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Defensive Coloration and Behavior in Insects: Function, Sensory Perception Mechanism, and Evolution

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Defensive Coloration and Behavior in Insects: Function, Sensory Perception Mechanism, and Evolution

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General Abstract

Cryptic color patterns in prey are classical examples of adaptations to avoid predation, but we still know little about behaviors that reinforce the match between animal body and the background. From observations of geometrid moths, *Hypomecis roboraria* and *Jankowskia fuscaria,* I determined that the positioning behavior, which consists of walking and turning the body while repeatedly lifting and lowering the wings, resulted in new resting spots and body orientations in *J. fuscaria,* and in new resting spots in *H. roboraria*. The body positioning behavior of the two species significantly decreased the probability of visual detection by humans, who viewed photos of the moths taken before and after the positioning behavior. This implies that body positioning significantly increases the camouflage effect provided by moth's cryptic color pattern regardless of whether the behavior involves a new body orientation or not. However, this positioning behavior is not always performed: some moths stay put on the initial landing position. We hypothesized that the moth's decision of whether or not to re-position itself is related to its crypticity at the landing spot. We determined the crypticity from a detection task experiment, in which 'human foragers' searched for the moths in photos of moths at their landing spots. Moths that landed on the less cryptic positions were more likely to reposition themselves to the more cryptic positions. In contrast, moths that already landed on substantially cryptic positions were less likely to reposition themselves. Next I explored how a moth finds an appropriate resting position and orientation. Here, I used a geometrid moth *Jankowskia fuscaria* to examine i) whether a choice of resting orientation by moths depends on the properties of natural background, and ii) what sensory cues moths use. We studied moths' behavior on natural (a tree log) and artificial backgrounds, each of which was designed to mimic one of the hypothetical cues that moths may perceive on a

tree trunk (visual pattern, directional furrow structure, and curvature). We found that moths mainly used structural cues from the background when choosing their resting position and orientation. Then I tested through which sensory organs (which are directly related to sensory information types) moths perceive bark structures to find adaptive resting orientations. We amputated (or blocked) one of the hypothetical sensory organs from moths (antennae, forelegs, wings, and eyes) and tested whether they were still able to perceive bark structures properly. We found that moths use visual information from eyes and tactile information from wings to perceive bark structure and to adopt cryptic resting orientations. These studies collectively show how behavior mediates the camouflage of moths in nature and how moths perceive background information to find a cryptic spot.

Next. I tackled the questions about a new strategy of chemically defended prey to show warning coloration to predators. Aposematic coloration in prey promotes its survival by conspicuously advertising unpalatability to predators. Although classical examples of aposematic signals involve constant presentation of a signal at a distance, some animals suddenly display warning colors only when they are attacked. Characteristics of body parts suddenly displayed, such as conspicuous coloration or eyespot pattern, may increase the survival of the prey by startling the predator, and/or by signaling unpalatability to the predators at the moment of attack. The adaptive value of such color patterns suddenly displayed by unpalatable prey has not been studied. We experimentally blackened the red patch in the conspicuous red-white-black hindwing pattern displayed by an unpalatable insect *Lycorma delicatula* (Hemiptera: Fulgoridae) in response to predator’s attack. There was no evidence that the presence of the red patch increased prey survival over several weeks. Next I asked the adaptive significance of this hidden-warning coloration and explored the mechanistic question how it provides survival benefits

to the bearer. The main advantage of aposematism is that it enhances learning by predators to avoid the prey. Using wild birds (*Parus minor*) and novel prey models, I tested whether hidden conspicuous display of defended prey accelerates the avoidance learning rate of predators and how does it compare with the typical conspicuous/non-conspicuous signal. We found the evidence that hidden aposematic signal indeed accelerates the avoidance learning rate of predators by two stages: i) enhancing the learning rate of the association between non-conspicuous (normal) state of the prey and prey defense, ii) promoting rejection after an attack has occurred by showing hidden conspicuous coloration. We show here the unique defensive coloration of prey which may provide dual benefits of crypsis and aposematism and highlight the specific mechanisms that hidden-aposematic signal provide the prey with survival benefits.

Keywords: predator-prey, animal coloration, camouflage, aposematism, startle display, insects

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General Introduction

The colors of animals affect many aspects of animal life. The animal colorations are usually a product of natural/sexual selection and have diverse functions such as deterring predators, thermoregulation, and attracting sexual partners (Endler 1978). In animals that evolved under strong selection for avoidance of visually hunting predators color patterns function as anti-predatory devices to avoid predation (Poulton 1980; Cott 1940; Ruxton et al. 2004).

Broadly speaking, there are three functional types of defensive coloration: crypsis (camouflage), aposematism (warning coloration), and mimicry. Crypsis makes the prey hard to detect or recognize against background. Cryptic animals accomplish crypsis by several mechanisms such as background matching (the color or pattern that resemble those of the background), disruptive coloration (disrupting the outline of animal body), or countershading (Cott 1940; Stevens & Merilaita 2012). Aposematism deters predators by having noticeable bright colors which signals the defense of prey (such as toxins or spines) to predators (Ruxton et al. 2004). Mimicry is a defensive mechanism by means of which non-toxic prey gains protection by mimicking the color patterns of toxic prey (Ruxton et al. 2004).

To effectively signal their color patterns to appropriate receivers, animals not only possess adaptive color patterns, but they should also present them against proper background. For example, a cryptic leaf-resembling prey looks cryptic near leaves, but reveals itself on sand or tree bark. Therefore, behavioral elements of animals adaptations to avoid predation are crucial, and they determine the final protection against predation. . However, to date, the contribution of behavioral elements has largely been neglected in the study of animal coloration. Therefore, I studied behavior of insects that co-evolved with their morphology to effectively signal (or conceal) their body color patterns to predators.

In this thesis, I mainly studied two research themes: 1) behaviorally mediated camouflage in moths, and 2) a strategy of prey that normally remains cryptic but displays warning coloration when being attacked (hereafter, hidden-aposematic coloration). Chapter one to four consist of the research on behaviorally mediated camouflage in moths, and chapter five to six consist of the research on hidden-aposematic coloration.

