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Interacting Effects of Eyespot Number and Ultraviolet Reflectivity on Predation Risk in *Bicyclus anynana* (Lepidoptera: Nymphalidae)

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Abstract

Small marginal eyespots on lepidopteran wings are conspicuous elements that attract a predator's attention to deflect attacks away from the body, but the role of ultraviolet (UV) reflectivity at the center of these patterns and variation in eyespot number in altering the function of eyespots remains unclear. Here, we performed a field-based predation experiment with artificial prey items based on the appearance of squinting bush brown butterflies *Bicyclus anynana* (Butler, 1879). We tested how two visual properties of the wing pattern affect predation risk: i) the number of eyespots on the ventral forewing surface—two or four; and ii) the UV reflectivity of eyespot centers—normal (where the UV reflectivity of the centers contrasts strongly with that of the darker surrounding ring) or blocked (where this contrast is reduced). In total, 807 prey items were deployed at two sites. We found a significant interaction between the number of ventral forewing eyespots and UV reflectivity in the eyespot centers: in items with fewer eyespots, blocking UV resulted in increased predation risk whereas in items with more eyespots, blocking UV resulted in decreased predation risk. If higher predation of paper models can be equated with higher levels of wing margin/eyespot conspicuity, these results demonstrate that UV reflectivity is an important factor in making eyespots more conspicuous to predators and suggest that the fitness of particular butterfly eyespot number variants may depend on the presence or absence of UV in their centers and on the ability of local predator guilds to detect UV.

Key words: predation experiment, UV, lepidoptera

Eyespots are color pattern elements with contrasting concentric rings (Stevens 2005, Skelhorn et al. 2016) that can be found on various animal taxa such as some fish (Neudecker 1989, Winemiller 1990) and in multiple insect orders including lepidopterans (Stevens 2005, Monteiro 2008, Kodandaramaiah 2011). In lepidopterans, in addition to a role in sexual signaling (Robertson and Monteiro 2005, Prudic et al. 2011, Westerman et al. 2014), eyespots are generally thought to deter predators: either intimidating them by mimicking the eyes of the predator's enemies (Vallin et al. 2005), or deflecting attacks to less important body parts by being conspicuous markings (Stevens 2005, Olofsson et al. 2010, Prudic et al. 2015). In butterflies, various aspects of eyespot appearance are known to affect this anti-predator function, including size (Ho et al. 2016), degree of eye mimicry (Blut et al. 2012, De Bona et al. 2015), pairedness (Mukherjee and Kodandaramaiah 2015), symmetry (Forsman and Herrstrom 2004), conspicuousness (Stevens et al. 2007, 2008a), and the larger visual context (Lyytinen et al. 2004a, Stevens et al. 2008b). However, the effects of many properties require further study, including eyespot number (i.e., the number of eyespots found on the wing surface)

and ultraviolet (UV) reflectivity (i.e., how the eyespots reflect light in the UV wavelengths).

There is large variation in eyespot number across wings, across butterfly taxa, and also across individuals within a species. Comparative data on 480 nymphalid genera indicate that ventral hindwings have twice as many eyespots as ventral forewings (Tokita et al. 2013), but variation in total eyespot number as well as in their distribution across forewings and hindwings is also present across closely related species within a genus, and within a species. Within the *Mycalesis* genus alone, for example, *Mycalesis intermedia* has eight ventral eyespots (Corbet and Pendelbury 1934); *Mycalesis anaxias* has 10 (Bingham 1905); *Mycalesis anapita* has 11 (Kirton 2001); and *Mycalesis malsarida* has 12 (Bingham 1905). Among *Bicyclus* spp.: *Bicyclus anynana* (Butler, 1879) and *Bicyclus auricruda* have 9 ventral eyespots (Butler 1868, Brakefield et al. 2009), *Bicyclus funebris* has 10 (Guérin-Méneville 1844), and *Bicyclus lamani* has 11 (Aurivillius 1901). When comparing species with the same number of eyespots, their distribution across the fore- and hindwings may differ, e.g., *B. anynana* has two and seven

eyespots on the ventral fore- and hindwings whereas *B. auricruda* has three and six, respectively. In addition, polymorphism exists within individual species, even after discounting differences between wet and dry season forms (Brakefield and French 1993, Westerman et al. 2014). For example, different *Mycalesis perseus* individuals have between 9 and 11 ventral eyespots (Bingham 1905); *Mycalesis gotama charaka* have 8 to 12 ventral eyespots (Varshney 1994); and *Mycalesis mineus* have between 7 and 11 ventral eyespots, 2 to 4 on the forewing, and 5 to 7 on the hindwing (Bingham 1905). However, whether predation pressures play a role in maintaining this variation in ventral eyespot number in general (Kodandaramaiah 2011) or in creating eyespot number asymmetry across forewings and hindwings (Tokita et al. 2013) is not clearly understood.

