More conclusively, we have shown previously that the iridescent blue coloration on the ventral wing surface of the pipevine swallowtail butterfly, *Battus philenor*, can be learned and recognized by predators (Pegram, Lillo, & Rutowski, 2013; Pegram & Rutowski 2014). Also, Fabricant, Exnerová, Ježová, and Stys (2014) found that predators can learn to associate the blue iridescent coloration of the hibiscus harlequin bug, *Tectocoris diophthalmus* with their unpalatability. These studies did not separate the potential effects of the color from the iridescence, but they do successfully demonstrate that iridescent colors can serve as warning signals. No studies have examined or carefully tested the potential effects of the iridescence *per se* on warning signal efficacy.

The angle-dependent shifts in appearance of iridescent colors have the potential to influence warning signal learning rates by predators through several mechanisms. First, if predators approach noxious prey from different angles, then signal appearance may vary

with each attack, and this inconsistent presentation could slow the rate of signal learning by predators (but see Rowe, Lindström, & Lyytinen, 2004; Ham, Ihalainen, Lindtröm, & Mappes, 2006; Ihalainen, Lindström, & Mappes 2007). Second, if predators approach from consistent angles, then iridescent colors may be brighter (and more contrasting/conspicuous) than diffusely reflecting signals, which could facilitate warning signal recognition and learning (Gittleman & Harvey, 1980; Roper & Wistow, 1986; Lindström, Alatalo, & Mappes, 1999; Riipi, Alatalo, Lindström, & Mappes, 1999; Aronsson & Gamberale-Stille, 2009). To my knowledge, there is only one study providing evidence that increased brightness (luminance contrast) facilitates learning, but here a predator that lacks color vision was used (Prudic, Skemp, & Papaj, 2007). Third, iridescent prey may vary not just in brightness but in hue (i.e. true color, such as blue vs green). In some systems, hue can be more important in warning signal learning than other signal features (Aronsson & Gamberale-Stille, 2008; Kazemi, Gamberale-Stille, Tullberg, & Leimar, 2014). Depending on predator visual system and ambient lighting conditions, some short-wavelength hues may be more effective than others and affect how, for example, an iridescent prey might position itself in relation to ambient light and/or affect warning signal learning.

I explored the effects of variation in brightness and hue of an iridescent warning signal on predator learning rates using naïve domestic chickens (*Gallus gallus domesticus*). First, I hypothesized that an iridescent warning signal is less effective than a non-iridescent version of the same color due to angle-dependent signal variation. This hypothesis would be supported if predators required more attacks on unpalatable prey to learn to associate the color with unpalatability (slower learning rate) and made more

errors when the iridescent unpalatable prey appears different with every presentation as opposed to when the iridescent unpalatable prey is unchanging throughout the learning process. Such a result could be due to variation in hue, intensity, or both, so I tested the effects of variation in both of these parameters separately. Second, I tested the hypothesis that a more intense (brighter) warning signal is more effective than a less intense signal. If a brighter signal is more effective, learning will be faster and fewer errors will be made by predators when the prey's iridescent signal is more intense. Third, as with intensity, iridescent signals may have different colors reflected and, therefore, I examined the ability for predators to learn three different blue hues, all within the range of *B. philenor* reflection seen in nature (440-570 nm). As the role of short-wavelength hue in warning signals has not previously been tested, I have no prediction as to the direction of potential effects of hue. If there is an effect of hue, predators learning one hue will be faster and make fewer errors than predators learning another short-wavelength hue.

Methods

Predators

I purchased Barred Cochin Bantam chicks from commercial hatcheries (Murray McMurray Hatchery, Webster City, IA, USA; Stromberg's Chickens and Game Birds, Pine River, MN, USA; Cackle Hatchery, Lebanon, MO, USA). The birds were shipped the day they hatched and arrived at the facilities at 2-3 days old. Chickens were housed in large white tubs with pine bedding and access to water and brown food pellets. Chickens were individually marked using numbered aluminum leg bands. Chicks were 3-20 days old when tested (mean = 9.6 days).

At the end of the experiment, most of the chickens were adopted for personal use but some males were humanely euthanized. All procedures used in this study were approved by the Arizona State University Institutional Animal Care and Use Committee (Protocol # 14-1349R).

Prey Items

The prey items consisted of an 8 mm x 24 mm piece of cardstock, folded in half to make a 8 mm x 12 mm tent covering a piece of mealworm about 5 mm long. Mealworm pieces were either untreated (and thus palatable) or rendered unpalatable by soaking them for 20 min in a solution of 4% quinine hydrochloride and 2% mustard powder, a concentration previously used for experiments with domestic chicks (Rowe & Guilford 1996; Hauglund, Hagen, & Lampe, 2006). The prey stimuli (consisting of both a tent and mealworm piece) were presented in 55mm diameter plastic petri dishes.

To create the specific prey appearances, I constructed three types of tents to cover the mealworm pieces: (1) black, (2) blue, and (3) pearl. All three were made from white cardstock that was covered with black paper on the lower surface and painted on the upper surface. The black tents were painted with black acrylic paint on the upper surface (Golden Artist Colors Series 1 #1200-3, Mars Black, New Berlin, NY, USA), and a stripe of black enamel paint (Testors Semi-Gloss Black #1139, The Testor Corporation, Rockford, IL, USA) on the lower surface (to control for enamel paint on other tents). The blue tents were painted with blue enamel paint (Testors Glossy Light Blue #1108) and a stripe of black acrylic paint on the back. The pearl tents were painted with interference

blue acrylic paint (Golden Artist Colors Series 7 #5004030-2, Interference Blue Fine) and a stripe of black enamel paint on the back.

Intensity Treatments

To measure the effect of intensity and variation in intensity on warning signal learning, birds were randomly placed into one of three treatment groups (Table 3). In the first, the intensity of the unpalatable stimulus varied in three levels: low, medium, and high (IntVary). In the second, the intensity of the unpalatable stimulus was kept constant at the high level (IntHigh, Fig. 10). In the third, the intensity was again constant but at the low level (IntLow, Fig. 10). For all three treatment groups, the palatable stimuli were black (Fig. 10) and hue was the same at all intensity levels (i.e. all stimuli except for black).

The levels of intensity were chosen to fall within the normal range of variation in the blue iridescent, ventral coloration of *B. philenor*. All prey stimuli were illuminated in the presentation cages by a halogen fiber optic light source (Dolan-Jenner Fiber-Lite High Intensity Illuminator Series 180, Dolan-Jenner Industries, Inc., Boxborough, MA, USA) filtered with a bandpass filter (Edmund Optics BG-39, Edmund Optics Inc., Barrington, NJ, USA) that reduced the amount of red and infrared light reaching the stimulus. I used an Ocean Optics USB2000 spectroradiometer (Dunedin, FL, USA) and collimating lens to measure the radiance of the stimuli and *B. philenor* ventral hindwings under this illumination. I measured the intensity as the photon flux (photons/s/sr) of reflected light at the peak wavelength.

Under these conditions, the photon flux from the *B. philenor* iridescent area ranges from about $3.5x10^{12}$ photons/s/sr at the highest intensity (i.e. angle of peak reflectance) to $1x10^{12}$ photons/s/sr at the lowest intensity. I therefore created three levels of intensity that fell within this naturally occurring variation in the radiance of the *B. philenor* ventral iridescence, all while keeping the hue around 510 nm (Table 4). Chroma does change with the shifts in intensity, but chroma would also change as intensity shifts in nature. I used the light blue tents and manipulated their radiance by altering the intensity of the light reaching the stimulus with neutral density filters. I used no neutral density filter for the high-intensity stimulus, a 0.4 neutral density filter (FRQ-ND04, Newport Corporation, Irvine, CA, USA) for the medium-intensity stimulus, and a 1.0 neutral density filter (FRQ-ND10, Newport Corporation) for the low-intensity stimulus.

Modeling of chicken color vision suggests that these stimuli should be discriminable by the chickens. I used the color discrimination model (1) in Avicol v. 6 (Vorobyev & Osorio, 1998) in Avicol v. 6 (Gomez, 2006) and the visual sensitivities of the chicken (Osorio, Vorobyev, & Jones, 1999) to determine the discriminability of the stimuli. For all possible pairs, the JND (Just Noticeable Differences) were more than 1.0, which indicates that two stimuli are discriminable by the animals with the reference visual system (Vorobyev et al., 1998). For achromatic contrast, between the high intensity and medium intensity stimuli, the discriminability was 31.5 JNDs, 30.5 JNDs between the high intensity and low intensity stimuli, and 12.8 JNDs between the medium intensity and low intensity stimuli. All were also discriminable from the black palatable stimulus (between 1.8 JNDs and 42.5 JNDs), and for all chromatic contrasts as well. The summed response of all four color photoreceptors and the double cones, which are

thought to be used for achromatic discrimination (Osorio, Vorobyev, & Jones, 1999), and based on the visual sensitivity of the domestic chicken (Osorio, Vorobyev, & Jones, 1999) are found in Figure 10.

Hue Treatments

To measure the effect of short-wavelength hue and variation in hue on warning signal learning, birds were randomly placed into one of five treatment groups (Table 3). I had a treatment in which the hue of the unpalatable stimulus varied in three levels (HueVary) and three different treatment groups in which the hue was constant at three levels (HueHigh, HueMed, HueLow; Fig. 11). For these treatment groups, black was the palatable stimulus. The last treatment group was a reverse control, where black was unpalatable and a medium hue stimuli was palatable (Reverse). For all hue stimuli (i.e. all except for black), intensity was kept constant.

I chose hue values that fell within the range of variation of the iridescent blue of *B. philenor*. Prey stimuli were illuminated generally as in the manipulation of intensity, with the fiber-optic illuminator and bandpass filter, and measured using the aforementioned spectroradiometer set-up. I measured hue as the wavelength at peak reflectance. Hue of *B. philenor* ventral hindwing iridescence shifts from approximately 440 nm to 510 nm as incident light-angle varies from 20-60° (Rutowski, Nahm, & Macedonia, 2010). Using color filters and the pearl tent, I created three stimuli whose hue fell within this range, while keeping intensity constant (Table 4). I created the high-hue stimulus (hue = 514 nm) using Roscolux Teal Gel Filters (Roscolux #395, Rosco Laboratories, Stamford, CT, USA), medium-hue stimulus (hue = 489 nm) using Roscolux

Brilliant Blue Gel Filters (Roscolux #69), and the low-hue stimulus (hue = 460 nm) using Roscolux Medium Violet Gel Filters (Roscolux #359). Because the three hue values could potentially stimulate different photoreceptors in the eyes of the predators, I also multiplied the radiance values by the spectral sensitivities of the domestic chicken (Osorio, Vorobyev, & Jones, 1999) to affirm that my three hue stimuli were perceptually similar in quantum catch. The medium-hue stimulus had a higher quantum sum, so I also added a 0.4 ND filter to this stimulus, which resulted in a similar quantum sum to the other stimuli as perceived by a chicken (Table 4; Fig. 11). The chroma value (calculated as the reflectance at each nm 50nm above and below the peak reflectance divided by the total reflectance over the entire 300-700 nm range), had only minor variations (high-hue=0.82, medium-hue=0.72, low-hue=0.76).

As with the intensity stimuli, visual modeling suggests that all of the hue stimuli were discriminable from one another. In chromatic contrast, the high-hue stimulus was discriminable from medium-hue (18.6 JNDs) and low-hue stimuli (45.1 JNDs). The medium-hue stimulus was discriminable from the low hue stimulus (27.11 JNDs) and all were discriminable from the black stimulus (between 54.5 and 57.2 JNDs).

Presentation Chamber

To control for detection distance and angle of approach of the prey stimulus, I presented the stimuli in a presentation chamber (10 cm x 11 cm x 12.5 cm) attached to a clear, open cage for the chicken (50.8 cm x 25.4 cm x 25.4 cm; Fig. 12). The presentation chamber was opaque black to prevent any ambient illumination from reaching the prey stimulus. This also provided a flat, black background with no radiance from 300-700

under the experimental conditions. At the start of a trial, the prey item on the petri dish was placed in the presentation chamber through a door on the back to minimize disturbance of the chick. Events in the chamber were recorded with a video camera through a port on one side of the chamber. Another port on the top of the chamber accommodated a fiber-optic from the light source that illuminated the prey item. This port also had a slot for filters to manipulate the illuminating beam.

Outside the presentation chamber, the room was illuminated by a 100w incandescent bulb (GE Soft White) that was positioned behind the opaque presentation chamber, to prevent the room light from directly illuminating the tent within the presentation chamber. During the trials, the only food available was the mealworm pieces, but I did not deprive the chickens of food before the experiment. Water was available at all times. To reduce stress on the chicks during the learning phase, a companion chick was placed in an adjacent cage and was visible to the experimental chick. Companion chicks had food pellets available.

Pre-training Phase

Following the methods of Pegram and Rutowski (2014), before formal testing I taught the chicks to eat from the petri dish and then access the mealworm piece from underneath a brown tent (i.e. associating the tent with food). This pre-training occurred in 52 cm x 26 cm x 20 cm cages within the environmental chamber in which the chickens were housed. Once they were able to complete this task three times in a row, they were trained to retrieve the mealworm from underneath the tent in the presentation chamber in the testing room. I started with the brown tent and mealworm in the middle of the cage

and then moved it back in two presentations into the presentation chamber with the bar across the bottom of the presentation chamber removed. Then, I moved the bar so that it was halfway covering the stimuli. If the bird did not attack the tent, I removed the bar again. I continued until the bird could complete the task with the bar completely across the opening. If they did, to ensure they were hungry and motivated, birds had to eat a mealworm from under the brown tent in three consecutive presentations before I started the learning phase. Pre-training sometimes occurred on the day before the learning phase depending on how quickly they learned and time available for testing, but this motivation test always occurred immediately before testing.

Learning Phase

The learning phase consisted of 24 presentations to each bird, each 3 minutes in duration based on the methods used in Pegram and Rutowski (2014). I followed a discrimination learning regime that included both palatable (S+) and unpalatable (S-) stimuli. Prey stimuli were presented sequentially with 15 s between each presentation. The order of the stimuli presented was pseudorandomized (Smith, Abramson, & Tobin, 1991; Gerber et al., 1996; Fan & Hansson, 2001; Fernandez et al. 2009; Pegram & Rutowski 2014), in this sequence: S+/S-/S-/S+/S-/S+/S+/S- three times in a row. This sequence has a pattern that is assumed to be difficult for the predators to learn (as opposed to an alternating pattern) and prevents the birds from generalizing their responses from presentation to presentation, as could happen if the same stimulus order was used repeatedly. For the variable treatment groups, to simulate changeable appearance of iridescent coloration (see above) I varied the unpalatable (S-) stimuli,

while keeping the pattern the same. I grouped the S- stimuli into groups of three and then randomly assigned the three different unpalatable stimuli, giving the sequence: $S+/S-_{H}/S-_{M}/S+/S_{L}/S+/S+/S_{M}/S+/S-_{H}/S-_{L}/S+/S-_{L}/S+/S+/S-_{H}/S+/S-_{M}/S-_{L}/S+/S-_{M}/S+/S+/S-_{H}$, where the letter subscripts designate the high, medium, and low stimuli.

Video Analysis

Observers were behind a divider during the learning phase and only appeared to change the stimuli. Hence, I recorded each bird's behavior during the learning phase using two digital video cameras (JVC Everio GZ-HM300; JVC Kenwood Corporation, Wayne, NJ). One had a macro lens attached and was focused on the stimulus through a port in the presentation chamber. The second was outside of the cage with the whole cage in view (but not the inside of the presentation chamber). Videos were analyzed by two different observers to determine if the prey item was attacked by the chick (defined as the beak of the chicken touching the tent), the time at which the chick viewed the stimulus, and the time between when the chicken viewed the stimulus and attacked the prey (i.e. attack latency). The chick was scored to have viewed the stimulus when its head was within 4 cm of the opening of the presentation chamber (so that the chick could see over the bar), and observers used scale bars on the side of the cage to make this determination. The data for a trial was not counted if during the trail (1) the bird was never within 4 cm of the opening of the presentation chamber (i.e. did not see the stimulus), (2) the bird uncovered the mealworm with another body part other than the beak (e.g. chick kicked tent), or (3) the light did not hit the tent before the chick attacks (due to chick blocking light). If there was any discrepancy between the two viewers (e.g. different

determinations on whether or not a prey was attacked, more than 5 s difference in attack latency), the video of that presentation was reviewed by a third viewer. Each video reviewer was blind to the treatment group and the ratings by the other reviewers.

Data and Statistical Analysis

I excluded birds from analysis if they did not view the stimulus (i.e. get within 4 cm of the presentation cage) for more than half of the trials (n=23) and did not include the results of any trials after the bird did not view the stimulus for three trials in a row (n=12 birds, 79 trials).

Following the methods of Pegram and Rutowski (2014), to determine if variation in intensity of the unpalatable stimuli or the intensity of the unpalatable stimuli influenced how readily the chickens learned to discriminate between the prey, I analyzed the decisions made by the chickens in the treatments IntVary, IntHigh, IntLow. I measured the proportion of correct decisions (attacked palatable and did not attack unpalatable prey) made during learning presentations 3-24 using an ANOVA. The dependent variable was the number of correct decisions, independent variable was treatment, and all correct decisions data were normally distributed. I used Tukey post-hoc comparisons to determine significant differences between treatment groups. I also used this analysis to determine the effect of variation in hue and the effect of short-wavelength hue by testing the differences between the HueVary, HueHigh, HueMed and HueLow treatment groups. Additionally, to determine that responses to the stimuli changed over time (i.e. birds were learning), I analyzed the attack latency. The attack latencies did not fit the assumptions of repeated measures analysis, so I used the non-parametric

Friedman's 2-way Analysis of Variance by Ranks to look at changes across all treatment groups. All analyses were performed in SPSS v. 21 (IBM, Somers, NY, USA) with a 0.05 level of significance.