Specifically, in chapter one, I tested how bark-like moths behaviorally modify their resting positions and body orientations to find locally most cryptic spots after landing on tree trunks. I reported ‘positioning behavior’ in moths for the first time and showed that this behavior actually reinforces the crypticity of moths. However, based on the observations in the field, moths seem to selectively perform this positioning behavior. In chapter two, I tested whether the decision of moths whether re-position or not after landing on tree trunks depends on the crypticity of the moths at landing position. Throughout chapter one and two, I show the ability of moths to seek out a cryptic resting positions and body orientations in a fine scale for effective camouflage.

In chapter three, I tackled two questions that are related to the sensory perception mechanism of moths: 1) do moths rely on background information when choosing body positions and orientations on tree trunk? and if they are, 2) which information from tree bark (visual patterns, furrow structures, roundness of tree trunk) moths perceive and use when choosing body orientations? The results suggest that moths perceive furrow structure when choosing body orientations. In chapter four, I investigated the sensory organs that responsible for the perception of furrow structure. As hypothetical sensory organs, I considered antennae, forelegs, wings, and eyes and tested which sensory organ(s) is responsible when perceiving furrow structure and adopting adaptive resting orientations. These questions are classic, but fundamental to understand how morphology, behavior, and sensory mechanisms co-evolved to achieve camouflage.

In chapter five, I tested whether an aposematic lantern bug *Lycorma delicatula* in natural population gains survival benefits by possessing bright hindwings. This insect has cryptic forewings and brightly colored (red/white/black combined) hindwings. The behavior that I mainly concerned for the study is that it remains cryptic normally but suddenly shows bright hindwings when disturbed (typically called “startle display”). I hypothesized that bright hindwings of *L. delicatula* may function to deter predators and tested this hypothesize by manipulating their hindwing colors. In chapter six, I tested whether hidden-aposematic coloration of chemically defended prey enhances avoidance learning rate of predators as typical aposematic signal. As far as I know, the adaptive significance of hidden-aposematic coloration and the mechanism by means of which prey increases survival has never been tested empirically. During my Ph.D., I attempted to reveal the effectiveness of this strategy for deterring predators and tried to reveal the mechanism by which hidden-aposematic coloration gains a protection from predators.

Chapter 1. Camouflage through an active choice of a resting spot and body orientation in moths

ABSTRACT

Cryptic color patterns in prey are classical examples of adaptations to avoid predation, but we still know little about behaviors that reinforce the match between animal body and the background. For example, moths avoid predators by matching their color patterns with the background. Active choice of a species-specific body orientation has been suggested as important function of body positioning behavior performed by moths after landing on the bark. However, the contribution of this behavior, to moths' crypticity has not been directly measured. From observations of geometrid moths, *Hypomecis roboraria* and *Jankowskia fuscaria,* we determined that the positioning behavior, which consists of walking and turning the body while repeatedly lifting and lowering the wings, resulted in new resting spots and body orientations in *J. fuscaria*, and in new resting spots in *H. roboraria*. The body positioning behavior of the two species significantly decreased the probability of visual detection by humans, who viewed photos of the moths taken before and after the positioning behavior. This implies that body positioning significantly increases the camouflage effect provided by moth's cryptic color pattern regardless of whether the behavior involves a new body orientation or not. Our study demonstrates that evolution of morphological adaptations, such as color pattern of moths, cannot be fully understood without taking into account a behavioral phenotype that coevolved with the morphology for increasing the adaptive value of the morphological trait.

INTRODUCTION

Evolution of adaptations through natural selection is the central theory in biology (Darwin 1859; Fisher 1930), and crypticity of moths (morphological phenotype) has been the icon of morphological adaptations to avoid predation throughout the history of evolutionary biology (Thayer 1909; Cott 1940; Kettlewell 1955a,b; Endler 1984). Most non-toxic and non-mimetic moths are inconspicuous in their natural habitat due to color-patterns on their wings that provide camouflage when the moths rest in species-specific sites (Poulton 1890; Thayer 1909; Cott 1940; Endler 1978; Endler 1984). Whereas morphological aspects of crypsis, such as disruptive coloration (Cuthill *et al*. 2005; Fraser *et al*. 2007; Cuthill & Székely 2009) and background matching pattern (Summer 1934; Pietrewicz & Kamil 1977; Stuart-Fox et al. 2004) have attracted the attention of researchers, some crucial aspects of behavioral adaptations are poorly understood. The importance of previously unexplored aspect of behavior in moth crypticity has became apparent in recent discussions (Majerus 1998; Wells 2000) of Kettlewell's classic research (Kettlewell 1955a; Kettlewell 1955b), as well as in popular attempts to criticize evolution as a scientific discipline (Wells 2000; Hooper 2003).

While the preference of moths for landing on substrates that generally resemble the moth's color has been well studied (Sargent 1966; Sargent 1973; Steward 1976; Kettlewell & Conn 1977; Endler 1984), it alone cannot explain how the final cryptic match between the patterns on the moth wings and the patterns on the background such as tree bark is achieved. This is because the landing spots and body orientations at the moment of, and immediately after, landing on a trunk are different from the spots and body orientations in which the moths finally rest on a substrate. The final resting positions and orientations, which result from body positioning behaviors after landing (Sargent 1969b), are species-specific (Endler 1978) and have been hypothesized to be the key adaptation to achieve crypticity (Sargent & Keiper 1969). The positioning behavior is more crucial than the general preference for trunk coloration, because even if a moth landed on a bark with a coloration or a pattern different from its own, the behavioral positioning may in principle lead to considerable crypsis through disruptive coloration or matching between the wing and background patterns. Therefore, the positioning behaviors performed by moths after landing are essential to account for the almost perfect match between the pattern on the moth wing and the pattern of the bark. However, the body positioning behavior has only been approached from the proximal perspective (Sargent 1969b), and no direct test of adaptive role of this behavior has been conducted.