Another area in particular that would benefit from further investigation is the role of UV reflectivity within eyespots in altering predation risk, as known predators of butterflies such as birds (Cuthill et al. 2000), lizards (Font 2014), and mantids (Sontag 1971) are all able to see UV light. To date, no predation study on eyespot number has specifically investigated the effects of UV and little is known regarding how it may influence predation risk. Evidence supporting its importance, however, does exist: Olofsson et al. (2010) discovered that the presence of UV wavelengths in low ambient lighting is important for the UV-reflective white eyespot centers of the woodland brown butterflies *Lopinga achine* to deflect attacks by blue tits *Cyanistes caeruleus*. Conversely, Brunton and Majerus (1995) provided indirect evidence (i.e., a high degree of variability in UV reflectivity within many different species) suggesting that UV may be more important for intra-specific communication than predation. Butterflies are indeed known to be able to perceive UV (Arikawa et al. 1987, Koshitaka et al. 2008, Bybee et al. 2012) and there is evidence that UV reflectivity within eyespots is important in sexual selection (Robertson and Monteiro 2005, Huq et al. 2019). Furthermore, dry season forms of *B. anynana* lower the UV-reflectivity of their hindwing ventral eyespots relative to that of wet season forms, presumably to decrease conspicuousness, but maintain UV-reflectivity in forewing ventral eyespots (Monteiro et al. 2015) which are used by females in the dry season to signal to males (Huq et al. 2019). Hence, it remains unclear whether UV reflectivity within butterfly eyespots plays a role in deterring predators or serves primarily as a sexual signal. In this study, we employ a field-based predation study with artificial prey items to test whether two properties of eyespots on butterfly wings affect predation risk: i) eyespot number on ventral forewings; and ii) the contrast between the UV reflectance of the eyespot center and that of the surrounding ring.

Materials and Methods

Artificial Prey Items

Bicyclus anynana (Butler, 1879) was chosen as the model species for the artificial prey items in this experiment for three reasons: a) previous predation experiments on their eyespots have been carried out and some potential predators are known (e.g., Lyytinen et al. 2004a, Vlieger and Brakefield 2007, Prudic 2015), but it is still unclear how eyespot number variation on the ventral forewings affects survival in the wild; b) the wet season form of the species bears eyespots with UV-reflective centers on the ventral surfaces of its fore- and hindwings which are naturally displayed at rest; and c) members of the genus display variation in forewing eyespot number, whose function remains unclear.

To test the effect of eyespot number on predation risk in the wild, we first imaged two different phenotypes of *B. anynana* butterflies: i) the wild type (wt) with two eyespots on the ventral forewing (Fig.

1A), and ii) the mutant Spotty (sp) line with four eyespots on the ventral forewing (Fig. 1B; Brakefield and French 1993, Monteiro et al. 2007). Both lines have seven eyespots on the ventral hindwing. Using Adobe Photoshop CC 2018, we moved the sinuous area containing the four eyespots on the forewing of the sp image to the wt image to create the sp model. The images were printed on acid free Fabriano Tiziano Bianco 160gsm paper (which we found to have the highest UV reflectance after comparing over 80 types of paper) using a Brother DCP-J562DW printer and corrected using Adobe Photoshop to match the hue, saturation, and brightness of actual butterflies.