Results

Overall, chicks successfully learned not to attack the unpalatable stimuli, indicated by a more than two-fold increase in the latency to attack unpalatable stimuli (Fig. 13) as the learning trials progressed for all Intensity treatments including the variable group (Friedman's 2-way ANOVA by rank: $\chi^2 = 29.798$, $df = 11$, $p = 0.002$) and the Hue treatments including the variable group (Friedman's: $\chi^2 = 38.280$, $df = 11$, $p < 0.001$).

Intensity Treatments

There was no effect of treatment (ANOVA, Treatment: $F = 3.019$, $df = 2$, $p = 0.057$; Fig. 14) on the number of correct decisions made by chickens in the three intensity treatment groups: IntVary, IntHigh and IntLow (see Table 3 for sample sizes), indicating that variation in intensity during learning or a difference in intensity within the range of the *B. philenor* iridescent blue wings did not affect warning signal learning.

Hue Treatments

Treatment significantly influenced the proportion of correct decisions made by chickens in the five hue treatment groups (ANOVA, Treatment: $F = 3.667$, $df = 4$, $p =$

0.008): HueVary, HueHigh, HueMed, HueLow and Reverse (Table 3; Fig. 14). Birds trained with more violet hues made fewer mistakes because birds in the HueLow treatment group made fewer discrimination mistakes than birds in the HueVary treatment group ($p = 0.040$), HueHigh treatment group ($p = 0.030$), and the Reverse treatment group ($p = 0.009$). Birds in the HueVary group learned as effectively as those in HueMed ($p = 0.079$), HueHigh ($p > 0.999$) and Reverse ($p = 0.984$), indicating that hue variation of the unpalatable stimulus did not seem to affect learning.

Discussion

Variation in warning signals

Iridescent warning signals have the potential to appear different with each approach from a predator, as the hue and intensity change with the angle of view and illumination relative to the surface. I hypothesized that this variation would slow the learning process, at a cost to both predators and prey. I found no effect of variation in either hue or intensity during predator discriminatory learning. This is consistent with previous tests of the effect of signal variation on warning signal learning, in the context of Müllerian mimicry. Two experiments found no effect on the learning abilities of great tits (*Parus major*) when presented with unpalatable stimuli that varied in pattern (Rowe et al., 2004; Ihalainen et al., 2007). Also, variation in long-wavelength color did not affect the learning abilities of great tits (Ham et al., 2006). It is possible that variation due to directional iridescence could function to deter experienced predators recognizing previously learned stimuli (Ihalainen, Lindström, Mappes, & Puolakkainen, 2008), but

my experiment revealed that the levels of variation in appearance due to iridescent signals may not be great enough in nature to influence learning by predators.

Natural selection on warning signals is expected to result in reduced phenotypic variation (Guilford & Dawkins, 1993; Mappes & Alatalo, 1997; Beatty, Beirincky, & Sherratt, 2004; Rowland et al., 2007). However, the potential negative effect of this variation, whether intraspecfic phenotypic variation (e.g. Sandre et al., 2007; Svádová et al., 2009; Crothers & Cummings, 2013; Pegram, Nahm, & Rutowski, 2013) or the intra-individual variation possible with iridescent coloration, on warning signal effectiveness may be reduced by predator generalization. In response to novel stimuli, predators can generalize their learned experience with aposematic prey to other prey with a similar but not necessarily identical signal (e.g. Gamberale & Tullberg, 1996; Ghirlanda & Enquist, 2003; Ruxton, Franks, Balogh, & Leimar, 2008; Sandre, Stevens, & Mappes, 2010). Such generalization has been shown for domestic chicks trained on long-wavelength colors in a warning signal context (e.g. Gamberale-Stille & Tullberg, 1999; Svádová et al., 2009) and could also be possible for short-wavelength signals. This could diminish any potential effects of variation due to directionality on the learning of an iridescent warning signal, and could be the reason for the lack of effect in this experiment.

Effect of differences in intensity

Conspicuous warning signals are often suggested to be more effective at deterring predation (Gittleman & Harvey, 1980; Roper & Wistow, 1986; Lindström et al., 1999; Riipi et al., 2001; Aronsson & Gamberale-Stille, 2009). The intensity, or brightness, of a color can influence conspicuousness, with higher-intensity colors generally standing out

more against the background (Crothers & Cummings, 2013) and having a higher luminance contrast than lower-intensity colors on the same background (Fleishman & Persons, 2001; Uy & Endler, 2004). I hypothesized that more intense (brighter) warning signals (i.e. with higher luminance contrast and conspicuousness) could be one adaptive benefit of iridescent warning signals. However, within the levels of intensity found in an iridescent warning signal under laboratory conditions (i.e. with chicks as predators), I did not find any effect of the intensity on warning signal learning. The birds that leaned to avoid the high-intensity unpalatable stimulus (IntHigh) did not learn to discriminate any better than birds learning the lowest intensity unpalatable stimulus (IntLow). Levels of intensity in nature could be different than what was recorded in the laboratory using a spectroradiometer and point source of light, and I encourage further investigation into the effects of natural light on iridescent warning signals.

While measures of the effect of conspicuousness on predator learning are numerous (see above), to my knowledge there is only one direct study of luminance contrast and predator learning (Prudic et al., 2007). The predator in that study, the Chinese mantid (*Tenodera aridifolia sinensis*), is not thought to have color vision and so iridescent colors could be more effective when viewed by predators with monochromatic vision. Also, the luminance contrast generated by a brighter signal could be more important for detection rather than learning, as domestic chicks have been shown to learn a color more accurately and rely on visual contrast for prey detection (Osorio, Jones, & Vorobyev, 1999). Since the hue values of my intensity stimuli were constant, this could have explained the lack of differences in learning between our intensity groups.

Effect of differences in short-wavelength hue

The feature of iridescent colors that sets them apart from other structural colors is that they can shift in the hue of the reflectance as the angle of illumination and angle of view change (Land, 1972; Vukusic et al., 2001; Doucet & Meadows, 2009). This experiment examined how relatively minor differences (about 30 nm) in short-wavelength hue would affect how well colors were learned by predators because iridescent colors can change in the hue reflected. Previous studies have shown that the hue of long-wavelength warning colors can influence warning signal effectiveness (Exnerová et al., 2006; Svádova et al., 2009; Lindstedt et al., 2011). I found here too that short-wavelength hue, controlled for intensity, can influence the effectiveness of a warning signal. Birds that received the low-hue unpalatable stimulus learned better than birds that were asked to discriminate the medium-hue or high-hue unpalatable stimulus from the black palatable stimulus. The low-hue stimulus did have a slightly higher quantum sum, but the difference is much less than the differences between the intensity stimuli which did not affect learning.

The greater effectiveness of blue-violet color could have several implications for the effectiveness of iridescent warning signals and even diffusely reflecting static signals. First, iridescent warning signals may be more effective when approached from some angles than others. The ventral surface reflectance of *B. philenor* will have a peak wavelength around 460 nm (the hue value of the low hue stimulus) when the angle of incident illumination is approximately 45° (angle between viewer and light source approximately 90°; Rutowski et al., 2010). So, for example, if the butterfly is perched and the sun is approximately 45° above the horizon, the signal may be most effective if

predators approach from below the butterfly. However, caution should be taken in directly interpreting these values. Illumination in nature (light coming from many directions in addition to the sun) may be very different from laboratory point-sources of light, the iridescent signal may even be effective when the sun has set (Pegram, Han, & Rutowski, 2012), or prey may move in response to predators. Second, this could influence one potential effect of iridescent coloration that I did not test: the ability for iridescently colored animals to become camouflaged when viewed from a distance or at certain angles (e.g. Pérez i de Lanuza & Font, 2014). The blue-green hue could be more camouflaged and less distinctive from surrounding vegetation. Therefore, iridescent animals like *B. philenor* may have the potential to display an effective warning signal from some angles and be camouflaged with a less effective warning signal from others. Third, when animals display non-iridescent short-wavelength warning signals, the signals could be more effective if in the blue-violet range, as the prey colors for these treatments were static and could also represent diffusely-reflecting prey colors.

Conclusions

Before the study presented here, the costs and benefits of displaying an iridescent warning signal had not been investigated. Here I tested the ability of predators (domestic chicks) to learn a warning signal that varies with each presentation, in both hue and intensity, as is likely to happen with an iridescent warning signal in nature. I found no support for this hypothesis. The intra-individual variation caused by an iridescent signal, within the range of a natural iridescent warning signal, did not slow the learning of the predator and was likely mitigated by predator generalization. I also hypothesized that,

because iridescent signals have the potential to be much brighter than diffusely reflecting signals, this could be an adaptive advantage of displaying the signal. I found that wing color intensity did not affect warning signal learning by chicks. Third, I hypothesized that differences in short-wavelength hue, within the ranges seen in iridescent colors, may influence warning signal learning. I found that shorter wavelength (blue-violet) unpalatable stimuli were more effectively learned than blue-green stimuli. This is interesting because angle-dependent hue sets iridescent coloration apart from other colors and has implications for both iridescent signals and static warning signals. I conclude that, in the properties of iridescence tested here, there is not likely to be a cost to displaying an iridescent warning signal compared to a diffusely-reflecting signal in terms of predator response. However, there is a potential benefit in interactions with predators, in that animals displaying a short-wavelength iridescent warning color may be able to display a hue that is more effective at deterring predators.

In addition to the potential effect of angle- or distance-dependent camouflage, I also did not test the potential for the iridescent patch to combine with prey movement and create a flashing, attention-grabbing signal. This could also be another adaptive advantage of displaying an iridescent warning signal because the flashing could startle predators or make the signal easier to learn because it is now more distinguishable from a static signal (Sargent, 1990; Hinton, 1973; DeCock & Matthysen, 1999; Long et al., 2012), and *B. philenor* sometimes open and close their wings in response to disturbance when perched (M. Shillingburg and R. Rutowski, unpublished data). These potential effects should be investigated further in future studies to understand all of the adaptive advantages of displaying an iridescent warning signal.

I have shown through previous research that the iridescent blue coloration of *B. philenor* can be recognized by experienced predators (Pegram, Lillo, & Rutowski, 2013) and can be learned by avian predators (Pegram & Rutowski, 2014), but may not be effective at deterring naïve predators (Pegram & Rutowski, 2014) or predators with a range of experiences in the field (Pegram, Han, & Rutowski, submitted ms). I also investigated the role of iridescence in the field (Pegram, Han, & Rutowski, submitted ms) and found no effect, but my models had one hue value, above 500 nm. Here, I examined if the iridescent nature of the signal affected the ability for predators to learn and found only an effect of hue. This is interesting because an angular dependence of hue sets iridescent coloration apart from other colors and has implications for both iridescent signals and static warning signals. I also, importantly, found no effect of signal intensity, which is not consistent with the one other study of warning signal brightness and intensity (Prudic et al., 2007). This indicates that warning signal intensity may not be as important for predators with color vision. There is a great diversity in the components that make up warning signals, including iridescence, hue and intensity, and the implications of these different forms on warning signal effectiveness should continue to be investigated.

Table 3. Intensity and Hue Treatment Groups. Each treatment had either had a variable or constant unpalatable stimulus. In groups in which intensity is variable, hue was held constant. In groups in which hue was variable, brightness was held constant.

treatment	unpalatable stimulus/stimuli (S-)	palatable stimulus (S+)	sample size
INTENSITY			
IntVary	high-intensity medium-intensity low-intensity	black	19
IntHigh	high-intensity	black	19
IntLow	low-intensity	black	21
HUE			
HueVary	high-hue medium-hue low-hue	black	19
HueLow	low-hue	black	19
HueMed	medium-hue	black	18
HueHigh	high-hue	black	17
Reverse	black	medium hue	19

Table 4. Descriptions of stimuli presented to the chicks. For details of filters, calculations of hue and brightness, and paint on the tents, see text. All stimuli had IR Filter applied.

stimulus	Tent	color filter	neutral density filter	hue (nm)	intensity (photons /s/sr)	quantum sum (photons/cm^2)
low intensity	light blue	none	1.0	511	4.5×10^{11}	4.2×10^{11}
medium intensity	light blue	none	0.4	510	1.9×10^{12}	1.2×10^{12}
high intensity	light blue	none	none	509	4.6×10^{12}	2.8×10^{12}
low hue	Pearl	Rosco #359	none	460	1.8×10^{12}	1.08×10^{12}
medium hue	Pearl	Rosco #69	0.4	489	1.6×10^{12}	8.8×10^{11}
high hue	Pearl	Rosco #395	none	514	2.3×10^{12}	8.8×10^{11}
black	Black	none	1.0	452	4.4×10^{11}	5.2×10^{10}

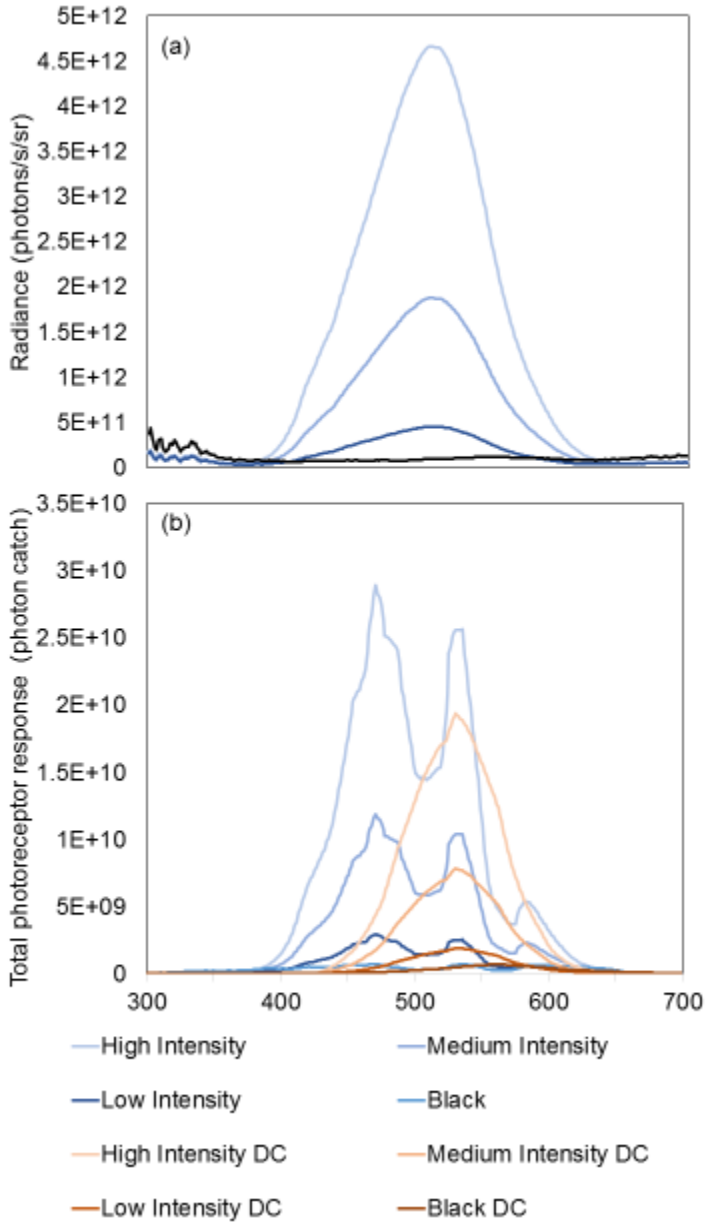

Figure 10. (a) Radiance spectra and (b) Photoceptor response for the intensity stimuli and black stimulus. Photoreceptor response is shown for both the four color photoreceptors and double cone (DC). The background is not shown because there was no radiance under the expermental conditions from 300-700 nm. I measured radiance in the experimental set-up and calculated quantum catch using the visual sensitivities of domestic chickens. See text in Methods section for more detail.

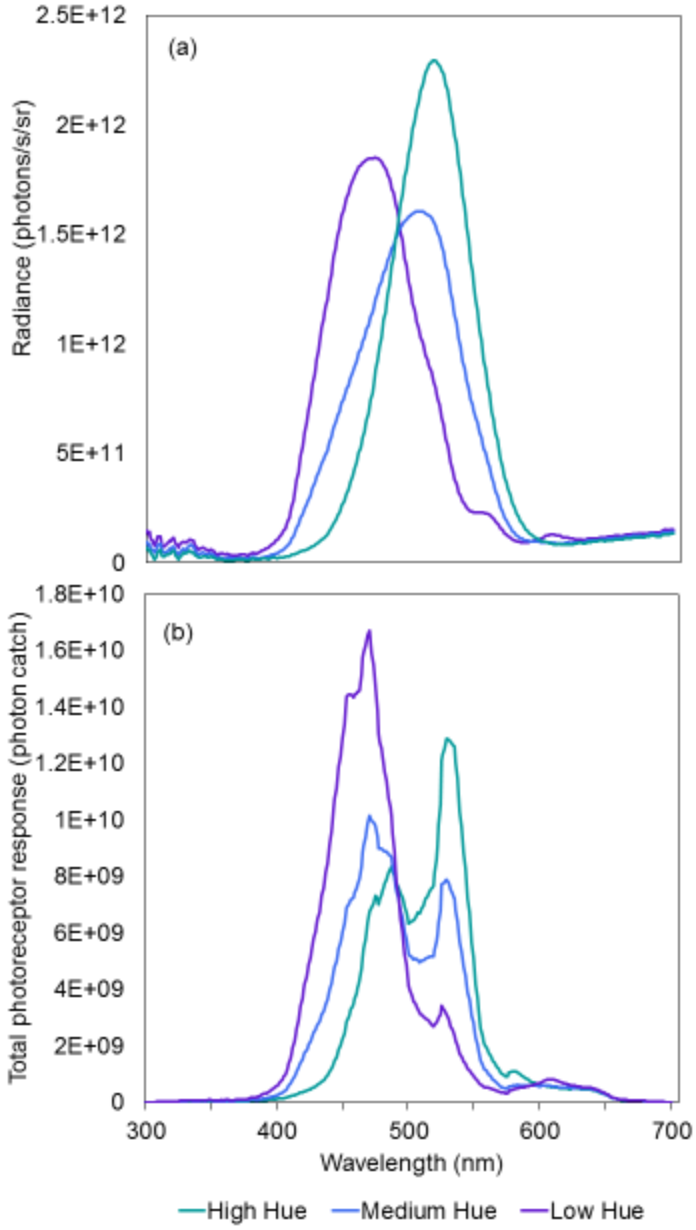

Figure 11. (a) Radiance spectra and (b) Photoreceptor response for the hue stimuli. I measured radiance in the experimental set-up and calculated quantum catch using the visual sensitivities of domestic chickens. See text in Methods section for more detail.