The effect of final resting orientation (resulting from the body positioning behavior) on crypsis has been experimentally confirmed by Pietrewicz & Kamil (1977), Webster et al. (2009), Wang & Schaefer (2012). Pieterwicz & Kamil (1977) showed that the camouflage effect of species-specific body orientation is the largest on a bark of tree species that provides the typical species-specific resting sites for the moths. Researchers interested in more general principles of crypsis also confirmed that orientation of patterns in artificial prey models relative to the orientation of patterns in the background has strong effect on the degree of crypsis (Wang & Schaefer 2012). However, the adaptive role of behavioral adjustments of a resting spot by moths after landing has been largely neglected.

Although, in his classical experiment, Kettlewell (Kettlewell 1955b, but see also Majerus 2005) used wild birds to measure detectability of live moths after the moths landed on natural substrates, the experimental paradigm that has been employed over the last 50 years can be summarized as "*an experimenter arranges the prey – a predator attempts to detect it*". Researchers used specimens pinned on a tree bark (or photographs of thereof), or images copy-pasted onto a tree bark image, in body orientations chosen by the experimenter based on knowledge on how the moths position their bodies (Pietrewicz & Kamil 1977; Webster et al. 2009; Wang & Schaefer 2012). This paradigm ignores that moths may choose landing spots on a bark differently than humans do. This paradigm also ignores that the moths after landing actively search for a "suitable" resting spot, and that the body orientation in nature functions as adaptation for camouflage only when matched with the natural behavior to seek the resting spot. An additional consequence of this typical experimental paradigm was inability to realistically imitate the variation in body orientations of the moths and in the choice of the resting spot.

We decided to switch back to the experimental paradigm originally proposed by Kettlewell (1955b): *a moth positions itself – a predator attempts to detect it*. We focused on measuring the direct consequences of moth behavior in their natural habitat. Rather than artificially arranging moths in positions that are believed to imitate their naturally achieved resting spot and orientation we allowed the moths to do this for us. To determine whether moths' positioning behavior after landing increases their crypticity, we studied two geometrid moths, *Hypomecis roboraria* (Denis & Schiffermüller 1775) and *Jankowskia fuscaria* (Leech 1891), with wings that resemble color patterns of a tree bark (Fig. 1.1; Fig. 1.2).

MATERIALS AND METHODS

Field observations

We studied two geometrid moths, *Hypomecis roboraria* and *Jankowskia fuscaria,* with wings that resemble color patterns of a tree bark (Fig. 1.1; Fig. 1.2). The observations were conducted in Aug-Sep 2010 at Mt. Baek-woon, South Korea (N35º,01',54.30"; E127º,36',22.30"). Moths were collected at night with a black light, kept at 4 ℃ in plastic containers and released one by one in the next morning after waiting at least 30 min for their bodies to warm up to ambient temperature. The moths were released in the same forest habitat in which they were captured comprising mostly pine trees (more than 95% trees were *Pinus taeda*). The release point was surrounded by at least several trees so that the moths could actively choose a tree to which they fly. We watched the behavior of moths in response to being released from containers during day light hours. The species-specific resting sites and postures of moths after such a disturbance do not differ from the postures of undisturbed moths found naturally in the same habitat (pers. obs; Sargent & Keiper 1969). Most of the released moths (except few which fell down to the ground) landed on *Pinus taeda* and the trunk of this tree was used as the background in the subsequent experiments. The exact location of release site varied among the moths.

Preparation of moth images

We used humans as experimental predators of the moths in digital photos taken at the study site. As the moth landed, we took photos of the trunk with a moth at the moth's initial position (*real initial,* i.e. photo before positioning) from 60 cm away using a camera (CanonPowershot S5IS; ISO400, F3.2, 1/125s–1/250s shutter speed and 3264×2448 resolution). After one hour, which is sufficient for the moth to finish positioning, we took photos of the same moth again at the final position (*real final*, i.e. photo after positioning) and collected the moth. Immediately after each moth has been collected, the tree bark area containing both the initial and final location of a moth was photographed again to obtain photo of exactly the same illumination as the photo of the moth in the final position. These three photos (moth on a bark in initial position, moth on a bark in final position, and the bark only containing both spots of the initial and the final position) were used to generate *control* images (see below). We photographed 63 *H.roboraria* and 32 *J. fuscaria*. Among them, 32 *H. roboraria* (51%) and 25 *J. fuscaria* (78%) changed their resting position from the inial spot where they landed to a new one. We excluded the photos where part of the moths' wing was concealed by tree bark. We additionally excluded the moths for which the initial and final positions cannot be included in one background image either because the moth moved over especially long distance or because it moved to another side of the tree trunk. In these cases we were unable to create *control* image because we were unable to overlay the two positions (*initial* and *final*) of the moths on the same background photo. For the experiment, we used photographs of 14 individuals of *H. roboraria* and 16 individuals of *J. fuscaria* at both initial and final positions. All photographs were taken under a thick canopy of forest between 0900-1400 h, and there is no reason to believe that differences in lighting conditions between *initial* and *final* photos were biased. However, because moth detection by humans can also be influenced by the difference in light conditions between the *initial* and *final* photos, we created a pair of photos that differed only in the position and body orientation of a moth, but not in the lighting conditions. Using Photoshop 10.0 CS3 (Adobe), we manually cut out the image of a moth from the *real final* photo, saved it as PNG file, and overlaid it onto a separate bark-only photo of the same area of a trunk (taken after moth collection) at the initial (*control initial* photo) and, in a separate file, at the final (*control final* photo) site/body orientations (see below for examples of photos). Thus, in these *control* photos, *initial* and *final* photos represented similar brightness.