To test the effect of eyespot UV reflectivity and contrast with the surrounding area, an Edding 8280 Securitas UV permanent marker pen was used in half of the printed images to block the UV reflectivity of the eyespot centres ('blocked UV'); in the remainder, the pen was used to trace the surrounding black ring in order to both control for the odor of the ink and enhance the contrast between the ring and the unblocked center (hereafter called 'normal UV'). To verify the effects of this blocking, we viewed the prey items using a Canon VIXIA HV40 UVcorder (Fig. 1C and D). One result is that the black rings in items with normal UV were slightly more UV-absorbent than those in items with blocked UV. This enhancement of the contrast between the centers and the black ring actually better approximates the control models to the visual properties of real *B. anynana* wings (Supp Fig. 1A–C [online only]), and the images show that we were successful in creating one category of prey item with strong UV contrast and one with no UV contrast between the eyespot center and the black surrounding ring. In total, four types of artificial prey items were created: i) wt with normal UV, ii) sp with normal UV, iii) wt with blocked UV, and iv) sp with blocked UV. The additional eyespots in the final sp prey items (Fig. 1B) measured 3 and 3.5 mm in diameter (measured along an axis parallel to the veins, crossing the center, and up to the end of the gold ring), and the two eyespots which are common to both sp and wt items measured 2.5 and 5.5 mm in diameter. Each printed image was cut out using a Jinka YS380 desktop vinyl cutter (based on vector templates made with Adobe Illustrator CC 2018) and attached using Blutac onto a mealworm (i.e., a *Zophobas morio* larva) to create a complete prey item (Fig. 2A).

Predation Experiments

The artificial prey items were deployed in secondary forests at two sites: Koh Lon in Thailand (7°47′29.62″N, 98°22′09.9″E; Fig. 2B) and Green Park in Sri Lanka (6°41′29.2″N 80°48′32.9″E; Fig. 2C). They were positioned at least 3 m apart and fixed using green florist wire coiled around wooden sticks in a manner which allowed them to sway in the wind (a movement hypothesized to be important for attracting predators; Ho et al. 2016; Fig. 2A). Prey items in Sri Lanka were exposed to predators for 24 h and those in Thailand for 48 h (determined by pilot studies to be the amount of time required for at least 25% of the models to have been preyed upon). Those items with a missing mealworm or obvious damage to the mealworm or wings at the end of the stipulated period were considered to have been preyed upon.

Statistical Analysis

The results were analyzed using the lme4 package (Bates et al. 2015) in R v3.6.0 (R Core Team 2019). We used generalized linear mixed models (GLMM) with a binomial error distribution, with prey status (whether the prey item had been attacked or not) as the response variable, eyespot number and UV reflectivity as fixed effects that
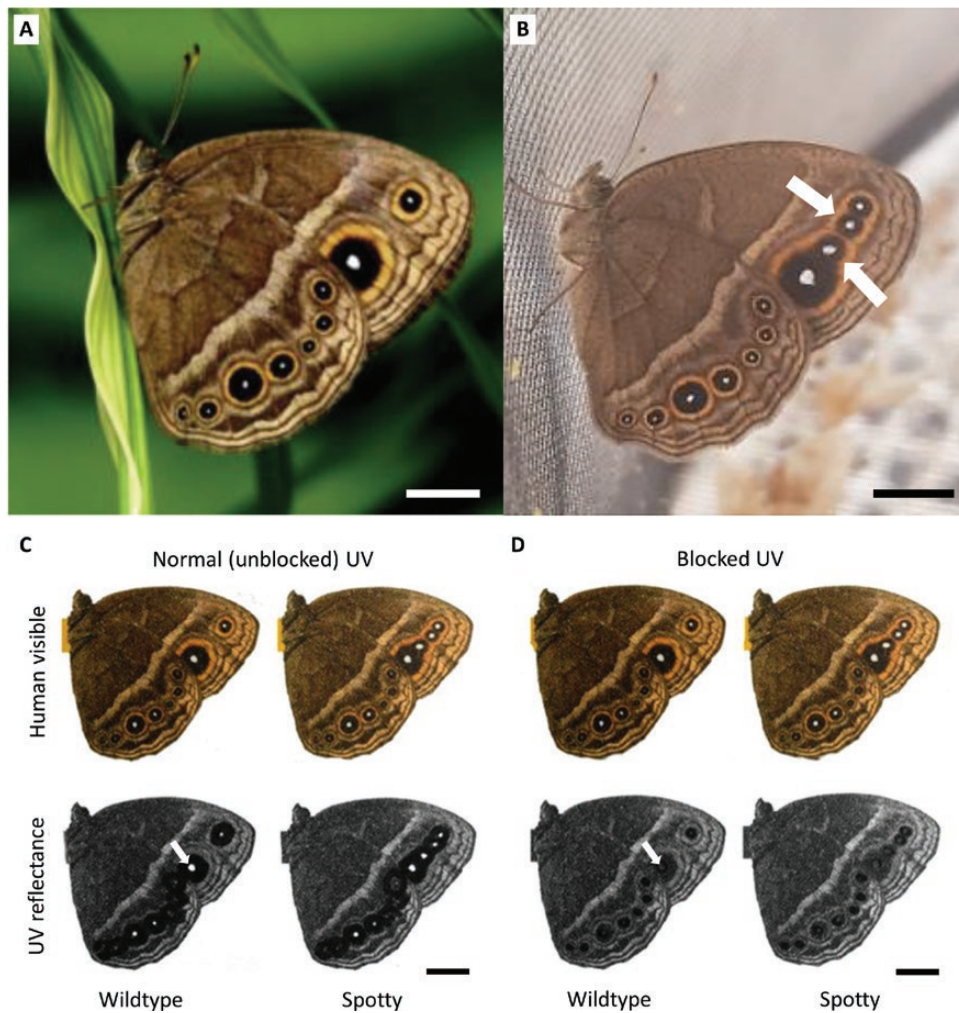
Fig. 1. (**A**) The wild-type and (**B**) Spotty images used to design the artificial prey items, with, respectively, two and four spots on the ventral surface of the forewing (white arrows in B indicate the two extra eyespots in the Spotty line). To test the effects of UV reflectivity at the eyespot centers, artificial prey items with normal (i.e., unblocked) UV reflectivity (**C**) were deployed alongside prey items with blocked UV reflectivity (**D**) (white arrows in C and D indicate examples of the same eyespot center blocked or unblocked). All scale bars are 4 mm.