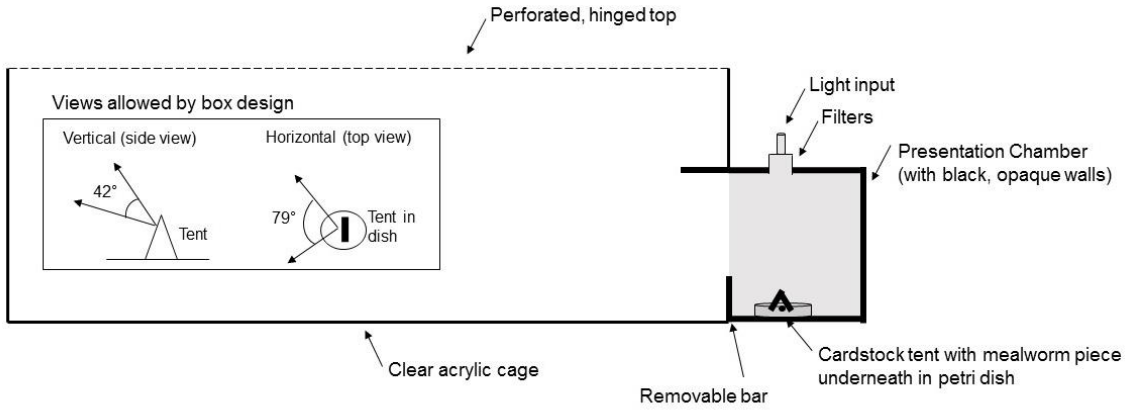

Figure 12. Diagram of presentation chamber and cage in cross section. Chickens were placed in the clear cage and the prey stimuli were placed in the opaque presentation chamber from a door on the back. The removable bar was used to train birds to find the stimuli in the presentation chamber, but was always in place during testing so that detection distance and angle of viewing was held constant.

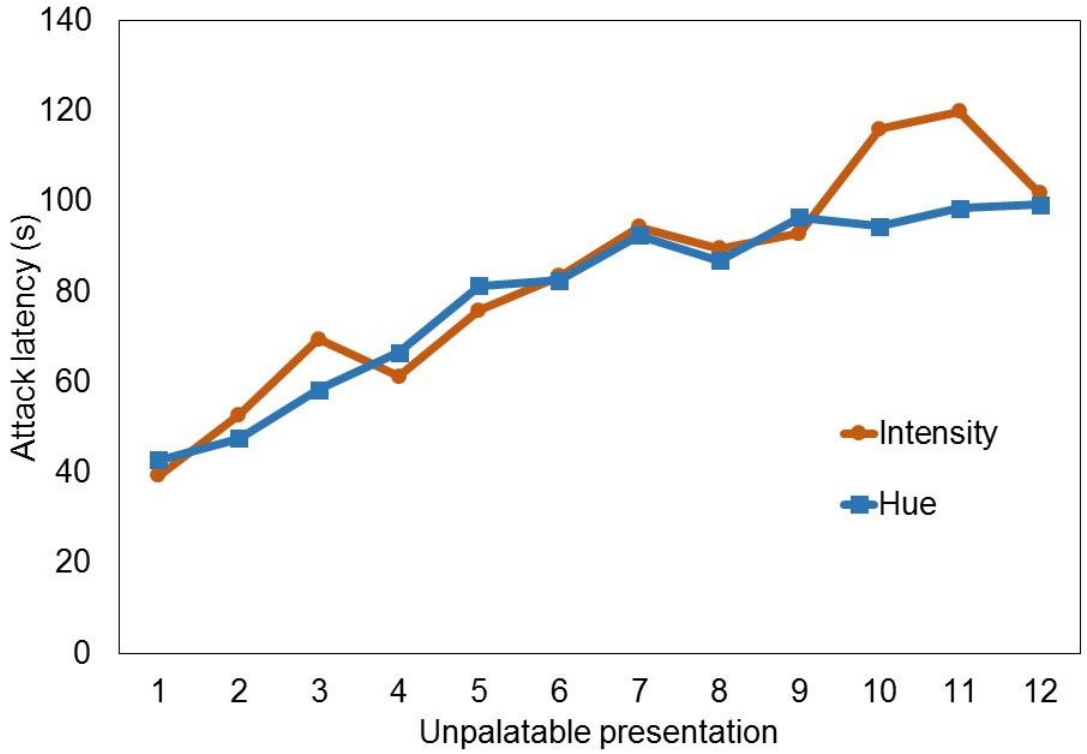

Figure 13. Average attack latencies for the intensity and hue treatments. Attack latencies varied amongst the unpalatable presentations, indicating that birds were learning as the trials progressed.

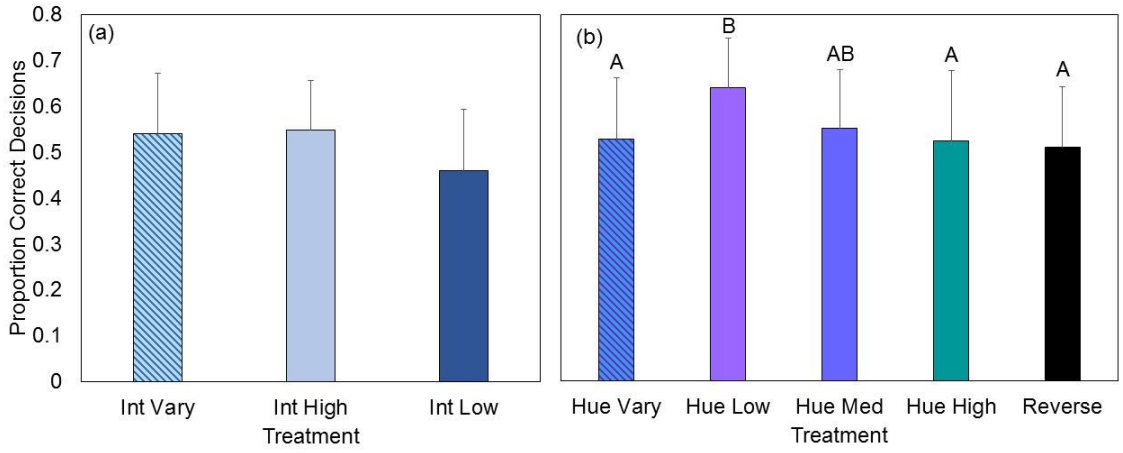

Figure 14. Proportion of correct decisions made by birds during the learning phase. I defined correct decisions as attacking the palatable prey and not attacking the unpalatable prey. (a) Correct decisions by birds in the Intensity treatment group were not significantly different from one another. (b) Correct decisions by birds in the hue treatment groups were influenced by treatment. Shared letters indicate that treatments are not significantly different.

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APPENDIX A

OVERNIGHT PERCHING AGGREGATIONS OF THE APOSEMATIC PIPEVINE SWALLOWTAIL (*BATTUS PHILENOR*: LEPIDOPTERA: PAPILIONIDAE): IMPLICATIONS FOR PREDATION RISK AND WARNING SIGNAL USE

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Overnight perching aggregations of the aposematic Pipevine Swallowtail (*Battus philenor*: Lepidoptera: Papilionidae): implications for predation risk and warning signal use

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Abstract. Aposematic butterflies, those that are unpalatable and warningly colored, may aggregate during overnight perching to reduce the risk of predation. The conditions under which they aggregate and the postures assumed by perching butterflies may indicate how aggregations are a useful defense against predators, including the use of the warning signal. Additionally, studying these aggregations allows for a better understanding of the conditions under which their warning signal may be used. We investigated the overnight perching behavior of the aposematic Pipevine Swallowtail (*Battus philenor*) in both the field and in an enclosure. We found that the butterflies begin perching very close to sunset, when their blue iridescent warning coloration may still be effective, and the aggregations consist of between two and 21 individuals, which may accelerate warning signal learning by naïve predators. In both the field and enclosure, aggregated butterflies perched with the plane of their wings surfaces in parallel which suggests they perch in ways that increase the size of the warning signal. Additionally, *B. philenor* individuals perch in conspicuous locations which may facilitate warning signal detection, learning, and recognition. Our investigations of *B. philenor* aggregations lend support to the hypothesis that aposematic butterflies aggregate to increase the effectiveness of the warning signal against visually hunting predators.

Keywords: Warning coloration, aggregations, perching, *Battus philenor*

Introduction

Aggregations of aposematic animals, such as the overwintering and overnight aggregations of Monarch and *Heliconius* butterflies, are thought to provide enhanced protection against visually hunting predators (e.g. Turner, 1975; Sillén-Tullberg & Leimar, 1988; Gamberale & Tullberg, 1998). When aposematic butterflies aggregate, individual risk of predator attack can decrease through several mechanisms (Mappes & Alatalo, 1997; Gamberale & Tullberg, 1998; Lindström *et al.*, 1999). First, regardless of whether a predator's association of unpalatability with warning coloration is learned or innate, aggregations can present a larger and, so, more effective warning signal (Gamberale & Tullberg, 1996a,b; Gamberale-Stille & Tullberg, 1999; Forsman & Merilaita, 1999). Second, aggregations may facilitate learning by naïve predators by 1) providing the opportunity for predators to see warningly colored individuals during or immediately following perception of distastefulness (Gagliardo & Guilford, 1993; Alatalo & Mappes, 1996), or 2) allowing predators to sample more prey in each encounter (Sillén-Tullberg & Leimar, 1988; Riipi *et al.*, 2001). By accelerating the learning process, fewer butterflies will be attacked and the individual risk for butterflies in the aggregation is reduced ("dilution effect"; e.g Bertram, 1978; Foster & Treherne, 1981). All these mechanisms rely on the predators seeing the butterflies and therefore may not be in force after dark for overnight aggregations.

Aggregations may also reduce the risk of attack by predators without the influence of the warning coloration. A naïve predator that attacks a group of aposematic butterflies may leave the aggregation after determining that prey are unpalatable (Alatalo & Mappes, 1996; Riipi *et al.*, 2001) and the risk of an individual being attacked is again reduced through the dilution effect. Predators will also be less likely

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to encounter aggregated prey than solitary prey scattered throughout an area, because a finite amount of prey aggregated into larger groups will form fewer groups, decreasing the chance of encountering prey (Turner & Pitcher, 1986; Ioannou *et al.*, 2011).

With these potential benefits in mind and in order to better understand the conditions under which warning signals are used, we made observations on the dynamics and structure of overnight aggregations in the Pipevine Swallowtail butterfly, *Battus philenor* (Linnaeus, 1771). We suspected that *B. philenor* adults may aggregate because of their unpalatability, anecdotal reports of overnight aggregations (Scott, 1992; J. Fordyce and L. Gilbert, pers. comm.), and reports of feeding aggregations (Otis *et al.*, 2006). In March 2009, during a search for perching adult *B. philenor*, we observed overnight aggregations in the Mazatzal Mountains of Arizona, USA, and used this as an opportunity for further study of *B. philenor* overnight perching over two months. However, field observations were limited by access to the butterflies and so we expanded our observations and understanding of the aggregations by studying *B. philenor* perching behavior in an enclosure.

B. philenor is distasteful to predators due to the sequestration of aristolochic acids by the larvae (Sime *et al.*, 2000; Fordyce *et al.*, 2005). The ventral hindwing surface functions as a warning signal (Brower, 1958; Codella & Lederhouse, 1990) and displays both iridescent blue and orange spots (Fig. 1; Rutowski *et al.*, 2010). Both the iridescent blue and orange spots are recognized by predators as a warning signal and the most common predators of *B. philenor* in Arizona are insectivorous birds (Pegram *et al.*, unpublished observations).

We aimed to better understand how aggregations may reduce the risk of predation as well as the environmental conditions under which the warning coloration may be used by pursuing answers to four questions. First, do aggregations form and disband at times of day when visually hunting predators are active and when the warning signal is effective? If so, we expect that butterflies would aggregate before sunset or when ambient light is still available and disband after sunrise. Second, do aggregations form in locations that facilitate learning and recognition? To facilitate learning and recognition, we expect butterflies to perch in locations that make them conspicuous. Third, do butterflies position themselves in a way that increases the size of the warning signal? If so, we predict that the butterflies will orient themselves so that more wing surfaces are visible to an approaching predator. Finally, does the size of aggregations indicate that the butterflies aggregate to facilitate warning signal learning? If this is the case, we expect that there are more butterflies in aggregations than the number of butterflies required to be sampled by the predator for the predator to learn. Answers to these questions will help us to determine why these animals aggregate and how aggregations may influence the effectiveness of the warning signal.

Figure 1. A *B. philenor* perched after sunset and illuminated with only indirect solar radiation. Even without the solar orb present in the sky, the blue iridescence of the ventral hindwing is visible.

Materials and methods

Field observations

We observed overnight aggregations of *B. philenor* from March to May 2009 at the confluence of Mesquite Wash and Sycamore Creek in the Mazatzal Mountains of Arizona, USA (N 33°43.784', W 111°30.997'; Fig. 2). Here, the riparian vegetation includes Sycamore (*Platanus wrightii*), Willow (*Salix* spp.), and Cottonwood (*Populus fremontii*) trees. The streamside area in which we made our observations was approximately 9000 m^2. On observation days, we arrived at the field site before sunrise or sunset and visually scanned trees with binoculars until we spotted *B. philenor* individuals.

Because some of the benefits of aggregation, such as increased signal size, can be realized with only two individuals, we considered two or more *B. philenor* butterflies perched together to be an aggregation. For each aggregation found, we determined how many individuals were clustered together (within a cubic

Figure 2. Riparian forest at the confluence of Sycamore Creek and Mesquite Wash in Arizona where aggregations of *B. philenor* were observed.

area of about 2 m on a side) and recorded the time at which the aggregation was first observed. On two mornings, we also recorded the time at which each individual left the aggregation. We obtained the sunrise or sunset time for each observation day from the NOAA calculator (http://www.srrb.noaa.gov/highlights/sunrise/sunrise.html), and compared all observed times to sunrise (for morning observations) or sunset (for evening observations).

We estimated height from the ground to the lowest butterfly for each aggregation using a known height as a reference. For 20 aggregations, we observed the orientation of each butterfly with binoculars and described it as the compass bearing of the azimuth of the line going from the wing tips to the body of the butterfly. This was always done before we observed any movement in the morning and after no more movement was observed in the evening.

To better understand how aggregations form and the activity around the time in which they perch, on four evenings we also counted individuals flying amongst the trees (at least 3 m above ground) every 15 minutes from a distant observation point that allowed us to observe the whole stand of trees. We stopped recording around sunset to observe the aggregations from a closer vantage point and take the above measures. Throughout the study, we also took notes on any interactions we observed among the butterflies.

Enclosure study

Due to limitations of the field study, in the summer of 2011 we also investigated the perching aggregations of *B. philenor* in a 10 m wide x 24 m long x 4.5 m high enclosure, the Maxine and Jonathan Marshall Butterfly Pavilion at the Desert Botanical Garden in Phoenix, AZ, USA. This enclosure is covered with 65% shade cloth and contains a large variety of vegetation and nectar sources, including Mexican Orchid trees (*Bauhinia mexicana*) and *Lantana* spp., but no hostplant. We populated the pavilion with lab-raised *B. philenor* that were either collected as eggs or larvae from the field site described above, or as eggs from females that mated in this pavilion and oviposited in the lab. Animals were raised to adulthood in an environmental chamber as described in Rutowski *et al.* (2010). We released individually marked *B. philenor* adults into the pavilion within 0-4 days of eclosion, and maintained a population of 6-20 individuals in the enclosure throughout the study. We always released butterflies at least two hours before sunset. The butterflies were an unstructured mix of males and females, and we recorded the sex of each before release.

To facilitate the assessment of the distribution of perched butterflies within the pavilion, we created a map of the interior of the enclosure, plotted on it the

location of perched individuals, and noted whether they perched in aggregations or individually. As with the field study, we defined an aggregation as two or more individuals perched within a cubic area of approximately 2 m on a side.

We measured the height of each perched individual with a tape measure. Also, as in the field we described the orientation of perched butterflies using the compass bearing of the azimuth of the line going from the wing tips to the body of the butterfly. These measurements in the enclosure are likely to be more accurate than those made in the field because we were able to more closely observe the butterflies.