To randomize the position of a moth within the screen presented to the observer, and to include only the bark surface in each photo, we cropped each photo to the size 750×1000 pixels. Cropping made the position of the moth within the screen, and the characteristics of the visible background, dissimilar among four photos of the same individual (*real initial*, *real final*, *control initial* and *control final*). In this way we avoided temporal memory retention (in the human subjects) of moth position on the screen that might have been observed if we used the same background for all four photos (for each moth). Size of the moths was same in all photos (lengths of the longest axis of moth body were 140 pixels for *H. roboraria* and 124 for *J. fuscaria*, which reflects the real size ratio of the two species: 4.24 cm and 3.75 cm (mean of wingspan of 5 females) for *H. roboraria* and *J. fuscaria* respectively). ImageJ 1.43u (National Institute of Health) was used for cropping and resizing the photos. Total 56 photos of *H. roboraria* (14 moths × 4 images) and 64 *J. fuscaria* (16 moths × 4 images) were prepared.

Human predator system

We developed a pictorial puzzle program in Java that presents the photos on the monitor in a specific order, and records the latency until the moth image is clicked. The program also presents the next photo if a participant clicks or spends 10 sec without any click. Sixty participants (30 for *H. roboraria* and 30 for *J. fuscaria*), who were unaware of our experimental purpose and design, were tested. Each participant was asked to find a moth in the photo and click on it. A training session of 6 photos, of additional 6 moths different from those used in the test, was given before the actual test to ensure that the volunteers were accustomed to the shape of the moth and to the testing system. Photos were presented in a random order on the LG Flatron 24” widescreen LCD monitor (1920×1080 resolution which corresponded to the 16:9 ratio) at a distance of 60 cm from the subject’s eyes. To prevent formation of memory about the features of the background, which could affect the perceived crypticity of the moth, the photos of the same moth (in different positions and orientations on the same trunk), were separated by at least four images of other moths.

Statistical analyses

For each species separately, we used generalized linear mixed models (GLMMs) to examine the effects of two fixed factors, the position (*initial* versus *final* position) and the type of photo (*real* versus *control*), as well as the interaction between them, on the probability of a moth being detected (binary) and on the latency to the detection of those moths that have been detected in all four photos (*real initial*, *real final*, *control initial*, *control final*). Identities of human participant and the moth were used as random factors. The latency to the detection of a moth was measured as the time from the beginning of a trial until the moment of moth image being clicked on. This variable was power-transformed to meet the assumptions of GLMMs (Sokal & Rohlf 1995). The orientations of moths were measured as angle from 0-360 degrees and non-parametric circular statistics [Watson’s two sample test for homogeneity (Zar 1999); data did not follow the assumption of parametric test] was performed to compare two groups of angles (*initial* vs *final*). R 2.12.0. (http://www.r-project.org) was used for all statistical analyses.

RESULTS

After a moth was released, it flew towards a neighboring tree, landed on the bark and assumed the initial body orientation (Figs. 1.1a,d). Later, many of the moths repositioned themselves by walking and turning the body while repeatedly lifting and lowering their wings. The positioning behavior started within several minutes after landing (for *H. roboraria*: 224 [98-317]s; median [lower-upper quartile], *n*=12; for *J. fuscaria*: 201[167-241]s, *n*=9). After several attempts at repositioning (1-3 times, average 1.5 times for both species), the moth eventually (after 509 [234-739]s for *H. roboraria*; 208[178-303]s for *J. fuscaria*) remained motionless in the final body position and orientation (Figs. 1.1b,c,e,d).

The final position was always different (within a distance of 50 cm) from the initial position. The final body orientation of *H. roboraria* (93 % of moths oriented horizontally, within 20 degrees from the horizontal plane; Fig. 1.2b) did not differ from the initial body orientation (85% horizontally; $U^2_{14,14}$=0.04 P>0.1; Fig. 1.2a). The final body orientation of *J. fuscaria* significantly differed from the initial orientation (88% vs 13% of individuals oriented horizontally; $U^2_{16,16}$=0.64, $P<0.001$; Fig. 1.2c,d).

Once a moth re-positioned itself, the probability of being detected significantly decreased (effect of position [*initial* or *final*]: χ^2_1=31.69, $P<0.001$ for *H. roboraria*; χ^2_1=49.91, $P<0.001$ for *J. fuscaria*; Figs. 1.3a,b), and the latency to moth detection increased (effect of position: χ^2_1=11.96, $P<0.001$ for *H. roboraria*; χ^2_1=165.02, $P<0.001$ for *J. fuscaria*; Figs. 1.3c,d). For *H. roboraria*, there was no effect of photo type (*control* vs *real*) on the probability of being detected (effect of photo type: χ^2_1=0.37, $P=0.54$; interaction between photo type and position:

χ^2_1=2.91, P=0.09) or on the latency to the detection of the moth (effect of photo type: χ^2_1=1.36, P=0.24; interaction between photo type and position: χ^2_1=0.01, P=0.93).

Although in *J. fuscaria*, the increase in crypticity due to positioning behavior was stronger for real than for control photos in both, the probability of being detected (interaction between positioning and photo type χ^2_1=15.96, P<0.001; effect of photo type: χ^2_1=4.31, P=0.04) and the latency to moth detection (interaction between positioning and photo type χ^2_1=10.10, P<0.001; effect of photo type: χ^2_1=17.11, P<0.001), all the results clearly show that the body positioning behavior resulted in better background matching than the initial position taken by a moth right after landing on the tree trunk.

Figure 1.1. Examples of moths on a bark of *Pinus taeda* before (a,d) and after (b,c,e,f) body positioning behaviours. *H. roboraria* is presented in (a-d). *J. fuscaria* is presented in (d-f). Arrows in (a) and (d) point to the head of a resting moth. Contour lines in (c) and (f) are drawn to make detection of the moth in the final position easier. Original, unmarked versions of (c) and (f) are in (b) and (e).