were allowed to interact, and site as a random effect. Model suitability was assessed using binned residual plots drawn using the arm package (Gelman and Su 2018) to check whether the residuals fell within ±2 SEs (Gelman and Hill 2006). Where simplification was performed, models were compared using a chi-squared test with the anova function in base R. All data and R code used to perform the analysis are included in the Supp Material (online only).

Results

Of the 658 prey items deployed in Sri Lanka (Fig. 3A), 39.8% were attacked: among the 318 wt items deployed, 126 (39.6%) were attacked, and items with normal UV (19.8%) were attacked less often than those with blocked UV (48.8%); among the 340 sp items deployed, 136 (40.0%) were attacked, and items with normal UV (46.4%) were attacked more often than those with blocked UV (34.9%). Of the 159 prey items deployed in Thailand (Fig. 3B), 27.7% were attacked: among the 75 wt items deployed, 21 (28.0%) were attacked, and items with normal UV (22.6%) were attacked less often than those with blocked UV (31.8%); among the 84 sp items deployed, 23 (27.4%) were attacked, and items with normal

UV (33.3%) were attacked more often than those with blocked UV (21.4%).

The GLMM (Fig. 4) showed that prey items with more eyespots (i.e., the sp) were 1.8 times ($e^{0.5699}$) more likely to be attacked ($P < 0.01$), and items with blocked UV reflectivity were 1.6 times ($e^{0.4885}$) more likely to be attacked ($P = 0.016$). The analysis also showed that these effects were interdependent: the interaction between the two factors was highly significant ($P < 0.001$), and blocking UV decreased attack rates in sp items (with more eyespots) but increased attack rates in wt items (with fewer eyespots) (Fig. 3).

Discussion

Eyespots in butterflies are known to have an antipredator function (Lyytinen et al. 2003, Stevens 2005, Olofsson 2010, De Bona et al. 2015, Prudic 2015), but the effects of forewing eyespot number and UV reflectivity of the white center within eyespots on predation risk remain poorly studied. Here, we tested the influence of these two properties by performing a predation experiment using four types of artificial prey items: with two versus four forewing eyespots, and with versus without UV reflectivity contrast between the eyespot center and