We also focused on the formation and disbanding of aggregations. On five evenings, we plotted the location and recorded the height of every perched individual every five minutes, starting a half hour before sunset and ending a half hour after sunset. To understand how the aggregations disband, on five mornings, we recorded when each individual left the perch. We started this at sunrise and ended one hour after sunrise. In addition, we made qualitative observations on flight behavior and interactions among individuals forming aggregations at night or disbanding in the morning.

Statistical analysis

To determine whether perching individuals in the field and enclosure were oriented in a haphazard fashion we used circular statistics (Batschelet, 1981) using Oriana v.3 (Kovach Computing Services, Anglesey, Wales). We calculated: the mean angle; the Rayleigh statistic, which determines if the orientations are significantly different from random orientations; and the *V* test, which tests whether the butterflies were significantly clustered around specific compass bearings, with 180° and 0° as the given angles. We chose 180° and 0° as the given angles because we hypothesized that the butterflies may be perching with their wing surfaces perpendicular to the rays of the rising and setting sun. For the enclosure, we first sorted the orientation observations into those that were taken from aggregations and those that were taken from butterflies perched individually. We then calculated the mean orientation angle for each individual and ran the tests described above on these mean angles to control for multiple measurements on the same individual.

We determined whether height and propensity to aggregate were consistent among individuals using repeatability calculations. We calculated the repeatability (or r-) and p-values (with a significance of 0.05) using one-way ANOVAs and the calculations described in Lessells and Boag (1987). To determine whether individuals were consistent from day to day in their orientation, we used second-order circular statistics on the mean vector lengths, because linear statistics are not appropriate for angular measurements (Batschelet, 1981). We calculated the mean vector length for each individual using Oriana v. 3 (Kovach Computing Services, Anglesey, Wales) and then compared the distribution to the circular uniform distribution using the Kolomogorov's one-sample test (Batschelet, 1981).

The number of males and females in the enclosure on any given day was not equal. Therefore, to determine whether males and females perch in aggregations at the same rate, we used a t-test to compare the observed number of males in each aggregation to an expected number of males in each aggregation based on the sex ratio in the enclosure and the total number in the aggregation.

Results

Field observations

We recorded data on 27 natural aggregations from 12 March – 5 May 2009 during 13 field visits (six in the early morning and seven around sunset). Nine of the aggregations were found at dusk and 18 were found at dawn. All aggregations were either found at the top or the outer edges of deciduous trees (Fig. 3). Heights ranged from 5.4 m – 10.6 m (mean = 7.9 m). Individuals started arriving at the site and flying around about 1 hour before sunset, and started to settle right around sunset. Counts of individuals in each aggregation ranged from 2-21 (mean = 5.8). Additionally, we found 10 individuals perched alone (10.5% of all butterflies observed), but our efforts in the field were focused on finding aggregations and so could easily have missed many solitary perchers. By 5 May, the trees had leafed out to an extent that made it difficult to scan for perched butterflies. We also found aggregations during future trips to the field site during other parts of the year when *B. philenor* was active (approximately March - October) suggesting that aggregations are not seasonal.

Aggregated butterflies measured in the field (n=85), were significantly oriented with the mean at 215° (Rayleigh: z=13.9, p<0.001; Fig. 4). We also did a V-test, which measures whether the observed orientations are clustered around a given angle. The *V*-test for 180° was significant (p<0.001) while the *V*-test for 0° was not (p>0.999), which means that the orientations of the butterflies were significantly clustered around 180°, that is their wings tended to point to the north.

Figure 3. An aggregation of six *B. philenor* high in a tree in the morning just before the animals disbanded. Note that three of the animals are dorsal basking.

Enclosure study

In June and July of 2011, we observed the overnight perching behavior of *B. philenor* in 38 visits to the enclosure on 33 different days, on some days visiting both in the morning and the evening. Our observations in the enclosure, as in the field, revealed individuals perching within aggregations as well as individuals perching alone (not within about 2 m of another butterfly). The mean percentage of individuals aggregating was 43% over all nights with a maximum of 65% on 21 July 11 and a minimum of 0% on 27 June 11 when only six individuals were present in the enclosure. The mean size of aggregations was 2.8, ranging from two to six individuals, and aggregations were composed of both males and females. The sex ratio of these aggregations was not biased toward either sex (t-test, $p = 0.464$).

Butterflies perched in aggregations (n = 57 individuals, 144 observations) were significantly oriented (Rayleigh test $z = 8.398$, $p < 0.001$) with a mean angle of 227.14° (Fig. 4). Also, as in the field, the orientations of aggregated individuals were significantly clustered around 180° (*V*-test, $v = 0.261$, $p = 0.003$) but not 0° ($v = -0.261$, $p = 0.997$), that is, with their wings pointed toward the north. Interestingly, butterflies perched individually (not in aggregations; n = 57 individuals, 173 observations) were not significantly oriented overall (Rayleigh test $z = 0.402$, $p = 0.669$; Fig. 4). In the enclosure, butterflies perched at heights ranging from 0.05-3.9 m (mean = 2.02 m), much lower than in the field and no doubt constrained by the height of the pavilion's roof. As in the field, aggregations were found at the top or outer edges of trees and plants (Fig. 5), but were also found on the shade cloth and other structures within the pavilion.

In the enclosure, we could identify individuals and therefore determine repeatability or consistency in perching behavior among individuals. We found height ($r = 0.967$, $p < 0.001$) and whether they perched in aggregations or individually ($r = 0.814$, $p < 0.001$) to be consistent among individuals. However, orientation angle was not consistent among individuals (Kolmogrov's one-sample test; T = 0.717, $p = 0.762$).

We noticed that during their search flights in the evenings, individuals often landed on multiple perching spots before settling on a final perch between a half hour before sunset and a few minutes after sunset. Movements varied from slightly shifting their orientations to leaving for a new perching location up to several meters away. The mean number of times an individual landed on a perch before their final location was 3.1 (min = 0, max = 6). We also noticed that individuals already perching within an aggregation sometimes left after another butterfly arrived and flew around the perch, interacting with those already perched. Our observations ended about 45 minutes after sunset and, on 13 nights, we made observations the following mornings. On two occasions out of the 13, we found that the individual moved overnight and, on eight occasions, we were not able to find the individual anywhere in the pavilion and suspected they were attacked overnight. All of these individuals were perched alone. Predation may have been due to lizards (*Sceloporus* spp.) or roof rats (*Rattus rattus*), which were both spotted in the enclosure.

In the mornings, individuals opened their wings to bask, made small movements, or took off from their perches starting from a few minutes to one hour after sunrise. Most individuals moved to a different perching location after leaving their original night perch. We counted the number of perches until an individual started flying continuously or began feeding. The mean number of perches that individuals made after leaving their night perch was 1.4 (min = 0, max = 4). Aggregations disbanded one individual at a time, similar to how they formed. The shortest time from the first individual leaving to the last departure was 17 minutes for an aggregation of two individuals and the longest time from the first individual leaving to the last departure was 48 minutes for an aggregation of four individuals. However, we never observed any interactions between individuals within an aggregation during disbanding.

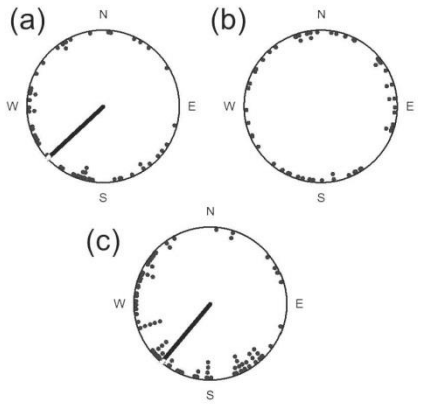

Figure 4. Orientation of a) aggregated butterflies in the enclosure, b) butterflies perched individually in the enclosure, and c) aggregated butterflies in the field. For the field observations, each dot represents one butterfly orientation, measured as the azimuth of the line going from the wing tips to the body. For the enclosure observations, these are averaged for each individual, so that each dot represents an individual. Butterflies in aggregations were significantly oriented (mean vector = 227° in (a) and 215° in (c)). The azimuths of the sunsets during measurement periods ranged from 270° to 299° and from 62° to 90° for sunrise.

Discussion

In this study we set out to answer four questions about *B. philenor* aggregations and how they might influence predation rates: when and where the butterflies aggregate, the way the butterflies position themselves, and the size of the aggregations.

Do aggregations form and disband at times of day when visually hunting predators are active and when the warning signal is effective?

Insectivorous birds, the most common predators of *B. philenor* in Arizona, are active throughout the day, but may hunt more intensely around sunset or sunrise (e.g. Morton, 1967; Hutto, 1981). We found that *B. philenor* started perching around sunset but left their perches well after sunrise, which may indicate they are perching when their predators are most active.

The timing of the formation and disbanding of aggregations is likely to influence the effectiveness of warning coloration. The transmission and perception of color signals are influenced by light environment (Endler, 1990; 1993). Under low light conditions, color signals become difficult to discriminate by birds (Cassey, 2009). Therefore, whether or not the solar orb is still present in the sky influences if and how predators learn or recognize the warning signal. The formation of aggregations around sunset or after the sun had set could limit the effectiveness of the visual signal. However, during field observations, the iridescent blue of the ventral hindwing was visible to the human eye even for some time after sunset but while there was still skylight (Fig. 1). This may be an advantage of displaying an iridescent warning signal. We also found that aggregations disbanded well after sunrise, so the warning coloration may be more effective at deterring insectivorous birds in the morning than in the evening.

Additionally, aggregating individuals may benefit from reduced predation through dilution or fewer predator encounters, as discussed earlier. Therefore, even though the aggregations are forming after sunset and diffusely reflecting warning colors may not be effective, iridescent warning colors may still be effective and aggregations may still reduce the risk of predation.

Do aggregations form in locations that facilitate learning and recognition?

A more conspicuous and larger signal may facilitate predator learning and recognition of a warning signal (Guilford, 1986; Gamberale & Tullberg, 1996b; Gamberale-Stille & Tullberg, 1999; Forsman & Merilaita, 1999; Gamberale-Stille, 2001; Prudic *et al.*, 2007). We found that *B. philenor* aggregations in the field average 5.8 individuals and form very high in trees. The area in which our observations took place is surrounded by mountains, and the sunshine clearly hits the tops of the trees first. This may allow for both the diffusely reflecting and iridescent warning colors to be effective earlier, as light becomes available to reflect off of the wings. Higher perching locations may also discourage predation by nocturnal, ground dwelling animals that may not be visually oriented and therefore not deterred by the warning coloration. In the enclosure, the average height of perching was only about 2 m off the ground but was likely constrained by the fact that the maximum height in the enclosure is only 4.5 m. We also found that aggregations were often formed on the outer edges of trees, which may also increase conspicuousness and, thus, warning signal effectiveness.

Despite perching in locations that may facilitate learning and recognition of warning signals through increased conspicuousness and signal size, microclimate could also be a factor driving *B. philenor* perch

Figure 5. An aggregation of four *B. philenor* in the enclosure taken in the evening.

selection and aggregations. Other butterfly species (e.g. *Danaus plexippus*) choose their perching location based on temperature and protection from wind and precipitation (Brower *et al.*, 2008; Salcedo, 2010). In *B. philenor*, perching high in trees may allow the butterflies to start basking earlier and therefore leave their perch, where they are most susceptible to predation (Rawlins & Lederhouse, 1978; Lederhouse *et al.*, 1987), earlier. An indication that *B. philenor* individuals are seeking specific conditions for perching is found in their evening activity. In the field and enclosure, we regularly observed interactions between perched and patrolling individuals in the trees during the evening. Individuals often settle on several perches before selecting their overnight perch. In the enclosure, most individuals landed on at least one perch before settling on a perch overnight. In the mornings, there is less interaction, but the butterflies still land on several perches before becoming fully active.

Do butterflies position themselves in a way that increases the size of the warning signal?

Butterfly orientation can have several implications for signaling behavior because the iridescent color on the wings will only be visible from certain angles and predators approaching on a path in the plane of the wing surface will not see any of the wing colors. We found that butterflies both within and among aggregations were similar in their body orientation in both the field and enclosure, but that non-aggregated butterflies were not. This suggests that butterflies may aggregate and position themselves to increase the size and, therefore, effectiveness of the warning signal. If all of the butterflies in an aggregation are facing in the same direction, the warning signal they display is much larger to any potential predator approaching from a direction perpendicular to the plane of the wings and, in general, a larger warning signal is a more effective signal (Gamberale & Tullberg, 1996b; Gamberale-Stille & Tullberg, 1999; Forsman & Merilaita, 1999). An alternative hypothesis is that *B. philenor* butterflies could also be orienting themselves in order to increase the sun rays hitting the wings for warmth, but then we would expect to find that all perched butterflies significantly orient themselves to a direction perpendicular to the sun. This was not the case as butterflies perched individually were not significantly oriented.

Does the size of aggregations indicate that the butterflies aggregate to facilitate warning signal learning?

If a naïve predator is sampling prey from the aggregation and learning to avoid the animals based on the warning coloration, then the number of individual butterflies in the aggregation should increase with the number of prey the predator needs to sample to learn to avoid that prey item (Sillén-Tullberg & Leimar, 1988). For *B. philenor*, one experiment demonstrated that it takes an average of 2.67 butterflies for Blue Jays (*Cyanocitta cristata*) to learn not to attack this species using the ventral surface in a captive setting (Codella & Lederhouse, 1990). Considering the mean size of the observed aggregations was 5.8 for the field and 2.8 in the enclosure, predator sampling during learning could have influenced the size of *B. philenor* aggregations.

Conclusions

Our study provides information on the environmental conditions in which the warning signal of *B. philenor* is likely to mediate interactions between them and their predators and the ways in which by forming aggregations they may increase the effectiveness of their warning signal. We now know that *B. philenor* forms aggregations, selects postures within aggregations that may maximize the size of the warning signal, forms groups of a size that may facilitate predator learning, perches in locations that may facilitate learning and recognition, and forms aggregations at times during the day when iridescent warning coloration may be effective. Our observations revealed that the iridescence is still visible when the solar orb is not present in the sky, giving us a potential reason for why an iridescent warning signal might evolve. Our observation that the only

animals that disappeared from the pavilion overnight were individuals that were perched individually may also support the idea that *B. philenor* aggregations reduce predation risk.

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APPENDIX B

WARNING COLOR CHANGES IN RESPONSE TO FOOD DEPRIVATION IN THE PIPEVINE SWALLOWTAIL BUTTERFLY, *BATTUS PHILENOR*

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Warning color changes in response to food deprivation in the pipevine swallowtail butterfly, *Battus philenor*

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Abstract

Predation on distasteful animals should favor warning coloration that is relatively conspicuous and phenotypically invariable. However, even among similarly colored individuals there can be variation in their warning signals. In butterflies, individual differences in larval feeding history could cause this variation. The warning signal of the pipevine swallowtail butterfly, *Battus philenor* L. (Lepidoptera: Papilionidae) consists of both a blue iridescent patch and pigment-based orange spots on the ventral hindwing. *B. philenor* males also display a dorsal surface iridescent patch that functions as a sexual indicator signal. A previous study of iridescence in *B. philenor* found that the iridescent blue on both the dorsal and ventral hind wings is variable and significantly different between lab-reared and field-caught individuals. These differences could be the result of larval food deprivation in the field. Through experimental manipulation of larval diet, larval food deprivation was evaluated as a potential cause of the differences observed between lab and field individuals, and if food deprivation is a source of inter-individual variation in warning signals. *B. philenor* larvae were food restricted starting at two points in the last larval instar, and one group was fed through pupation. Adult coloration was then compared. Food deprivation led to poorer adult condition, as indicated by lower adult body mass, forewing length, and fat content of stressed individuals. As the level of food deprivation increased, the hue of the iridescent patches on both the dorsal and ventral hind wing shifted to shorter wavelengths, and the chroma of the orange spots decreased. The shifts in iridescent color did not match the differences previously found between lab and field individuals. However, the treatment differences indicate that food deprivation may be a significant source of warning color variation. The differences between the treatment groups are likely detectable by predators, but the effect of the variation on signal effectiveness and function remains to be empirically explored.

Keywords: aposematic, coloration, food restriction, iridescence, pigment-based coloration
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Introduction

Warning coloration can function as a signal to predators of a prey's unprofitability (Poulton 1890; Cott 1940). Such signals are expected to be naturally selected by predation to facilitate learning and recognition by relevant predators (Guilford and Dawkins 1991; Ruxton et al. 2004) through increased conspicuousness (Gittleman and Harvey 1980; Gamberale-Stille and Tullberg 1999; Lindström et al. 1999; Riipi et al. 2001; Lindstedt et al. 2008) and/or reduced phenotypic variation (Guilford and Dawkins 1993; Mappes and Alatalo 1997; Beatty et al. 2004; Rowland et al. 2007). Either of these processes should lead to a reduction in genetic variation or the extent to which the structures that produce a warning signal respond to environmental variation during development. Nonetheless, warning colors often display surprising levels of inter-individual variation in a population (Brakefield 1985; Brakefield and Liebert 1985; Holloway et al. 1995; Grill and Moore 1998; Grill 1999; Ojala et al. 2007; Lindstedt et al. 2008; Borer et al. 2010; Rutowski et al. 2010). This indicates that in the face of stabilizing selection there are factors that maintain variation in warning signal expression (Endler and Mappes 2004; Ojala et al. 2007; Sandre et al. 2007; Maan and Cummings 2008; Lindstedt et al. 2010; Lindstedt et al. 2011). One such potential factor in nature is variation among individuals in the extent to which they experience food restrictions during growth and development. The effects of food restriction or deprivation on sexual coloration are well-studied (e.g., McGraw et al. 2002; Siefferman and Hill 2005; Kemp 2008; Punzalan et al. 2008), and reduced diet quality can affect warning coloration (e.g., Grill and Moore 1998; Grill 1999; Ojala et al. 2007), but the effects of food deprivation on warning coloration are unknown.