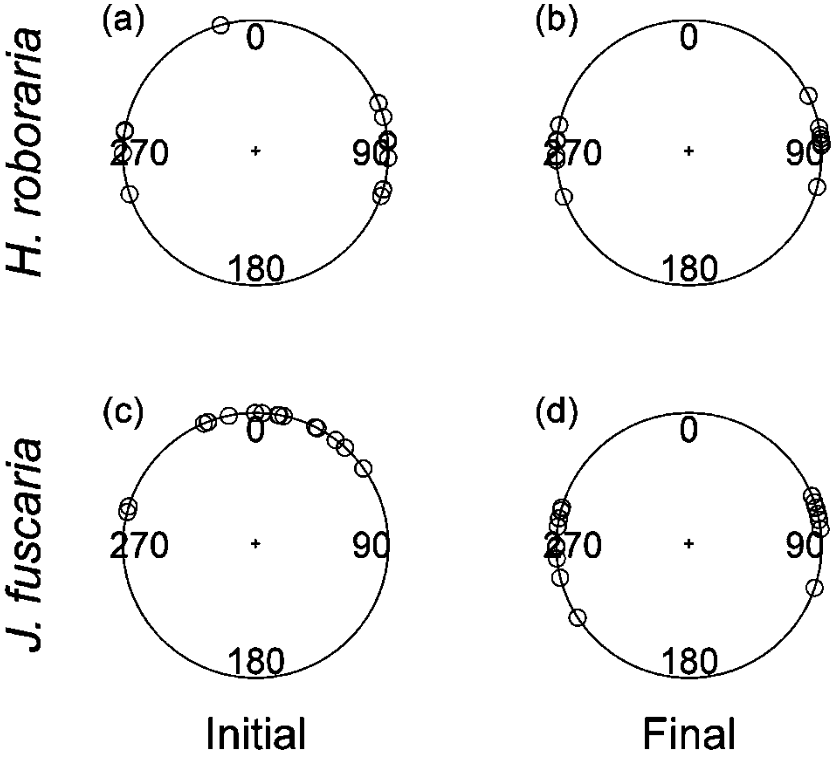


Figure 1.2. Angular distribution of body orientations of *H. roboraria* (a,b) and *J. fuscaria* (c,d) at immediately after landing (initial position; a,c) and after positioning (final position; b,d). Black circles indicate the orientation of the head relative to the orientation of the upright tree. At final position, both moth species orient their heads non-randomly but towards the side (towards 90 and 270 degrees in the graphs).

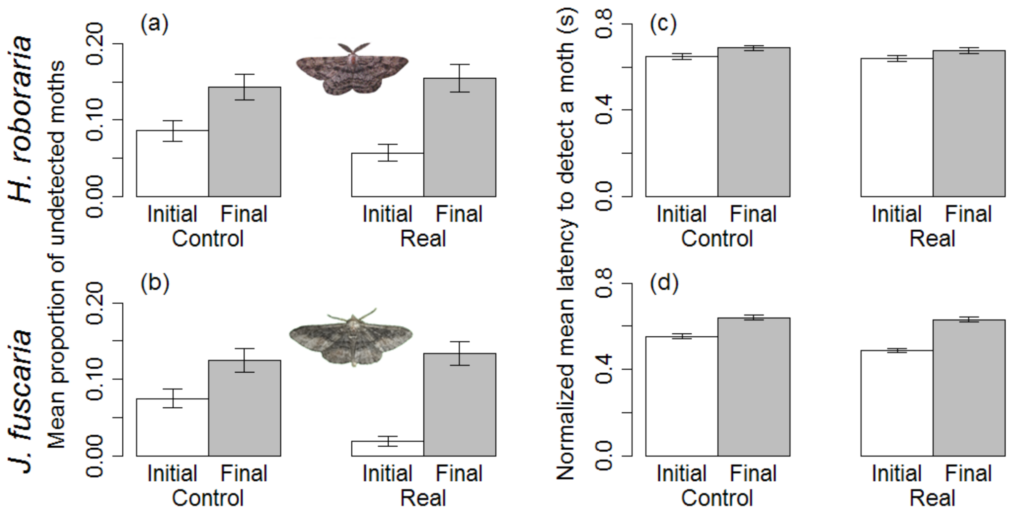


Figure 1.3. Effect of body positioning behaviour on the detectability of moths (mean±s.e.m.). Comparison of the proportions of undetected *H. roboraria* (a) and *J. fuscaria* (b), or the latency to the detection of *H.roboraria* (c) and *J. fuscaria* (d), between the *initial* position, at which the moth landed, and the *final* position, recorded after the moth performed body positioning and body orienting behaviors. Results from *control* and *real* photos are shown separately. The latency to the detection of the moth was power-transformed ($\lambda = -0.83$ for *H. roboraria*, -0.68 for *J. fuscaria*) to comply with normality assumption of the GLMMs.

DISCUSSION

This is the first study that directly measures the detection of moths by visual predators before and after a moth performed the body positioning behavior. The behavior includes finding an appropriate spot on a bark after landing and, at least in some species, adjusting body orientation. The results show that the natural body positioning behavior, involving both the choice of spot on a bark and the choice of body orientation (*J. fuscaria*), or only the selection of the new resting spot (*H. roboraria*), will lead to even greater crypticity against typical substrates than previously shown in experiments using photos of moths specimens on tree trunks (Pietrewicz & Kamil 1977) or moth images (Webster et al. 2009) placed by a researcher on a tree bark. This conclusion is valid for both the real photos, where the natural variation in illumination is present, and for control photos, where the variation in general illumination of the background is artificially minimized by using the same photo of a background similar to the final photo of a moth. The moth silhuette in the initial control photo originates from the final photo where, due to a different body orientation between initial and final positions, and perhaps due to different resting spot and time of picture taking, the moth wing pattern might have been illuminated in a different manner than the natural way expected at the initial position. This is especially crucial if initial and final body orientations differ (such like in *J. fuscaria*), and it might have led to lower crypticity of the initial than the final *control* photos of *J. fuscaria*, as well as to the significant interaction between positioning and photo type for *J. fuscaria*. Nevertheless, the effect of positioning was still clearly visible and significant even in those artificially designed photos, indicating that behaviorally-mediated change of resting spot (for both species) and body orientation (for *J. fuscaria*), rather than change of illumination, lead to significant increase in crypticity at the final resting spots.