Fig. 2. (**A**) Live prey items (mealworms) were attached onto sticks using green wire to allow them to sway in the wind, a movement which is thought to be important for attracting predators. The sites at which prey items were deployed in (**B**) Koh Lon, Thailand, and (**C**) Green Park, Sri Lanka were both secondary forests.
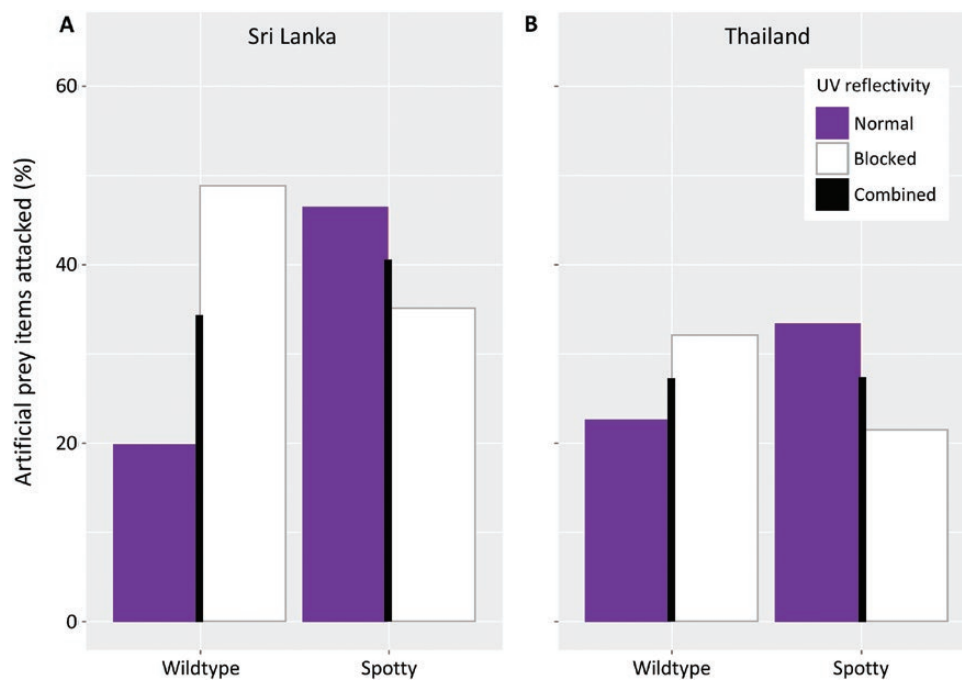

Fig. 3. The data on the proportion of prey items attacked show an interaction between eyespot number and UV reflectivity contrast within eyespot centers in both Sri Lanka (**A**) and Thailand (**B**). Blocking UV reflectivity increased the predation rate on prey items with fewer eyespots (wild type, with two ventral forewing eyespots) but decreased the predation rate on items with more eyespots (Spotty, with four corresponding eyespots). Combined percentages shown here are the mean of the normal and blocked percentages.

the surrounding black ring. Our results show that eyespot number and UV reflectivity had significant and interacting effects on predation risk: blocking UV increased attack rates on items with fewer eyespots but decreased attack rates in items with more eyespots.

The effects of eyespot number on predation risk are complex. Several studies, including those by Stevens et al. (2008a), Merilaita et al. (2011), Mukherjee and Kodandaramaiah (2015), Ho et al. (2016), and Halali et al. (2019), have examined the roles of eyespot number, eyespot size, and other factors but came to different

conclusions. This may be partly accounted for by the explanation that some eyespots intimidate (i.e., repel) predators, while others deflect them (i.e., attract them to less essential body parts), and that these two types of eyespots are likely to exhibit different visual characteristics (Kodandaramaiah et al. 2013). Furthermore, in the case of deflective eyespots, our results with models of *B. anynana* (whose eyespots are known to deflect predators; Lyytinen et al. 2004a, Prudic et al. 2015) suggest that UV reflectivity also plays a role in the ability of small eyespots to mitigate predation risk.
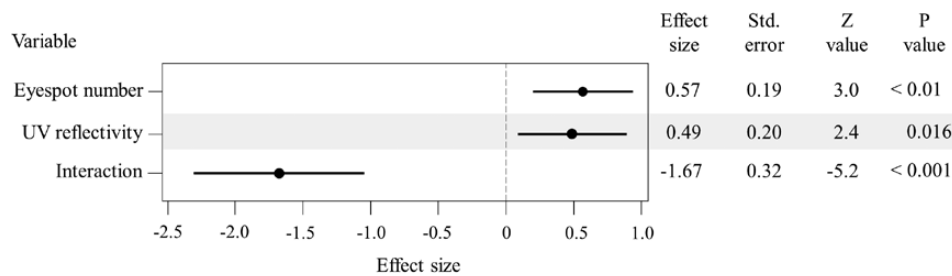
Fig. 4. The generalized linear mixed model showed that eyespot number, UV reflectivity and the interaction between the two variables had significant effects on the attack rate on the artificial prey items. Horizontal black bars represent the 95% CIs of the effect sizes.