Food deprivation in lepidopteran larvae is common in species that feed on small plants because they may have to travel between hostplants after complete defoliation of a plant (Stockhoff 1991; Fischer and Fiedler 2001). Larvae of the pipevine swallowtail butterfly, *Battus philenor* L. (Lepidoptera: Papilionidae) are especially susceptible to food deprivation during movement from one host plant to another. *B. philenor* larvae feed on plants in the genus *Aristolochia,* and individual hostplants rarely provide enough suitable foliage for complete larval development (Rausher 1979a, b, 1980; Rausher and Feeny 1980; Fordyce and Agrawal 2001), which can sometimes require more than 25 plants (Rausher 1980). The larvae also sequester aristolochic acids, which make them and the adults they produce unpalatable (Sime et al. 2000; Fordyce et al. 2005). To advertise this defense, adults display warning coloration on the ventral hind wing surface (Brower 1958; Codella and Lederhouse 1990), which consists of orange spots in a field of iridescent blue (Figure 1). Both colors are used by predators in recognizing *B. philenor* as distasteful (Pegram et al. 2013).

Figure 1. *Battus philenor* with ventral surface visible. High quality figures are available online.

In Arizona, the iridescent warning coloration of *B. philenor* on the ventral hind wings varies in ways that may be attributed to differences in larval diet. Rutowski et al. (2010) reported significant differences in the iridescent coloration between lab-reared individuals fed *ad libitum* and field-caught individuals. Therefore, variation in larval food availability could be a source of adult color variation, including warning color variation. As in the *B. philenor* populations previously studied, populations in Arizona are likely to experience food deprivation as larvae because their hostplant, *Aristolochia watsonii* Wooton and Sandley (Aristolochiales: Aristolochiaceae), is a small plant that larvae often completely denude of leaves before completing development (personal observation). Therefore, to evaluate the role of food restriction on warning coloration and to determine if observed natural variation in iridescent signals is due to food deprivation, the amount of food to which *B. philenor* larvae had access was varied among three different treatments. The adult coloration was compared among treatment groups. The effects of food deprivation were evaluated for three different color patches: the iridescent blue field and the orange spots of the ventral hind wing surface, which contribute to the warning signal, and the iridescent blue on the male dorsal hind wing. The male dorsal hind wing is a signal used by females, likely to assess either male quality or species identity (Rutowski and Rajyaguru 2013).

If food deprivation is a source of variation in the iridescent signals of *B. philenor*, it is predicted that food deprivation will cause increased brightness, shorter wavelength hues, and higher chroma for the ventral surface iridescence and higher intensities in the dorsal iridescence. These expectations are based on the difference between animals reared in the lab and those from the field reported in Rutowski et al. (2010). Additionally, if there are differences in the ventral surface iridescence and the orange spots between treatments, even if this variation does not match that found in Rutowski et al. (2010), it will indicate that food restriction can be a significant source of intraspecific variation in warning signals.

Materials and Methods

Rearing conditions and study of developmental time course

Early instar *B. philenor* larvae and eggs were collected in the field near the confluence of Mesquite Wash and Sycamore Creek in central Arizona, USA (N 33° 43.784', W 111° 30.997'). After collection, the animals were reared in an environmental chamber in which relative humidity was held at 55%. Temperature and light varied on a 16:8 L:D cycle, in which the temperature was 30° C with lights on for 16 hr and 24° C with the lights off for 8 hr. Larvae were fed cuttings from field-collected host plant, *A. watsonii*, *ad libitum* until treatment began. These conditions and the field site are those described in Rutowski et al. (2010).

In order to develop a protocol of food restriction the time course of development for *B. philenor* larvae from the source population was determined. To determine the number, duration, and growth rate of larval instars, 10 larvae were raised as above, and their body mass was measured every day from hatching to pupation. The timing of molts in the last two instars was determined by putting a spot of paint on the integument and noting when it disappeared (i.e., had been shed with the exoskeleton). Earlier instar larvae were generally not marked because of the difficulty of doing so without injury, but four larvae were marked through their entire development and revealed

that there are five larval instars. Data on body condition and adult coloration of these individuals were not used in the analysis.

Food deprivation treatment

Prior to the molt into the final larval instar, each larva was marked on the posterior dorsum with a small dot of green paint, placed in an individual cup with host plant, and randomly assigned to a treatment group. Because larvae and eggs were collected from many different plants in the field and across a 19-day-timespan, it is unlikely that they were closely related. Larvae were checked each day between 11:00 and 15:30. When the dot was no longer present on a larva, it indicated that it had molted into the 5th instar within the last 24 hours, and that day was set as Day 0 for that larva.

The final larval instar was chosen as the best developmental stage to restrict food availability because at this stage larvae consume the most host plant and are most likely to need to seek new plants in the field. Also, during the 5th instar they will likely attain a threshold mass above which they are able to pupate without additional food, as has been documented for other butterflies (Nijhout 1975; Jones 1976). After this threshold stage, larvae will not die of starvation if they do not find another host plant, but if food is available they will continue to feed and so may accrue additional resources that will be available during metamorphosis to produce morphological features, such as wing colors.

When each larva reached the 5th instar, it was placed into one of three treatment groups. In all three groups, larvae were fed *ad libitum* until food deprivation began. In the Day 3 treatment, larvae (n = 27) were given no food from the third day of the final larval instar until they pupated. This was the treatment group that experienced the highest level of food restriction. In the Day 4 treatment, food deprivation began on the fourth day of the last larval instar and continued until they pupated (n = 32). Deprivation in both groups began between 11:00 and 15:30 on the appropriate day. Larvae in the unrestricted treatment (n = 32) were provided with food *ad libitum* until they pupated. The third and fourth days of the final larval instar were chosen to begin food deprivation because no individual deprived of food from the second day or earlier survived to pupate (n = 5), and by the fifth day most larvae stopped feeding or otherwise started preparing for pupation.

Pupae were kept in the environmental chamber under the conditions described above until the adults eclosed, typically after 11–14 days. Individuals that had apparently entered diapause (n = 6), as indicated by the pupal stage lasting longer than 21 days, were not included in the analysis. Treatment did not have any apparent effect on whether they entered diapause, as there were two from each treatment. Upon eclosion, butterflies were placed in a refrigerator at 4° C for 24 hr and then freeze-killed.

Analysis of adults

Each adult's forewing length (wing tip to anterior point of wing insertion in the thorax) was measured using digital calipers to the nearest 0.01 mm. After removing the hind wings for mounting, the bodies were lyophilized for 24 hr, and the "dry" mass of each was measured to the nearest 0.1 mg. In addition, fat content of the bodies and forewings was measured as a percent of dry mass using the methods described in Fordyce and Nice (2008).

The hind wings were mounted on double-ply, museum-quality, black cardstock using photo

mount adhesive. The right hind wing was mounted dorsal side up, and the left hind wing was mounted ventral side up. Reflectance spectra were obtained relative to an MgO white standard, using an USB 2000 spectrophotometer with a PX2 xenon light source and OOIBase 32 software (all from Ocean Optics, www.oceanoptics.com). The ventral iridescent reflectance was measured from an area proximal to the orange and black spot in the cell between M_3 and Cu_1 veins on the hind wing (Borror et al. 1992), and the dorsal reflectance was measured in the same cell from an area proximal to the white spot on the hind wing. The angle between the light beam and the optical axis of the collector was 60° with the wing positioned so that its base pointed toward the light beam. The specimen was tilted around an axis perpendicular to the long axis of the wing until the reflectance of the iridescence around 500 nm was highest (see Figure 2 in Rutowski et al. 2010). Maximizing the iridescence of each wing in this manner allowed for consistent reflectance measurements, as some individuals may vary in the exact angle that produces peak reflectance (Kemp and Rutowksi 2007; Kemp 2008; Meadows et al. 2011) and measurements that are comparable to previous analyses of *B. philenor* iridescent coloration (Rutowski et al. 2010; Rutowski and Rajyaguru 2013). The orange spot reflectance was measured on the most anterior orange spot, with the collector and light beam positioned the same as for the iridescent patch measures and the wing surface perpendicular to bisector of the angle between collection and light beam.

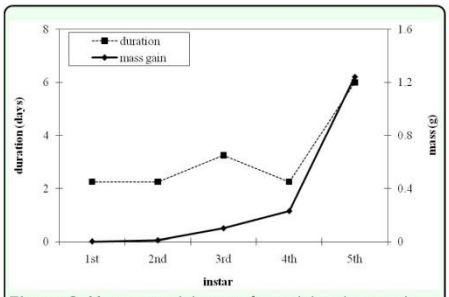

Figure 2. Mass gain and duration for each larval instar obtained by weighing *Battus philenor* larvae every day and recording molts (n = 10). Approximately 75% of total larval mass gain occurs in the final larval instar. The final instar lasts an average of 6 days. High quality figures are available online.

Each mounted hind wing was measured twice, in two separate rounds. All pairs of measurements on the same spot were within 5% of each other. The color parameters obtained from these two measurements were then averaged for the analysis. Only reflectance from 300–700 nm was used in the analysis. Spectra were characterized using three color parameters: brightness, hue, and chroma. Brightness is the amount of light reflected from the spot measured, and for both the orange and blue was calculated as the average percent reflectance from 300–700 nm (Montgomerie 2006). Hue identifies the wavelengths in which the most light is reflected and therefore contributes to the perceived color of the signal. Chroma (also known as saturation) is the spectral purity of the reflected light (Montgomerie 2006). Hue and chroma needed to be calculated differently for the iridescent blue and the diffusely reflecting orange because the orange reflectance lacks a clear peak. For the iridescent reflectance, hue is the wavelength at which the maximum reflectance is observed, and chroma is the summed reflectances between 50 nm above and below the peak wavelength, divided by the summed reflectances between 300 and 700 nm (Rutowski et al. 2010). For the reflectance of the orange spot, hue is the wavelength at which the percent reflectance is halfway between the minimum and maximum reflectance (Morehouse and Rutowski 2010) over all wavelengths (300–700 nm). Chroma is the difference between the minimum and maximum reflectance, divided by the average

reflectance over the 300–700 nm range (Montgomerie 2006).

To infer whether avian predators would be able to distinguish differences in the warning colors between the different treatment groups, AVICOL version 6 (Gomez 2006) and a physiological visual model based on Vorobyev and Osorio (1998) were used. The model used the spectral sensitivities and cone proportions for Blue Tits, *Cyanistes caeruleus*, a well understood passerine visual system (Hart et al. 2000). While Blue Tits are not predators of *B. philenor*, the visual sensitivities of birds are not highly variable (Hart 2001), and the specific visual sensitivities of known *B. philenor* predators (e.g., Cactus Wrens, *Campylorhynchus brunneicapillus*, and Curve-billed Thrashers, *Toxostoma curvirostre*) are not known. As such, the visual sensitivities of Blue Tits are a good surrogate of those of predators of *B. philenor*. The ambient light was a measure of downwelling irradiance from the field site, measured with the USB 2000 spectrophotometer described earlier fitted with a cosine-corrected probe on the end of the collector fiber. The model was used to obtain the chromatic contrasts between all three treatments for both the ventral orange spots and ventral iridescence. Chromatic contrast is reported in units of just noticeable differences (jnd), and a difference above 1 jnd is considered to be distinguishable by a bird (Vorobyev et al. 1998).

Statistics

MANOVAs were used to determine the overall effects of treatment on body condition (i.e., dry body mass, forewing length, and fat content) and the color parameters (i.e., hue, chroma, and brightness) of the two ventral color components. Each model included sex, treatment, and sex by treatment interaction as the factors. To sort out the effects of the individual variables in the significant MANOVAs, univariate ANOVAs and the standardized coefficients from discriminant analysis were used. If these tests revealed a significant treatment effect for a variable, the significance of differences in that variable between specific treatment groups was assessed using Tukey's posthoc tests. Three mixed models with restricted maximum likelihood were used to determine the response of male iridescence (i.e., brightness, hue, and chroma of both iridescent surfaces) and included surface, treatment, and surface by treatment interaction as fixed effects and individual as a random effect. For these models, Bonferroni corrections using $\alpha = 0.05$ requires $p \leq 0.016$ for a factor to be considered significant. All statistical analyses were run using SPSS version 19 (IBM, www.ibm.com) with a 0.05 level of significance where not otherwise indicated. Tests for normality revealed that brightness measurements were not normally distributed, and so those measurements were log transformed before analysis. Dependent variables in all models had equal variance.

Results

Study of developmental time course

Under the rearing conditions, *B. philenor* larvae from the Arizona population that was studied (1) have five instars, (2) acquire 75% of their maximum larval body mass during the 5th instar, and (3) spend an average of six days in the 5th instar before pupating (Figure 2). These results guided the design of the treatments. Interestingly, *B. philenor* larvae in other geographic areas have six instars (J. Fordyce, personal communication), which indicates that *B. philenor* may be another case of intraspecific variability in number of instars (e.g., Esperk et al. 2007).

Effects of food stress on body condition and pupation time

Treatment had an overall effect on body condition (MANOVA: Wilk's $\lambda = 0.353$, $F_{6, 154} = 17.29$, $p < 0.001$). Univariate ANOVAs revealed that treatment negatively affected adult dry body mass, forewing length, and fat content, and the highest level of food restriction produced smaller adults with a lower percentage of fat in their bodies (Table 1). Standardized discriminant coefficients indicated that forewing length explains more of the variance due to treatment than body mass or fat content. The dry body mass of adults in the Day 3 treatment (highest level of food restriction) was only 50–55% that of animals with unrestricted access to food (Table 1). Forewing length of males and females in the Day 3 treatment was reduced by 13% and 12%, respectively, compared to those in the Unrestricted group (Table 1). For fat content (as a percentage of body mass), Tukey's comparisons of differences among treatment groups revealed that the adults in the Unrestricted treatment had a significantly higher fat content than those in the Day 3 treatment ($p = 0.017$) and in the Day 4 treatment ($p = 0.011$). Fat content of the adults in the Day 3 and Day 4 treatments was not significantly different ($p = 0.999$).

Table 1. A summary of dry body mass, forewing length, and fat content values of *Battus philenor*.

Treatment	Sex	Dry body mass (g)	Forewing length (mm)	Fat content (% of body mass)
Unrestricted	Male	0.12 ± 0.02 (0.07 - 0.16)	48.49 ± 2.19 (45.77 - 51.4)	17.45 ± 2.68 (11.52 - 21.92)
	Female	0.15 ± 0.01 (0.13 - 0.17)	51.31 ± 1.65 (48.27 - 53.52)	15.73 ± 3.17 (9.5 - 21.07)
Day 4	Male	0.09 ± 0.02 (0.06 - 0.13)	45.92 ± 1.78 (40.69 - 48.19)	14.49 ± 2.54 (7.37 - 17.52)
	Female	0.12 ± 0.03 (0.07 - 0.17)	48.68 ± 2.54 (44.19 - 53.39)	13.74 ± 5.11 (4.54 - 25.41)
Day 3	Male	0.06 ± 0.008 (0.04 - 0.13)	42.23 ± 0.9 (38.18 - 49.12)	13.36 ± 3.28 (6.01 - 17.57)
	Female	0.08 ± 0.02 (0.05 - 0.12)	45.36 ± 2.73 (40.4 - 49.99)	13.83 ± 3.85 (8.17 - 21.39)

Statistics are given as mean ± standard deviation (minimum-maximum).

Table 2. Results of univariate tests and discriminant analysis for body condition measures of *Battus philenor*.

	Dry body mass				Forewing length				Fat content			
Factor	**df**	**F**	**p**	**SC**	**df**	**F**	**p**	**SC**	**df**	**F**	**p**	**SC**
Sex	**1**	**22.1**	**<0.001**	-0.54	**1**	**34.4**	**<0.001**	-0.46	1	55	0.217	-0.31
Treatment	**2**	**58.5**	**<0.001**	-0.34	**2**	**48.1**	**<0.001**	-0.66	**2**	**5.4**	**0.007**	-0.36
Sex x Treatment	2	0.2	0.855		2	0.3	0.741		2	0.8	0.461	

SC = standardized coefficients for discriminant analysis. Significant factors for univariate ANOVAs are noted in bold. Only the first root was significant in all discriminant analyses. The total sample size used in the analysis was 89 in the three treatment groups: Day 3 (males n = 14, females n = 13), Day 4 (males n = 16, females n = 14), and Unrestricted (males n = 18, females n = 14).

The sexes differed significantly in body size and fat content (MANOVA: Wilk's $\lambda = 0.693$, $F_{3, 76} = 11.24$, $p < 0.001$). In univariate ANOVAs, sex was a significant factor for both body mass and forewing length (Table 2); *B. philenor* males are smaller than females. However, there was no significant effect of sex on fat content (Table 2). In the discriminant analysis, males and females differed in all three measures of body condition, but mass and forewing length had the highest coefficients (Table 2). There was no sex by treatment interaction for the three measures of body condition (MANOVA: Wilk's $\lambda = 0.940$, $F_{6, 152} = 0.792$, $p = 0.577$).

Duration of the pupal stage (measured in days) was significantly affected by treatment, with food-restricted individuals spending less time in the pupal stage (Kruskal-Wallis $\chi^2 = 12.67$, df = 2, $p = 0.002$). The mean duration of the pupal stage was 13.3 days for the Unrestrcited treatment group, 13.1 days for the Day 4 treatment groups, and 12.7 days for the Day 3 treatment group.