While other studies of moth crypticity used both avian (Kettlewell 1955b; Pietrewicz & Kamil 1977; Cuthill & Székely 2009; Wang & Schaefer 2012) and human (Fraser et al. 2007; Cuthill & Székely 2009; Webster et al. 2009) predators, the human predator model is more convenient for experiments (Fraser et al. 2007; Cuthill & Székely 2009; Webster et al. 2009; Tsurui et al. 2010). In studies with simple images on the monitor screen, results were consistent between human and avian predators (Beatty et al. 2005; Fraser et al. 2007; Cuthill & Székely 2009). The main concerns for using humans as predators of color images of insects presented on the computer screen may be the lack of UV sensitivity in humans [unlike in many birds (Cuthill et al. 2000; Majerus et al. 2000)], and unnatural spectral properties of monitor-generated images. However, based on spectrometric data obtained in natural habitats (see Supporting Information), we believe that UV contributes little to the general crypticity of the moths as perceived by UV-sensitive birds.

The effect of body orientation on moth's crypticity has been already addressed in experiments where the moths (specimens or fairly nautralistic models) were presented at random locations on a tree bark to avian or human predators (Pietrewicz & Kamil 1977; Webster et al. 2009; Wang & Schaefer 2012). Additionally, Wang & Schaefer (2012) re-analysed Endler's (1984) dataset including information on moths' orientations at resting sites. These studies not only suggested that moths orient non-randomly in order to maximize their crypticity at resting spots (Pietrewicz & Kamil 1977; Webster et al. 2009; Wang & Schaefer 2012), but also that the camouflage effect of species-specific body orientation is the largest on a bark of tree species that provides the typical species-specific resting sites for the moths. Our results are consistent with these studies: natural body positioning behavior of *J. fuscaria,* which includes adjustments of body orientation, indeed increased moths' crypticity. However, we demonstrated that body positioning comprising only the selection of a new resting spot without significant changes in body orientations, like in *H. roboraria*, is sufficient to significantly increase the moth's crypticity. This suggests that not only a suitable body orientation, but also a choice of a cryptic spot critically reinforces moth crypticity. Although ability of moths to find locally most cryptic spot is generally admitted, no study has formally confirmed that moths actually move toward a cryptic spot from the spot on which they initially land.

The body positioning that occurs soon after a moth lands on a tree trunk is a behavioral mechanism that significantly contributes to moth crypticity in a finer scale, after larger scale processes of habitat choice (Endler 1984) and the choice of tree trunks that match the moth’s color (Sargent 1966; Sargent 1973; Steward 1976; Kettlewell & Conn 1977; Endler 1984) have taken place. It appears that tactile stimuli from the tree bark, rather than vision, are used by the moths for choosing the final position and orientation (Sargent 1969b; Sargent 1973; Lees 1975) in geometrid moths. Therefore, the positioning behavior (choice of spot and body orientation) may not increase moth crypticity if both the coloration and the structure of furrows on the bark are not properly combined in a manner which is characteristic for the typical resting substrate of a moth species. Many classical studies of the moths’ camouflage ignored the role of positioning behavior. Our results suggest that some studies might have overestimated the contribution of moths’ preferences for landing on a bark of a specific color to the crypticity because the camouflage was measured after both the landing preferences and the positioning behavior have been completed (e.g. Kettlewell 1955b). In studies using moth specimens pinned at random spots on a tree bark in body orientations imitating the species-specific orientations (Pietrewicz & Kamil 1977), the contribution of natural body positioning to crypticity might have been underestimated because resting spot selection have not been imitated. We suggest that in the future research the contribution of preferences for landing on a specific bark color should be differentiated from the contribution of the subsequent positioning behaviors to the final crypticity of moths.

In summary, by using one of the iconic examples of evolution, we showed how a morphological adaptation cannot be fully understood without taking into account a full behavioral phenotype responsible in natural situation for increasing the adaptive function of the morphological trait. Our experimental approach to moth crypticity (*a moth positions itself – a predator attempts to detect it*) provides an unexplored method for testing hypotheses about behaviorally mediated morphological evolution (McPeek 1995; Webster et al. 2009) and about cospecialization between morphological and behavioral adaptations (Dewitt et al. 1998). We expect that a quantitative comparative analysis of naturally observed body positioning behaviors among moths with various wing patterns, combined with tests of crypticity against species-specific resting substrates, will illustrate an adaptive coevolution between cryptic morphology and the corresponding behaviors.

SUPPORTING INFORMATION

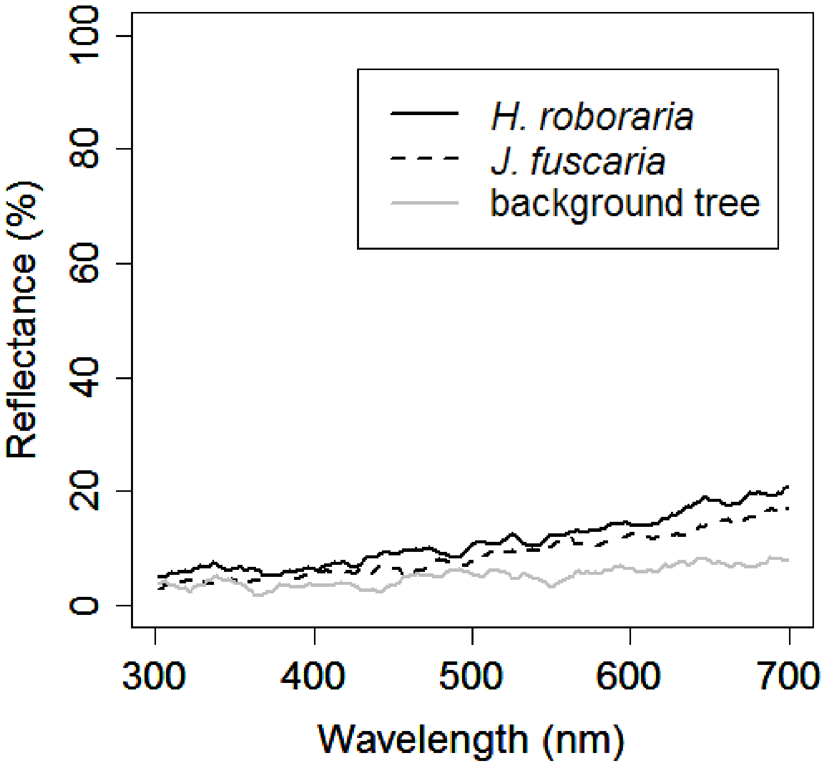


Figure S1.1. Reflectance spectra of *H. roboraria* (black solid line), *J. fuscaria* (black dotted line) and background tree (grey solid line)