Although the overall predation rates on wild-type and Spotty prey items in our study are similar (the black bars in Fig. 3), our data indicate that the effect of eyespot number on predation risk is dependent on UV reflectivity within the eyespots (the colored bars in Fig. 3). With eyespots whose center contrasts sharply against its immediately surrounding ring, prey items bearing more eyespots experienced higher attack rates, supporting the conclusions of Ho et al. (2016) who also used such eyespots (but where the UV was added to the paper wings with a special UV-reflective white paint; Supp Fig. 1D and E [online only]). We propose, as did Ho et al. (2016), that increases in the number of UV-reflective eyespot centers increases prey conspicuity, drawing more attacks from predators. Conversely, with eyespots whose center and surrounding ring are not contrasting in terms of UV reflectivity, an increase in eyespot number led to lower attack rates. This indicates that increases in forewing color pattern complexity (added eyespots) with wavelengths restricted to the visible range may make prey items more cryptic to predators, perhaps by better camouflaging them against the background. These results agree with the observation that exposed ventral patterns on many butterfly wings tend to be more detailed and complex, with many different earthy colors, than hidden patterns on dorsal surfaces which often display large swaths of single bright UV-reflective colors to attract mates (Allen et al. 2008, Oliver et al. 2009).

Hence, we propose that UV reflectivity in eyespot pattern elements of butterfly prey are essential in making eyespots more conspicuous to predators and should be taken into consideration in future experiments. It should be noted that, in our control UV-reflective prey items (i.e., with unblocked eyespot centers), the entire black ring surrounding the white eyespot centers were painted with a UV-blocking marker. Whilst this treatment made the eyespots more realistic (Supp Fig. B and C [online only]), it led to two factors (in addition to the main test factor, i.e., the presence or absence of UV in the center) being changed simultaneously: the relative conspicuity of the center increased (because UV-reflectivity in the surrounding black ring decreased), and more marker ink was used. It would be beneficial for future studies to control for the amount of ink used (e.g., by painting a spot of similar size on controls, ideally on an area with naturally low UV reflectance such as the black ring in our items) and to investigate whether the degree of conspicuity of the eyespot center relative to its surroundings also impacts predation risk.

Considering the nymphalids in general, most species have half as many eyespots on forewings compared to their hindwings (Tokita et al. 2013), suggesting that enhanced forewing conspicuousness, achieved by the addition of extra small and UV-reflective eyespots, may not increase prey survival in real life situations. When located on hindwing margins, small UV-reflective eyespots would deflect predator attacks to the butterflies' hindwings and allow them a greater chance of escape and survival, e.g., in the case of common evening browns *Melanitis leda* (Halali et al. 2019) and the wet season form of *B. anynana* (Prudic et al. 2015). The increased presence of such eyespots on forewings (e.g., in the case of Spotty wings), however, is likely to be disadvantageous because forewings are generally more important for powering flight (Dudley 2002, Jantzen and Eisner 2008) and forewing damage would make the butterflies more vulnerable to subsequent attacks. Hence, it would be interesting to test whether the asymmetric arrangement of eyespots (i.e., with fewer on the forewing than the hindwing) across the wings of most nymphalid butterflies might be an adaptation. It is important, however, to utilize live prey items in these studies because artificial models cannot predict prey escape behavior from nonlethal predator attacks targeting a less important 'body part'.

Our results add to the growing body of evidence supporting the importance of UV wavelengths in visual signaling by butterflies (Church et al. 1998, Lyytinen et al. 2004b; although see Blut and Lunau 2015). It would be interesting for subsequent work to examine how eyespot number and eyespot center UV reflectivity (which has not been examined in most nymphalids) may co-evolve. Furthermore, evolutionary shifts in eyespot number and/or their UV reflectivity would ultimately depend on the predator guild preying on local butterfly populations and their ability to see in the UV. It is reasonable to expect that other cues such as visible colors, and the shape, size, randomness, or repetition of markings (e.g., spots, stripes, and eyespots) are used by predators to identify prey from their resting background. However, whether predation risk is affected by how markings are repeated across an animal's body remains poorly understood as existing studies tend to measure pattern properties in general (e.g., Troscianko et al. 2016). While previous work has shown that the number of markings has some effect (e.g., Mukherjee and Kodandaramaiah 2015, Ho et al. 2016), this is only one aspect of repeatedness. The potential effects of specific properties—such as the relative positions of markings or the heterogeneity in their distribution—are rarely examined (although see Stoddard and Stevens 2010) and this is a promising area for future studies to explore.

Supplementary Material

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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