Effects of food deprivation on warning coloration

For the ventral iridescence, there was a significant effect of food deprivation treatment (MANOVA: Wilk's $\lambda = 0.826$, $F_{6, 162} = 2.71$, $p = 0.016$). Both discriminant analysis and univariate tests revealed that treatment affected hue the most (Table 3). Tukey's post-hoc comparisons revealed a significant shift in hue towards shorter wavelengths (towards blue) with increasing food restriction (Table 3), shifting almost 15 nm between adults in the Unrestricted and Day 3 treatments (Table 4;

Table 3. Results of univariate tests and discriminant analysis (SC, standardized coefficients) for color parameters of *Battus philenor*.

		Color Parameters											
		Brightness (Log Transformed)				Hue				Chroma			
Color Patch	Effect	df	F	p	SC	df	F	p	SC	df	F	p	SC
Ventral Iridescence	Sex	1	0.10	0.750		1	0.04	0.833		1	1.86	0.176	
	Treatment	2	1.00	0.371	-0.01	2	**3.43**	**0.037**	1.72	2	0.30	0.743	0.99
	Sex x Treatment	2	0.12	0.885		2	0.06	0.939		2	0.44	0.644	
Ventral Orange	Sex	1	**12.75**	**0.001**	-0.59	1	**11.51**	**0.001**	1.48	1	0.43	0.516	-1.44
	Treatment	2	1.48	0.235	-0.54	2	2.26	0.111	0.58	2	**6.88**	**0.002**	-1.65
	Sex x Treatment	2	0.26	0.772		2	1.03	0.362		2	1.818	0.17	
Male Iridescence	Surface	1	**967.56**	**<0.001**		1	**9.96**	**0.003**		1	**62.01**	**<0.001**	
	Treatment	2	2.46	0.097		2	13.78	0.027		2	0.16	0.854	
	Surface x Treatment	2	3.17	0.052		2	0.15	0.863		2	1.29	0.283	

For the two ventral surface colors, univariate ANOVAs and discriminant analysis were run to evaluate the effects of treatment and sex on each variable only after MANOVAs showed significant effects for both surface colors. Only the first root was significant in all discriminant analyses. Three mixed models with restricted maximum likelihood were used to evaluate the effects of surface and treatment on the properties of male iridescence. For the mixed models, only *p*-values less than 0.016 were considered significant after Bonferroni correction. Significant factors for univariate ANOVAs and mixed models are noted in bold.

Table 4. A summary of color parameter values obtained from reflectance spectra for all treatment groups of *Battus philenor*.

Treatment	Color	Surface	Sex	Brightness	Hue (nm)	Chroma
Unrestricted	Orange	Ventral	Male	14.5 ± 1.2 (12.9-16.3)	592.5 ± 5.7 (581-602)	3.40 ± 0.14 (3.23-3.79)
			Female	15.8 ± 0.93 (14.2-16.9)	586.8 ± 6.9 (576-599)	3.43 ± 0.21 (3.10-3.79)
	Iridescent Blue	Ventral	Male	46.8 ± 10.4 (31.0-63.7)	490.5 ± 16.9 (467-525)	0.44 ± 0.01 (0.42-0.46)
			Female	44.9 ± 13.1 (31.9-83.6)	491.2 ± 17.9 (461-516)	0.44 ± 0.01 (0.42-0.46)
		Dorsal	Male	12.8 ± 3.0 (8.2-18.6)	497.9 ± 21.7 (455-538)	0.43 ± 0.01 (0.41-0.45)
Day 4	Orange	Ventral	Male	14.1 ± 1.0 (12.4-16.2)	591.9 ± 5.7 (578-599)	3.55 ± 0.21 (3.05-3.83)
			Female	14.6 ± 1.3 (12.4-17.0)	589.3 ± 6.9 (578-603)	3.50 ± 0.23 (3.18-3.99)
	Iridescent Blue	Ventral	Male	42.1 ± 9.0 (32.2-60.7)	485.4 ± 11.8 (466-510)	0.44 ± 0.01 (0.42-0.46)
			Female	42.0 ± 8.1 (30.9-58.2)	484.6 ± 26.0 (448-526)	0.44 ± 0.02 (0.41-0.47)
		Dorsal	Male	13.2 ± 3.8 (7.4-24.1)	493.3 ± 11.9 (470-514)	0.43 ± 0.02 (0.38-0.45)
Day3	Orange	Ventral	Male	14.4 ± 1.5 (12.3-17.5)	587.4 ± 3.7 (581-593)	3.26 ± 0.25 (2.58-3.50)
			Female	15.2 ± 1.3 (12.5-16.8)	585.6 ± 5.2 (579-598)	3.35 ± 0.18 (3.01-3.70)
	Iridescent Blue	Ventral	Male	43.2 ± 10.2 (28.1-61.9)	477.5 ± 15.6 (455-509)	0.45 ± 0.01 (0.42-0.48)
			Female	42.6 ± 9.1 (28.1-64.3)	479.9 ± 14.9 (447-508)	0.44 ± 0.01 (0.42-0.47)
		Dorsal	Male	10.3 ± 2.4 (6.1-14.1)	482.6 ± 15.9 (454-508)	0.43 ± 0.02 (0.40-0.46)

Color parameters are described in the text. Statistics are given as mean ± standard deviation (minimum-maximum).

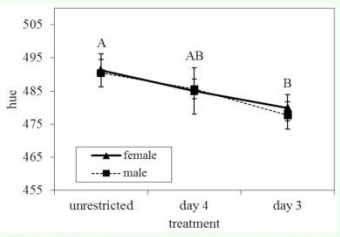

Figure 3. Hue measured on ventral iridescence of *Battus philenor* as the wavelength of highest reflectance. There was a shift towards shorter wavelengths in hue on both iridescent surfaces (dorsal not shown) with higher food restriction. Bars represent one standard error. Different letters represent significantly different values. Shared letters indicate responses are not significantly different. High quality figures are available online.

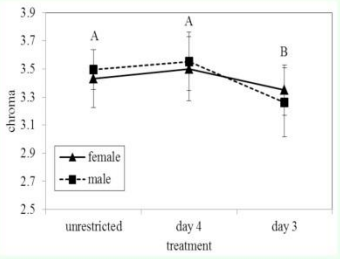

Figure 4. Mean chroma of ventral orange spots from reflectance spectra on *Battus philenor*. Chroma was measured as the difference between the minimum and maximum reflectance divided by the average reflectance over 300–700 nm. Bars represent one standard error. Different letters represent significantly different values. Shared letters indicate responses are not significantly different. High quality figures are available online.

Figure 3). The hue of the adults in the Day 3 treatment was significantly bluer than those of the Unrestricted treatment ($p = 0.028$) but was not different from those in the Day 4 treatment ($p = 0.340$). Univariate ANOVAs did not reveal an effect of food restriction on brightness or chroma (Table 3). Additionally, there was no difference between the ventral iridescence of the sexes (MANOVA: Wilk's $\lambda = 0.939$, $F_{3, 81} = 1.75$, $p = 0.164$) and no sex by treatment interaction (MANOVA: Wilk's $\lambda = 0.972$, $F_{6, 162} = 0.393$, $p = 0.883$).

Treatment also significantly affected the ventral orange spots (MANOVA: Wilk's $\lambda = 0.671$, $F_{6, 148} = 5.45$, $p < 0.001$). Univariate tests and the standardized coefficients revealed that chroma was the color parameter most affected by treatment (Table 3). Chroma decreased with increasing food deprivation (Table 4). Tukey's post-hoc comparisons revealed that the adults in the Day 3 treatment were significantly less chromatic than those in the Day 4 ($p = 0.002$) and Unrestricted treatments ($p = 0.019$). Individuals in the Day 4 treatment were not significantly different in chroma from those in the Unrestricted treatment (Figure 4). In addition to the effect of treatment, there was also a significant effect of sex on the ventral orange spots (MANOVA: Wilk's $\lambda = 0.652$, $F_{3, 74} = 13.17$,

$p < 0.001$). Univariate ANOVAs and discriminant coefficients revealed that sex mostly influenced brightness and hue (Table 3). On average, females' orange spots were brighter than males' and had a lower (shorter wavelength) hue than males' (Table 4). There was also no sex by treatment interaction for the ventral orange (MANOVA: Wilk's $\lambda = 0.929$, $F_{6, 148} = 0.928$, $p = 0.477$).

Models of avian color vision suggest that warning color differences between treatment groups would be distinguishable by an avian predator. For the ventral orange, the chromatic contrast between the color of the adults from the Unrestricted treatment and the Day 4 treatment was 5.76 jnd, between the Unrestricted treatment and the Day 3 treatment was 5.87 jnd, and between the Day 3 treatment and Day 4 treatment was 9.56 jnd. For the ventral iridescence, the chromatic contrast between the adults of the Unrestricted treatment and the Day 4 treatment was 2.96 jnd, between the adults of the Unrestricted treatment and the Day 3 treatment was 4.53 jnd, and between the adults of the Day 3 treatment and the Day 4 treatment was 1.59 jnd. Because all of these chromatic contrasts were above 1 jnd, they were all considered to be distinguishable by a bird.

Comparison of two iridescent surfaces

The statistical analysis on the color measurements for the iridescent areas from both male wing surfaces did not reveal an effect of treatment after correcting for multiple mixed model comparisons. However, Tukey's post-hoc comparisons revealed differences between the treatment groups in hue, where hue decreased (shifted towards blue) with increasing food restriction on both surfaces. Unrestricted individuals were significantly different from Day 3 individuals ($p = 0.008$) but not Day 4 individuals ($p = 0.320$). Day 4 individuals were also not significantly different from Day 3 individuals ($p = 0.068$). There was no significant surface by treatment interaction to suggest the two surfaces responded differently to food deprivation (Table 3). Also, all color parameters were significantly different between the two iridescent surfaces (Table 3). The ventral iridescence of males is brighter, has a lower hue, and is more chromatic than the dorsal surface. The difference in brightness and chroma were expected from the results reported in Rutowski et al. (2010). The variance accounted for by individual was significant for hue (Wald $Z = 3.269$, $p = 0.001$) and chroma (Wald $Z = 3.211$, $p = 0.001$), suggesting correlations between the two surfaces in these parameters.

Discussion

Measures of body condition

Compared to those with unrestricted access to food during development, food-restricted larvae developed into significantly smaller adults with smaller fat reserves. This result was expected based on previous studies of larval food limitation in butterflies (e.g., Bauerfeind and Fischer 2005; Boggs and Freeman 2005). The size of the adults produced from Day 3 treatment larvae was within the range of adult sizes observed in the field (Rutowski et al. 2010), indicating that the level of food deprivation induced in the Day 3 treatment was likely within the range of food limitation that this species experiences in nature. The effect of larval food deprivation on the level of sequestered aristolochic acids is currently under investigation.

Warning coloration

Food restriction produced significant variation in both the orange and blue components of the ventral warning coloration of *B. philenor*. The hue of the ventral blue iridescence shifted to

shorter wavelengths with increased food deprivation. The chroma of the orange spots decreased with increased food deprivation.

These results suggest proximate links between coloration, the structures and chemicals that produce color, and diet quantity, but these linkages are not clear at the moment (see Kemp et al. 2006). For *B. philenor*, this is true for both the orange spots and the blue patches, but there are some possible connections that could be tested. The diffuse reflection of the orange spots indicates that the pigments played a major role in shaping the reflectance spectrum by absorbing short wavelengths, which allows longer wavelengths to be reflected from the wing surface (Rutowski et al. 2005). The specific pigments involved are not known but are likely to be ommachromes or papilochromes synthesized de novo by the butterflies from the amino acid tryptophan (Nijhout 1991). The chroma of the orange spots should be positively related to the quantity of pigment in the scale, as more pigment means greater absorption of short wavelength light. During development, diet-restricted individuals whose orange is less chromatic may deposit less pigment in their scales due to a lower availability of tryptophan. On the other hand, the iridescent blue is likely a product of thin film interference, and the higher hue of diet-restricted individuals suggests a thicker film (Land 1972). If true, it is not clear how the film would be thicker in diet-restricted individuals who presumably experience restrictions in the materials needed to build these cuticular films. Again, questions about the potential proximate connections between diet and color phenotype remain untested but warrant investigation.

An experiment with captive Curve-billed Thrashers showed that the blue iridescence and the orange spots of the ventral hind wing were used by avian predators to recognize *B. philenor* as distasteful, and each component alone elicited a rejection response (Pegram et al. 2013). It is unknown whether the variation in the hue of the iridescent patches induced by food restriction would alter the effectiveness of the aposematic coloration of *B. philenor*. Although both reptiles and invertebrates (e.g., spiders and dragonflies) have been observed preying on *B. philenor* (Rausher 1979b, 1980), insectivorous birds are likely to be their most common predators in Arizona (Pegram, Han, and Rutowski, unpublished data). Visual models indicated that birds should be able to distinguish the spectral differences observed in adult coloration due to treatment. However, even though avian predators may be able to discriminate these colors, predators may generalize a learned warning signal to similar colors (Ham et al. 2006; Ruxton et al. 2008; Svádová et al. 2009) or the differences may not be detectable in complex and changing conditions of lighting and background (Lindstedt et al. 2011). Either way, the color shifts caused by food deprivation may not decrease signal effectiveness. Signal effectiveness could also be influenced if the observed responses altered conspicuousness (Gittleman and Harvey 1980; Gamberale-Stille and Tullberg 1999; Lindström et al. 1999; Riipi et al. 2001; Lindstedt et al. 2008). From these results, it is concluded that food deprivation did contribute to intraspecific variation in warning coloration, but determining if this variation correlates with signal effectiveness will require further study.

Response of iridescent coloration and comparison to natural coloration

The hue of the ventral and dorsal iridescent patches shifted to shorter (bluer) wavelengths with increased food deprivation. This was the opposite direction of what was predicted based on the results of Rutowski et al. (2010).

Rutowski et al. (2010) also found that lab-reared individuals had more intense ventral iridescence than did field-caught individuals, where no effect of rearing conditions on ventral iridescence brightness was found in our study. Therefore, differences in the lab and field individuals previously observed were not likely due to increased food deprivation in the field-caught *B. philenor*. The difference between the dorsal and ventral surfaces in male chroma observed in our study was expected based on Rutowski et al. (2010), but the lack of treatment effects and differences between the sexes was inconsistent with their results. Differences between male and female ventral hue were observed in the previous study, but not in our study. From these differences, it can be concluded that the differences observed between lab and field individuals in Rutowski et al. (2010) were not likely caused by food deprivation in field individuals.

However, differences in the results of these studies could be caused by at least two other factors. First, there were differences between the studies in seasons in which observations occurred (spring (Rutowski et al. 2010) vs. autumn (our study)). Second, the larvae of the field-caught individuals could have undergone food deprivation throughout the larval stage, while the larvae in our experiment only underwent food restriction in the last larval instar.

Also, the hue and chroma of an individual's dorsal iridescence were correlated with the hue and chroma of its ventral wing surface, which suggests a coupling of the iridescent surfaces. This is consistent with the findings of Rutowski et al. (2010), but the causes of this coupling are not understood at this time.

Because the dorsal coloration may serve as a signal of male quality (Rutowski and Rajyaguru 2013), we expected to see heightened condition dependence over a naturally selected signal (e.g., Andersson 1986; Cotton et al. 2004a, b; Kemp 2008). However, there were no significant surface by treatment interactions to suggest there is heightened condition dependence of the iridescence on the dorsal surface.

Conclusions

Food deprivation can be a common ecological occurrence for lepidopteran larvae that feed on relatively small hostplants, like the *Aristolochia* plants used by some populations of *B. philenor* (Rausher 1979a, 1979b, 1980; Rausher and Feeny 1980; Fordyce and Agrawal 2001), and may affect the development of adult color signals. In the case of *B. philenor*, spectral properties of the iridescent blue patches and diffusely reflecting orange spots that act as warning signals changed in response to a food deprivation, suggesting that food deprivation during the larval stage can be a significant source of intraspecific variation in coloration. However, because the findings were not wholly consistent with differences between field-caught and lab-reared individuals reported in previous studies (Rutowski et al. 2010), there are likely to be additional factors that explain the variation in adult coloration. The consequences of any of this variation for signal function remain to be explored.

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APPENDIX C

RELATIVE EFFECTIVENESS OF BLUE AND ORANGE WARNING COLOURS IN THE CONTEXTS OF INNATE AVOIDANCE, LEARNING AND GENERALIZATION

relative effectiveness of these colours at deterring naïve predators (i.e. those that had not previously learned to avoid a warning signal).

Warning colours may deter predation by initially naïve predators through two general mechanisms: (1) innate avoidance and (2) learning. First, naïve predators may not attack prey that display warning colours (i.e. aposematic prey) because of unlearned wariness or innate avoidance (e.g. Roper, 1990; Smith, 1975). Second, if predators do not innately avoid a specific colour, or the innate avoidance is overridden through experience or time, predators must learn to associate a warning colour (conditioned stimulus) with unpalatability (unconditioned stimulus) through a series of taste rejections (i.e. trial-and-error learning). Taste rejections are not always fatal for prey (Sillén-Tullberg, Wiklund, & Järvi, 1982; Wiklund & Järvi, 1982), but learning encounters are still potentially costly for both predators and prey. Therefore, predators should select for warning signals that increase learning rates (i.e. decrease the number of taste rejections required for predators to learn not to attack prey). A warning signal that is learned more quickly or is more likely to elicit innate avoidance can be considered a more effective signal.