Chapter 2. Moths on tree trunks seek out more cryptic positions when their current crypticity is low

ABSTRACT

Many animals use camouflage to avoid predation. Their crypticity, i.e. the degree of a visual match between the animal's body and the background, affects their survival. Therefore, they may develop the ability to choose a proper background, which matches the animal's own color pattern. Previously, we showed that moths, *Hypomecis roboraria,* have the ability to increase their crypticity by repositioning their bodies from the initial landing position to the final, more cryptic, position. However, this repositioning behavior is not always performed: some moths stay put on the initial landing position. We hypothesized that the moth's decision of whether or not to reposition itself is related to its crypticity at the landing spot. We determined the crypticity from a detection task experiment, in which 'human foragers' searched for the moths in photos of moths at their landing spots. Moths that landed on the less cryptic positions were more likely to reposition themselves to the more cryptic positions. In contrast, moths that already landed on substantially cryptic positions were less likely to reposition themselves. We suggest that the tactile cues received by moths from furrows and crevices, the elements of bark structure responsible for the color pattern of the bark, may play a role in mediating this adaptive behavior that results in improving the moths' visual crypticity.

INTRODUCTION

Camouflage decreases the probability of animal being detected or recognized and it is one of the most prevalent evolutionary outcomes of predator-prey arms races (Poulton 1890; Thayer 1918; Cott 1940; Stevens & Merilaita 2011). Visual camouflage can be achieved through a range of concealing mechanisms such as background matching, disruptive coloration or countershading (Endler 1978, 1984; Cuthill et al. 2005; Endler 2006; Fraser et al. 2007; Rowland et al. 2008; Stevens & Merilaita 2011) and depends on the visual patterns on the animal's body in relation to the characteristics of its background. Because the concealment of animals is strongly dependent on background, many animals have developed the ability to choose proper backgrounds and background choice often reinforces the crypticity of animals (Cott 1940; Kettlewell 1955a; Kang et al. 2012).

While the degree of concealment depends on background choice behaviors, it may also affect other behaviors. For example, the degree of crypticity may affect behavior of prey animals (Martin & Lopez 2000; Cuadrado et al. 2001; Cooper & Sherbrooke 2010, Briffa et al. 2008; Briffa & Twyman 2011, Kjernsmo & Merilaita 2012). Although this effect of crypticity may be related to the animal's awareness of its own conspicuousness as suggested in hermit crabs (Briffa & Twyman 2011), the actual perception and awareness of own crypticity in other prey animals that adaptively modify their antipredatory behavior according to their crypticity (e.g. fleeing/immobility of reptiles and finding a matching/complex background of killifish in response to predators' presence) has not been examined, and may not be needed. Recent studies on cuttlefishes suggest that these animals are able to modify their body pattern and colour in response to specific visual properties of the background (Chiao & Hanlon 2001; Barbosa et al. 2008; Allen et al. 2010; Barbosa et al. 2011). Although these behaviors result in higher visual crypticity as perceived by a potential predator (or prey), the underlying sensory processes appear to rely on simple responses to specific elements in the background (Chiao & Hanlon 2001; Barbosa et al. 2008; Allen et al. 2010) rather than on comparison and awareness of own versus background visual patterns.

Here, we ask if similar behavioral adjustments according to degree of crypticity may exist in an insect, the geometrid moth *Hypomecis roboraria* (Denis & Schiffermüller, 1775), and we discuss hypothetical sensory mechanisms that may be responsible for the observed behaviors. Moths actively select a resting substrate, location and body orientation, all of which contribute to the match between the color patterns of the moths' wings and those of the background (Kettlewell 1955a; Sargent 1966, 1973; Boardman et al. 1974; Kettlewell & Conn 1977; Pietrewicz & Kamil 1977; Webster et al. 2009; Kang et al. 2012; Wang & Schaefer 2012). We have recently shown that, after landing on tree bark, two species of geometrid moths, including *H. roboraria*, perform 'repositioning behavior' by walking on the tree bark in search of a final resting position that quite precisely matches wing patterns with those on the bark (Kang et al. 2012).

In our previous study, only half of the *H. roboraria* individuals repositioned their bodies (from the initial landing position to the final resting position) soon after landing (Kang et al. 2012). The other half remained at their initial positions. The individuals that repositioned themselves chose the spot and body orientation that provided a better match between the background and the moth wings than before repositioning. However it is still unclear why some individuals stayed put at their initial landing position without repositioning themselves. Here, we ask the follow-up question of whether their decision to reposition or not is related to the degree of crypticity in their initial (landing) position. If moths behave in an adaptive manner and if they improve their crypticity only when the current crypticity is low, we predicted that the landing positions of moths that did not decide to reposition their bodies (fixed positions) will be characterized by higher crypticity than the landing positions (initial positions) of moths that later decided to reposition themselves to the final positions.

We also predicted that the probability of repositioning will be higher for moths that landed at positions of low crypticity.

MATERIALS AND METHODS

Comparison of crypticity of moths using humans as predators – experimental design

We used a geometrid moth, the Great oak beauty (*Hypomecis roboraria;* Fig. 2.1 and 2.2), for testing our hypothesis. The Great oak beauty is a monomorphic species which has a color pattern resembling tree bark. After landing on a tree bark, the species is known to perform repositioning behavior, which reinforces its visual crypticity (Kang et al. 2012). The repositioning behavior (choosing a new position defined as a combination of location and body orientation) usually occurs a few times within one hour after landing. In our previous study, only 51% of *H. roboraria* repositioned their bodies after landing on tree bark while the others stayed put on the initial landing spots (Kang et al. 2012). We compared the crypticity of moths in three situations (treatments): Fixed, Initial and Final position. Fixed position is the position of a moth that did not reposition its body and remained at the landing spot and in the original body orientations assumed at landing. Initial position is the position of a moth just after landing for only those moths that later repositioned their bodies. Final position is the position of a moth after the repositioning behavior has taken place (only for those moths that repositioned themselves).