Once predators learn a warning signal, they may then generalize what they have learned to novel warning signals. This generalization can be narrow (Sillén-Tullberg et al., 1982), in which case they do not generalize very far from the learned signal on a stimulus gradient, or broad (Evans, Castoriades, & Badruddine, 1987), in which case predators generalize to warning signals that are very different from the learned signal. In addition, generalization can vary in the level of symmetry, such that predators generalize more in one direction of the stimulus dimension than in another (Gamberale & Tullberg, 1996; Gamberale-Stille & Tullberg, 1999; Lindström, Alatalo, Mappes, Riipi, & Vertainen, 1999; Svádová et al., 2009). The level and symmetry of the predator generalization may depend on the strength of the aversive prey stimulus (Darst & Cummings, 2006), and so have an impact on the evolution of warning colours and mimicry (e.g. Gamberale-Stille & Tullberg, 1999; Sillén-Tullberg et al., 1982).

The experiment described here measures the innate avoidance, learning and generalization of initially naïve predators to orange and blue warning signals, either alone or in combination, to measure the relative effectiveness of these components of prey coloration. We used naïve Gambel's quail, *Callipepla gambelii*, as predators because they are likely to be a more ecologically relevant alternative to domestic chickens, *Gallus gallus domesticus*, which are used frequently in studies of this kind (e.g. Aronsson & Gamberale-Stille, 2008; Gamberale & Tullberg, 1996; Miklósi, Gonda, Osorio, & Farzin, 2002; Roper, 1990; Rowe & Guilford, 1996). Gambel's quail chicks are insectivorous (Gorsuch, 1934) and thus may learn to avoid warningly coloured insects in nature. In addition, because *C. gambelii* have undergone limited, if any, domestication, their evolved innate preferences and aversions should still be more similar to natural predators.

Based on our previous experiment (Pegram, Lillo, et al., 2013), we hypothesized that blue would be an effective warning signal. If there is an innate aversion to blue, we expected that chicks would be less likely to attack blue prey when first presented. We also examined learning rates over repeated presentations to determine how fast and how well the chicks would learn to associate blue with prey unpalatability, compared to black or orange, or a combination of orange and blue. If blue is readily learned when it is associated with unpalatability, we expected that (1) predators would learn more quickly to make correct choices (i.e. attack palatable prey and not attack unpalatable prey) and (2) over repeated presentations, attack latencies would increase more rapidly and, at the end of the learning phase, they would be longer in response to blue unpalatable prey than to black unpalatable prey. Lastly, we measured the level of generalization by animals that completed the discriminative learning phase. Blue would be considered effective as a warning signal if experienced birds were less likely or just as likely to attack blue prey as a prey displaying a different colour that they had learned not to attack.

METHODS

Predators

We tested 100 Gambel's quail, at 1–4 weeks old, that we reared from eggs obtained from commercial hatcheries (Murray McMurray Hatchery, Webster City, IA, U.S.A.; CM Game Bird Farms and Hatchery, Calais, ME, U.S.A.). Upon arrival, eggs were incubated at 37.5 °C for approximately 3 weeks. Upon hatching, the quail chicks lived in temperature-controlled brooders with brown pellet food (Mazuri Gamebird Starter, PMI Nutrition International, St Louis, MO, U.S.A.) and water. In the second year, quail were housed with domestic chickens to facilitate acquisition of self-feeding skills. To ensure that chromatic stimuli were novel and avoid potential impacts of the rearing environment on innate avoidances (e.g. Miklósi et al., 2002; Roper, 1990; Roper & Cook, 1989), we reared the quail in environments free of objects with highly chromatic surfaces (e.g. all food and water dishes were clear, black or white; all humans coming into contact with the birds wore white laboratory coats and white or black gloves). Individual quail were marked using numbered aluminium leg bands. After quail had completed all phases of testing (see below), they were adopted for personal use, used for approved studies by other researchers or euthanized using standard and approved humane practices. All chickens were adopted. Our research was approved by the Arizona State University Institutional Animal Care and Use Committee (Protocol number 11-1175R).

Prey Items

Quail chicks were presented with prey items that consisted of a 6 × 18 mm piece of black cardstock, folded in half to make a 6 × 9 mm tent covering a piece of mealworm about 5 mm long. Four types of simulated prey were presented: (1) orange, (2) iridescent blue, (3) black, or (4) orange-and-blue. We chose these colours so that our results could be extrapolated to *B. philenor* and potentially to other species that display orange and blue warning coloration and thereby allow conclusions about how these types of colour function in nature. The tents were coloured with pieces of laboratory-reared *B. philenor* wings pasted onto the outward facing surfaces of the tent with nontoxic glue. We used *B. philenor* wing pieces because the iridescent coloration could not feasibly be reproduced artificially. For black prey, we used pieces from black areas of the ventral *B. philenor* forewing (Fig. 1). For orange prey, we pasted pieces from the orange spots on the ventral hindwing surface (Fig. 1). For the blue iridescent prey, we pasted pieces from the ventral surface blue patch, orienting the pieces so that the iridescent blue was bright when viewed from above and in front of the tent (the proximal–distal axis of the wing patch was laid perpendicular to the long axis of the tent; Fig. 1). The orange-and-blue prey items were made in a similar fashion to the orange-only and blue-only prey items, but the colours were split down the middle of the tent, on the long axis. This split resulted in each colour covering half the area as that of single-colour prey items, but the overall area of colour was exactly the same as the other prey items.

We compared the reflectance of the colours on the tents to those of the natural butterfly wings (Fig. 1). Reflectance spectra from 300 to 700 nm were collected with two separate optical fibres, one

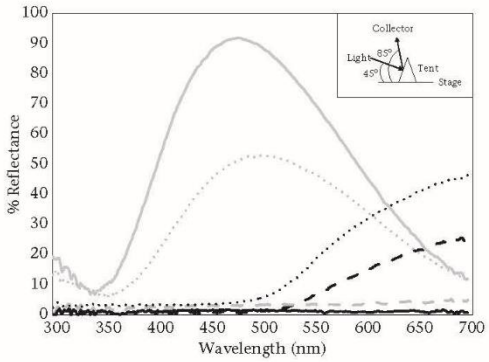

Figure 1. Reflectance spectra taken from the wings of *B. philenor* and the tents used in the experiment. Black (dashed grey line) areas of the wing were used to make black prey, and orange (dashed black line) areas of the wing were used to make orange prey and half of the orange-and-blue-prey. The blue ventral coloration of *B. philenor* is iridescent and reflectance changes with angle of view and illumination. Reflectance spectra displayed here are the average highest reflectance (solid grey line) and the average lowest reflectance (solid black line) for two specimens. Reflectance spectra averaged from two blue tents (dotted grey line) and two orange tents (dotted black line) are also shown. Blue prey stimuli were created in a way that would maximize the iridescence when viewed from above and in front of the tent, but the appearance could have varied during predator approach and under natural lighting conditions, as it may in nature. The inset diagrams how spectra were taken from the tents.

providing illumination from an Ocean Optics PX-2 xenon light source and the other delivering collected light to an Ocean Optics USB 2000 spectrophotometer (Dunedin, FL, U.S.A.). Our white standard was magnesium oxide. To measure the natural coloration, we used *B. philenor* hindwings from laboratory-reared specimens mounted on black cardstock. For these mounted wings, which were laid flat on our measuring stage, we set the angle between the illuminating and collecting beam paths at 60° with the specimen placed at the vertex of this angle. To demonstrate the change in reflectance due to the iridescent nature of the blue coloration, we took spectra at both the maximum reflectance and minimum reflectance. To achieve the maximum and minimum reflectance, we tilted the stage towards and away from the collector until the peak around 500 nm was at its maximum or minimum. For more details on the measurement and properties of the natural blue iridescent coloration of *B. philenor*, see Rutowski, Nahm, and Macedonia (2010). To measure the diffusely reflecting colours of *B. philenor* (black and orange), we used the same arrangement as above but kept the plane of the stage perpendicular to the bisector of the angle between the illuminating and collecting beams.

To measure reflectance spectra on the tents used in the experiment we could not use the same arrangement because the tents did not lay flat on the stage. Instead, we simulated an approaching position of the birds and positioned the collector 85° in elevation above the horizon to simulate a predator approaching from in front and above the tent (quail were always taller than the tents). We positioned the light source at 45° in elevation above the horizon (Fig. 1 inset). However, the spectrophotometer provides a point source of light, while the light in the greenhouse would likely be less direct, coming from many directions, which could affect the actual appearance. The spectra demonstrate that the iridescent blue on the tent was less reflective than the peak reflectance on the iridescent portion of the wings but within the natural range of variation of the iridescent colour and that the reflectance of the orange tent was higher than the reflectance off of the wing under our laboratory (darkroom) conditions (Fig. 1). This could have been due to the different settings under which the standing tents were measured as compared to the flat wings or differences in the specimens used in the analysis and to construct the tents as there is natural variation in the orange and blue of *B. philenor* wings (Pegram, Nahm, & Rutowski, 2013).

Mealworm pieces placed under the tent were either untreated or rendered unpalatable. We made mealworm pieces unpalatable by soaking them for 20 min in a solution of 4% quinine hydrochloride and 2% mustard powder (Hauglund, Hagen, & Lampe, 2006; Rowe & Guilford, 1996). Both the tents and the mealworms were presented in the centre of 55 mm diameter plastic petri dishes against the white background of the cage. Many of the unpalatable mealworms were taste rejected by the quail chicks, but we did not observe any negative health consequences in the quail that consumed the unpalatable mealworms. When either tasting or consuming unpalatable mealworms, we observed head shaking and bill wiping by the quail chicks.

Testing Environment

We performed all pretraining and testing in a greenhouse, so that prey items were illuminated by glass-filtered natural light. All learning and generalization phases occurred between 0630 and 1200 hours from May through August in 2011 and 2012 (the season in which *C. gambelii* eggs are available). For testing, quail chicks were individually held in 52 × 26 × 20 cm clear plastic cages with a white nonslip liner in the bottom of the cage that provided a consistent background. Contrast with the background can also be an important factor in warning signal effectiveness. Since the ecologically relevant background is likely to vary with species and environmental conditions, we chose a standardized white background. It is possible that using a less reflective background (e.g. green or brown) would have altered the effectiveness of the colour signals tested here.

The chicks were moved from their brooders to the test cage in the morning and allowed to acclimate for 30 min. During this acclimation period, chicks had one mealworm piece available in a petri dish to stimulate feeding from the petri dishes. There was no other food deprivation, and water was available at all times. To reduce stress on the quail chicks, a companion quail or chicken chick was placed in the adjacent cage. These companion birds were visible throughout all testing phases.

Pretraining Phase

During pretraining, we taught quail chicks to eat from the petri dish and then access the mealworm piece from underneath the tent (i.e. associating the tent with food). To do this we first placed palatable mealworm pieces in a petri dish (replaced every 30 min) until the bird ate the mealworms. This could occur over several mornings (maximum = 8 days) or the day of testing if birds were quick to learn. Birds that did not eat from the dish were excluded from subsequent testing. After birds ate from the petri dish, we added a cardstock tent painted brown with nontoxic tempera paint for a series of 3 min trials. We started with the brown tent placed next to the mealworm and moved it closer with each trial until the bird regularly ate the mealworm from underneath the brown tent within 3 min. Birds that could not learn to eat from beneath the tent were excluded from subsequent trials. Then, to ensure they were hungry and motivated, birds had to eat a mealworm from under the brown tent in three consecutive presentations before we started the learning trials. Pretraining sometimes occurred on the day before the learning trials, but this motivation test always occurred immediately before testing.

Learning and Generalization Phases

The learning phase consisted of 16 presentations, each 3 min in duration. We followed a discrimination learning regime that included both palatable (P, S+) and unpalatable (U, S−) stimuli. Prey stimuli were presented sequentially with 1 min between each presentation. The order of the stimuli was pseudorandomized (Fan & Hansson, 2001; Fernandez, Locatelli, Person-Rennell, Deleo, & Smith, 2009; Gerber et al., 1996; Smith, Abramson, & Tobin, 1991), so that they followed this sequence: PUUPUPPUPUUPUPPU. This sequence has a pattern that is presumed to be difficult for the predators to learn and reduces the probability of chance correct decisions (as opposed to an alternating pattern; Gellerman, 1993) and prevents the birds from generalizing their responses from presentation to presentation, as could happen if the same stimulus was presented repeatedly. Each bird was randomly placed into one of six treatment groups. We structured the treatment groups so that the three focal prey colours (blue, orange and orange-and-blue) would be paired with black. Then, to control for discriminability and for the effects of black, they were divided so that, for half of the birds, black was palatable (S+) and the focal colour was unpalatable (S−) and the reverse for the other half (focal colour was palatable (S+) and black was unpalatable (S−); Table 1). Since black was a stimulus in each treatment group, we named the treatment group by the focal colour presented and then whether that colour was unpalatable, U (e.g. Blue-U, Orange-U, Blue/Orange-U) or palatable, P (e.g. Blue-P, Orange-P; Blue/Orange-P).

Following the learning phase, birds were immediately given four presentations to test their level of generalization from the learned stimulus. This consisted of the four prey types (blue, orange, orange-and-blue and black) in random order, with a palatable mealworm for each. In the second year of the experiment, we included a post-testing motivation trial after the learning and generalization phases. We gave each bird eight palatable mealworm pieces in a petri dish. After 3 min, we removed the dish and counted how many pieces the chick had consumed. If the video record (see below) indicated that an experimental bird failed to eat any mealworm pieces and showed no clear interest towards prey tents (i.e. looking at the tent, walking up to the tent), we excluded that bird from the analysis. We excluded only three birds in year 1 and one bird in year 2 for this reason. Since we did not include a post-testing motivation trial in the first year, we excluded birds that showed no evidence in the video recordings of clear interest in the prey items.

Video Analysis

Observers were not present in the room. Instead, we recorded the learning and generalization phases using a digital video camera (JVC Everio GZ-HM300; JVC Kenwood Corporation, Wayne, NJ, U.S.A.). The videos were viewed at least twice, each time by a different observer to determine (1) whether the prey item was attacked by the quail chick, (2) the time between the presentation of the mealworm and the attack time (attack latency), and (3) any interest in the tent (i.e. walking up to the tent and examining it). If there was any discrepancy between the two viewers (e.g. different determinations on whether or not a prey was attacked or a difference of more than 5 s in attack latency), a third viewer watched the video for that presentation. Each video viewer was blind to the treatment group of the bird and the observations of the other viewers.

We defined an attack as the beak of the quail touching the tent. In some cases, quail inadvertently dismantled the stimulus, most often by running over the petri dish and knocking the tent away from the mealworm piece. If the bird did not eat the mealworm piece, even after the tent had been knocked away, this was considered a nonattack because we assumed that the bird had previously observed the colour of the tent. If the bird ate the mealworm piece after the tent had been moved away from the mealworm piece, we recorded no data for this presentation, as we could not be sure whether the bird had seen the colour of the tent or associated the mealworm piece with the colour of the tent.

Table 1
Treatment groups and sample sizes

Treatment	Unpalatable stimulus colour (S−)	Palatable stimulus colour (S+)	Sample size
Blue-U	Blue	Black	16
Orange-U	Orange	Black	16
Blue/Orange-U	Orange-and-blue	Black	16
Blue-P	Black	Blue	16
Orange-P	Black	Orange	15
Blue/Orange-P	Black	Orange-and-blue	16

Birds were randomly assigned to a treatment group and received one colour associated with palatable prey and one colour associated with unpalatable prey.

Data and Statistical Analysis

In the first presentation of the learning phase, the stimulus was always palatable and the structure of the treatment groups were designed such that a proportion of the birds first had each of the four prey stimulus types in the first presentation: black (49%; $N = 47$), blue (17%; $N = 16$), orange (17%; $N = 16$) and orange-and-blue (17%, $N = 16$). We thus measured innate avoidance by whether or not the bird attacked the first prey item. To determine whether colour influenced this attack response, we performed a generalized linear model with a binomial distribution and logistic link function. Bonferroni post hoc pairwise comparisons were used to determine whether there were significant differences in attack response between colours.

To determine whether treatment group influenced how readily the quail learned to discriminate between the prey, by not attacking unpalatable prey but attacking palatable prey, we analysed both the choices made and the attack latencies in the presentations. We first analysed attack latencies on unpalatable prey presentations with a repeated measures ANOVA. Attack latency was the dependent variable and presentation number, treatment and individual were the independent variables. For presentations in which the bird did not attack the prey item, 180 s was entered as the attack latency. We used Bonferroni post hoc pairwise comparisons to determine whether there were significant differences between treatment groups.

The second way we analysed learning was by measuring the proportion of correct choices (i.e. attacked palatable and did not attack unpalatable prey) made during the learning phase after the first two trials (Smith et al., 1991) using an ANOVA. The dependent variable was the proportion of correct choices and the independent variable was treatment. We determined differences between the treatment groups using Bonferroni post hoc comparisons. All nonbinary data sets were normally distributed and innate avoidance and learning analyses were performed in SPSS version 20 (IBM, Somers, NY, U.S.A.) with a 0.05 level of significance.

If a colour is to be effectively learned as a warning signal, it should be learned more efficiently than a control, nonwarning colour (black in this case). So, in our experiment, we predicted that the 'colour'-U group would learn better than the 'same colour'-P group since there would be the same level of distinctiveness or discriminability between the two types of stimuli (Merilaita & Ruxton, 2007). For example, to demonstrate that blue can be learned effectively, birds in the Blue-U group should show higher

attack latencies and be more likely to make correct choices overall than birds in the Blue-P group. In addition, to determine whether one colour is associated with unpalatability more quickly than another, we compared performance among the 'colour'-U groups. For example, if birds in the Blue-U group performed significantly better on the learning measures than birds in the Orange-U group, we would consider blue to be a more effective warning colour.

To analyse the level of generalization, we used a generalized linear mixed model with binary logistic distribution. Attack response for each presentation was entered as the dependent variable with colour and treatment entered as fixed independent variables and bird entered as a random effect. This analysis was completed in R version 2.15.2 (R Foundation for Statistical Computing) with a 0.05 level of significance.

For the learning and generalization phases, 95 birds completed all presentations and met the criteria for inclusion in the analysis (Table 1). All treatments except for Orange-P had 16 birds per treatment group and Orange-P had 15 birds.

RESULTS

Innate Avoidance

Inexperienced quail chicks responded differently to the four colour treatments offered (Wald $\chi^2_3 = 28.2$, $P < 0.001$; Fig. 2). More quail attacked black prey and blue prey than orange prey and orange-and-blue prey ($P < 0.001$). Attack frequencies did not differ between black and blue prey ($P > 0.999$) or between orange and orange-and-blue prey ($P > 0.999$), which suggests that innate aversions were greatest for orange prey and orange-and-blue prey.

Learning

To measure learning we analysed both changes in the attack latency and the number of correct choices made by the birds. There was a significant treatment effect on attack latency (repeated measures ANOVA: $F_{5,89} = 3.101$, $P = 0.013$; Fig. 3) and correct choices (ANOVA: $F_{5,89} = 15.098$, $P < 0.001$; Fig. 4), indicating that the treatments affected warning signal learning by the birds.

To determine whether blue was effectively learned as a warning signal, we compared the results of Blue-U to Blue-P. These two treatment groups did not differ significantly for either measure of learning (attack latency: $P > 0.999$; correct choices: $P = 0.250$). However, when compared with the other two colours, blue was learned at the same rate as orange, as measured by attack latency ($P = 0.103$) and correct choices ($P = 0.189$). When Blue-U was compared to Blue/Orange-U, there was no significant difference in learning rates for either of the measures: attack latency ($P > 0.999$) and correct choices ($P = 0.999$). To summarize, blue prey were not learned any better than black prey, but were also learned just as well as orange-and-blue prey and orange prey.

By both measures of learning, orange was learned more quickly and accurately than black. Birds in the Orange-U group increased their attack latencies more quickly ($P = 0.020$) and were more likely to make correct choices through the presentations ($P < 0.001$) than birds in the Orange-P group. As reported above, performance of

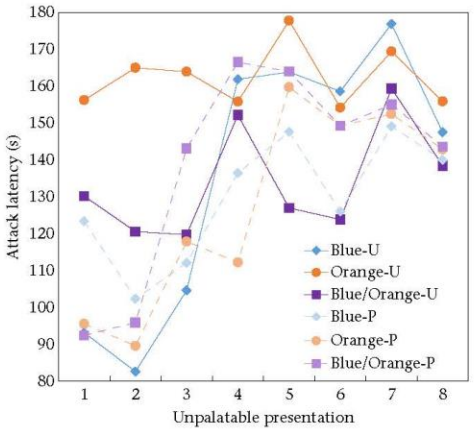

Figure 3. Mean attack latency for unpalatable prey, displayed by treatment group (P: palatable colour; U: unpalatable colour). Birds in treatments Blue-U, Orange-U and Blue/Orange-U had blue, orange and orange-and-blue as the unpalatable prey stimulus, respectively. Birds in treatments Blue-P, Orange-P, and Blue/Orange-P had black prey as the unpalatable stimulus. If birds learned not to attack the unpalatable prey, then we expected attack latencies to increase.

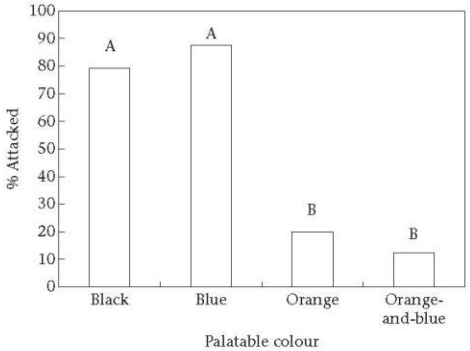

Figure 2. Innate avoidance of prey colour as measured by the percentage of quail chicks attacking the novel palatable prey on first encounter. Different letters above bars indicate significant differences between palatable prey colours.

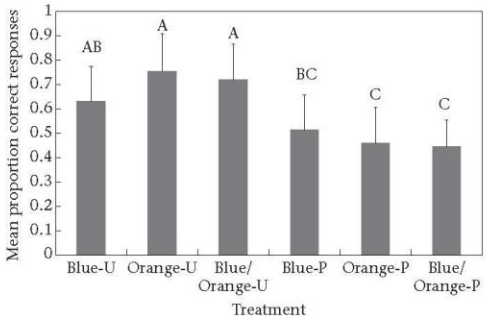

Figure 4. Mean proportion of correct choices made by quail chicks during the learning phase after the first two trials, displayed by treatment groups. Birds were considered to have made a correct decision if they attacked the palatable prey and did not attack the unpalatable prey. Error bars indicate standard deviation. Shared letters indicate that treatments were not significantly different.

birds in the Orange-U group did not differ significantly from those in the Blue-U group during the learning trials. Similarly, birds in Orange-U group learned just as quickly as birds in the Blue/Orange-U group when measured by correct choices ($P > 0.999$) and attack latencies ($P = 0.056$). These results indicate that, as expected, birds quickly learned to associate orange with a warning signal.

When orange-and-blue prey were associated with unpalatability, the birds learned more quickly than when black prey were associated with unpalatability in one measure of learning, correct choices ($P < 0.001$). However, birds in Blue/Orange-U and Blue/Orange-P treatments did not differ significantly in attack latency ($P > 0.999$). Also, as mentioned above, the performance of birds in the Blue/Orange-U treatment did not differ significantly from that of birds in the Blue-U treatment and the Orange-U treatment. To summarize, when blue and orange were displayed adjacent to one another, the birds learned the colour combination as a warning signal.

Generalization

We found no effect of treatment group on attack rates of the four stimuli presented in the generalization test following the learning trials ($z = -0.322$, $P = 0.322$; Fig. 5). We compared the result for each prey colour to black. Attacks on blue prey did not differ significantly from those on black prey ($z = 0.249$, $P = 0.803$). However, birds were less likely to attack orange prey ($z = -5.321$, $P < 0.001$) and orange-and-blue prey ($z = -3.842$, $P < 0.001$) than black prey.

DISCUSSION

Innate Avoidance

In our study, blue and black prey items were more likely to elicit attacks from inexperienced quail chicks than orange prey and orange-and-blue prey. This indicates that quail chicks have a fairly strong innate avoidance to orange and blue together and orange alone. Based on previous warning colour research, we expected that different colours would vary in their ability to elicit innate aversions; for example, predators are more likely to attack brown and grey prey, which are often associated with palatable and cryptic prey, than they are those bearing long-wavelength colours

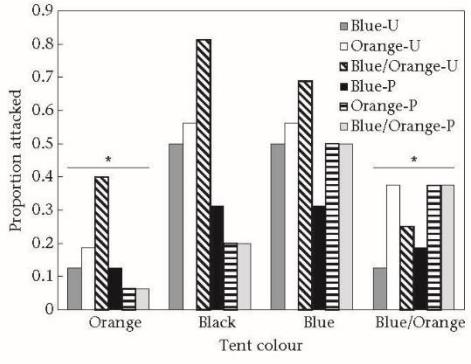

Figure 5. Results of generalization test, in which each bird was offered one of the four stimuli. An asterisk indicates a significant difference ($P < 0.001$) in the proportion of attacks received relative to black prey.

(Gamberale-Stille et al., 2010; Ham, Ihalainen, Lindström, & Mappes, 2006; Roper, 1990). The unknown factor was how effective blue and adjacent orange and blue colours would be at eliciting innate aversions. We found that blue elicited little innate avoidance, similar to the birds' responses to black prey, but that adjacent orange and blue elements were innately avoided.

The differences we observed in innate avoidance due to prey colour is important for our understanding of the role of blue coloration and reveals that it may not be as effective at deterring predation as orange coloration. Birds are common predators of warningly coloured animals (e.g. butterflies, frogs, beetles) and studies of avian vision thus far reveal relative uniformity in visual capabilities, with large variation only in the sensitivity of the UV- or shortest-wavelength cone (Hart, 2001). However, there may be differences in predator psychologies. For example, wild insectivorous birds may require more time or encounters to overcome unlearned aversions than quail or domestic chicks because of dietary conservatism (Marples & Kelly, 1999; Marples, Roper, & Harper, 1998). Also, bobwhite quail, *Colinus virginianus*, have previously been shown to prefer blue prey (Mastrota & Mench, 1995) while domestic chickens have shown some innate avoidance of blue prey (Roper, 1994). The innate responses to blue coloration should thus be measured in other avian predators and potential nonavian predators. For example, an aquatic fish predator, the bluehead wrasse, *Thalassoma bifasciatum*, has an innate aversion to blue prey (Miller & Pawlik, 2013). While not directly comparable to our results, because conditions surrounding the use of visual signals (e.g. perception or conspicuousness) differ in an aquatic environment, aquatic predators could encounter distasteful nudibranchs that display blue or adjacent orange and blue coloration, such as those in the genus *Chromodoris*.

Effects of Colour on Rates of Learning

The effects of colours on warning signal learning were not as clear as their effects on innate avoidance. Orange was clearly effectively learned as a warning signal and blue and orange-and-blue were learned as effectively as orange. However, quail chicks did not learn to associate blue with unpalatability significantly faster than black (when blue was the palatable colour), and orange-and-blue was only learned more quickly than black in one measure. Based on our results, we conclude that all three types of prey that we tested could effectively be associated with unpalatability, but that this association was learned most effectively and quickly when the prey items were orange.

Warning signal learning can be affected by several factors, such as conspicuousness or distinctiveness (Gamberale-Stille, 2001; Merilaita & Ruxton, 2007; Prudic, Skemp, & Papaj, 2007). Learning can also be affected by colour, that is, some colours may not be able to be learned or will be learned less effectively (e.g. Exnerová et al., 2006; Lyytinen, Alatalo, Lindström, & Mappes, 2001; Miller & Pawlik, 2013; but see Ham et al., 2006; Roper & Cook, 1989; Sandre, Stevens, & Mappes, 2010; Svádová et al., 2009). Previous studies, in the process of measuring warning signal patterns and conspicuousness, have demonstrated that blue can be learned when presented as an unpalatable stimulus (Aronsson & Gamberale-Stille, 2008; Gamberale-Stille & Guilford, 2003; Gittleman, Harvey, & Greenwood, 1980). However, to our knowledge, there has been no systematic comparison of the effectiveness of blue or adjacent blue and orange coloration to more common long-wavelength colours by avian predators. In a comparison of basic colours, aquatic bluehead wrasses learned to avoid blue, red and orange unpalatable stimuli but not yellow, purple and white prey stimuli (Miller & Pawlik, 2013). Our work builds on previous research by demonstrating that avian predators learn to

avoid orange unpalatable prey more effectively than they do blue unpalatable prey.

Generalization

Generalization by an experienced predator to novel stimuli may be influenced by three factors: (1) the warning signal they learned (e.g. Svádová et al., 2009), (2) the toxicity of the learned stimulus (e.g. Darst & Cummings, 2006) and (3) the effectiveness of the novel stimulus (e.g. Gamberale & Tullberg, 1996). If a bird learns to associate a more salient warning signal with unpalatability, or learns a signal associated with a highly unpalatable prey, it may be more likely to generalize to other stimuli. Alternatively, the novel stimulus itself may influence the level of generalization. Our results are consistent with this latter hypothesis because we found no treatment effect on the generalization response: all of our stimuli had the same level of unpalatability, but the colour of the novel stimulus was a significant factor in our experiment. Birds from all treatments were less likely to attack the orange prey and the orange-and-blue prey. This could be a result of the differences in innate avoidance, or a result of asymmetrical generalization. For example, Svádová et al. (2009) found that birds had asymmetrical generalization to warning colours: birds that learned red did not generalize to yellow or white, but birds that learned yellow generalized to red, even when all colours had been learned at the same rate. Previous experiments have found similar relationships with orange, red and yellow stimuli (e.g. Gamberale-Stille & Tullberg, 1999; Ham et al., 2006), but our experiment took into account short-wavelength coloration and adjacent orange and blue coloration. Orange and blue colours are spectrally different and thus our experiment may not be directly comparable to studies of generalization among long-wavelength colours, but we found that orange-and-blue provided a more effective novel stimulus to which predators generalized their learned responses.

Conclusions

Our results demonstrate that blue alone was not as effective as orange alone at deterring quails' predatory responses through innate aversion, learning or generalization. However, we previously found that a different avian predator (curve-billed thrasher, *Toxostoma curvirostre*) could recognize blue warning signals in the laboratory (Pegram, Lillo, et al., 2013). The reason for the differences between the studies is unknown. It could be due to differences among studies in the specific predators involved and their respective psychologies (Guilford & Dawkins, 1991; Ruxton, Sherratt, & Speed, 2004; Speed, 2000). Because we tested young quail in this study, but adult thrashers in the previous study, differences between these species, or their developmental conditions or prior experience might also have differentially influenced their feeding choices (e.g. Exnerová et al., 2007; Marples & Kelly, 1999; Marples et al., 1998). We note, however, that strong responses to orange were consistent across both studies, which means that orange is likely to be a more reliable warning signal if it is effective against different predators with different developmental histories.

The blue colour tested in our experiment was iridescent, which could have affected the results because of the directionality or brightness of the signal. The directionality could have resulted in varying appearances of the prey, as the brightness and hue of an iridescent signal will shift as the angle of view of the predator changes (Fig. 1). In the experimental design, we controlled the angle of the iridescent pieces so that all tents were bright from the same direction. However, there was no restriction on how the quail chicks approached the prey, so it may not have been as bright or may have looked different if they approached from different angles every time. This same sort of shifting appearance may also be a factor in the learning and recognition of prey in nature, potentially having negative consequences on learning, and future investigations on iridescent warning signals is warranted (Doucet & Meadows, 2009; Théry & Gomez, 2010).

The adjacent orange-and-blue prey item in our experiment was designed to model the natural coloration of *B. philenor* and other similarly coloured and mimetic species. This colour combination may be a unimodal multicomponent signal consisting of the long-wavelength element and the short-wavelength element. These two colours may create a more effective warning signal by creating a buffer against variation in predator visual capabilities or variation in conspicuousness under different environmental light conditions (Pegram, Han, & Rutowski, 2012). In the study reported here (with one predator and standard light conditions), we found no evidence of increased efficacy through display of both colours over just one colour, which complicates the determination of an adaptive advantage to displaying both colours. The structure of the prey with adjacent orange-and-blue patches in our experiment differed from that of the other three prey items, which were all single colours, in that it contained an internal contrast pattern. While some experiments have demonstrated that colour is more important than pattern and internal contrast in both innate avoidance (Roper & Cook, 1989) and learning (Aronsson & Gamberale-Stille, 2008, 2009) in birds, others have demonstrated that internal contrast can be important (Aronsson & Gamberale-Stille, 2013; Hauglund et al., 2006). In addition, the size of each individual colour was smaller than in the single coloured prey, and larger signals are known to increase warning signal effectiveness (Forsman & Merilaita, 1999), but the overall amount of the colour was the same.

From this study and the results of our other study, we conclude that blue displays can function as warning signals to avian predators, but they may not be as effective as long-wavelength colour signals. However, blue signals, and especially iridescent signals, could also be more effective than even long-wavelength signals if put into the right environmental context, such as low ambient light levels (Pegram et al., 2012). We encourage further exploration into the efficacy of short-wavelength warning signal components and how the interactions between predators and prey might be shaped by the visual environment and visual perceptions of predators.

Acknowledgments

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APPENDIX D

APPROVALS FROM INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number: **11-1175R**
Protocol Title: **Warning Signal Learning by Avian Predators**
Principal Investigator: **Ronald Rutowski**
Date of Action: **02/23/2011**

The animal protocol review was considered by the Committee and the following decisions were made:

- ☒ The original protocol was APPROVED as presented.
- ☐ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as presented.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENTS:
Total # of Animals: 640 **Pain Level: C** **Species: Birds**
Approval Period: 02/23/2011 – 02/22/2014

Signature: _____ Date: 2/24/11
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair

Institutional Animal Care and Use Committee (IACUC)
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Animal Protocol Review

ASU Protocol Number:	**14-1349R**
Protocol Title:	**Learning of Iridescent Warning Signals**
Principal Investigator:	**Ronald Rutowski**
Date of Action:	**01/23/2014**

The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved as presented.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**288**
Species:	**Birds** **Pain Level:** **C**
Protocol Approval Period:	**01/23/2014 – 01/22/2017**

Sponsor:	**National Science Foundation**
ASU Proposal/Award #:	**025456**
Title:	**Iridescent Color Signals in Context: Interactions among Behavior, Perception, Function and Light Environment**

Signature: C Johnson for D. Murphy Date: 1/27/14
IACUC Chair or Designee

Cc: IACUC Office
IACUC Chair

APPENDIX E

PERMISSION TO USE PUBLISHED ARTICLES

All co-authors have granted permission for published work to be included.

Koninklijke Brill NV has granted permission for the text of Chapter 1 to be included:

Pegram, K.V., Lillo, M.J., & Rutowski, R.L. 2013. Iridescent blue and orange components contribute to the recognition of a multicomponent warning signal. *Behaviour*, *150*, 321–336.

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