Although the comparison of crypticity between Initial and Final group was investigated in our previous paper (Kang et al. 2012) we included the two groups so that we could provide a direct comparison between Fixed-Initial and Fixed-Final groups. To quantitatively measure the degree of crypticity of moths, we used our previous method (Kang et al. 2012), which employs photographs of moths presented to humans as visual “foragers” (Kang et al. 2012).

Moths were collected at night near black lights, kept individually in small containers, tested the next morning. We released the moths in a forest (near the collection site) one by one and followed each moth until it landed on the initial landing position. All the tested moths landed on tree trunks of *Pinus taeda* (which comprised more than 95% of tree species near our releasing site). The choice of the landing tree and landing position was totally dependent on the moths. Then we took photographs of each moth twice using a Canon Powershot S5IS with one hour interval, which is sufficient time for the moths to reposition. From the photographs, we categorized the photos of moths into three groups: Initial, Final, and Fixed (see above methods for the description of each group). We successfully tracked and took photographs of 63 moths (32 moths that repositioned their bodies and 31 moths that remained at landing position). We discarded some photos where a part of the moth wing was concealed by furrow structure or photos were blurred. Then we resized the photos so that moths were of similar size in each. We cropped each photo to the size of 750×1000 pixels (corresponds to 21 × 28cm in the monitor screen). While cropping, we randomized the position of moth shape in each photo (i.e. the spatial position of moth shape in each cropped image was pre-determined by random x,y coordinates generation) to neutralize any effect of spatial position of moths in the monitor screen on their detectability. The size of moth shape (length of the longest axis of the moth body) was set to 125 pixels (corresponds to 35 mm in the monitor screen).

We used the custom-built pictorial puzzle program (previously used in Kang et al. 2012) to measure the performance of humans in detecting the moths in prepared photos on a monitor screen (24 inches LG widescreen LCD monitor with 1920×1080 resolution setting). This program presents the photos on the monitor in a designated order, and if a human participant clicks the target or spends certain time without a click, it presents the next photo. Each human participant, who was totally unaware of our experimental purpose, was asked to find a moth in each photo, and to click on it within 10 seconds. If participants clicked the wrong area or failed to click within 10 seconds, the next photo was presented and the moth was considered to have survived. Before real testing, all participants went through a training session to be accustomed to the testing system.

In each photo, we measured (the program automatically recorded) the latency to the detection (clicking) of a moth (continuous response variable) and whether the moth was detected or not within 10 seconds (binary response). A total of 49 real photos from 32 moths (15 moths at fixed position, and 17 moths at initial and final positions) were shown to 30 human participants. All participants were presented with all the 49 photos. The presenting order of the photos was randomized and was always different between participants. Therefore, although it is likely that the participants became better able to detect moth targets towards the end of the series of images (due to search image formulation), the effect of presenting order, if any, should not vary between treatment groups. All the photographs were taken in 2010 Aug-Sep at Choosan field station, Mt. Baekwoon, South Korea (N35º,01’,54.30”; E127º,36’,22.30”).

Because difference in photographing conditions between treatments may influence moth detection by human subjects, we first confirmed that colours in photos did not differ between treatment groups. For each photo, we used the RGB values extracted from the pixels of each color channel to compare the colour and intensity of photos. We randomly selected 100 points in each photo and extracted RGB values for each pixel. Then we averaged the RGB values of the 100 selected pixels (each channel separately) and this averaged value was used as the mean RGB value of each photo. These values of images in each treatment group were compared by multivariate analysis of variance (MANOVA; the mean RGB values as response variables, treatment groups as an explanatory variable). We found no difference in RGB values between treatment groups (MANOVA: *Wilk's lambda*=0.94, *approximate* $F_{6,88}$=0.43, P=0.86). Therefore we considered that there was no perceptual difference in colour properties between each treatment group.

ImageJ 1.44p (National Institute of Health, Bethesda, Maryland, USA) was used for pixel selection and RGB value extraction.

Although humans are not natural predators of moths, human predators have been successfully used in detection task experiments (Fraser et al. 2007; Cuthill & Szekely 2009; Webster et al. 2009; Tsurui et al. 2010; Bohlin et al. 2012; Kang et al. 2012). There are several advantages of using human predators over real predators: control of the factors that can influence the behaviour of real predators (previous experience, hunger level or the degree of tameness to experimental condition), and the wide applicability of various experimental design. Furthermore, the results of detection task experiments using human predators are congruent with those using natural predators (Beatty et al. 2005; Fraser et al. 2007; Cuthill & Szekely 2009). The most problematic issue of using humans over natural predators is that the visual sensory system of humans is different from those of natural predators, mainly lack of UV detection (Cuthill et al. 2000). In this experiment, both tree bark and wings of *H. roboraria* reflect low levels of UV (less than 7% throughout whole UV spectrum range) and we consider that UV contributes insignificantly to the crypticity of moths.

Comparison of crypticity of moths using humans as predators – statistical analysis

We used R 2.14.0 (http://www.r-project.org) for all statistical analysis. We employed Generalized Linear Mixed Models (GLMMs; using 'glmer' function in 'lme4' package) to compare the two response variables (the binary response of whether a moth was detected or not within 10 sec, and the latency to the detection of a moth in each photo if the moth was detected) between treatment groups. However comparison of the three treatment groups (Fixed, Initial and Final) in one analysis is statistically invalid because Initial and Final groups are paired photographs of the same moth whereas photographs at fixed positions are from different moths. For comparison between Initial and Final positions, we set human subject id and moth id as random factors. For the comparison between Fixed and Initial and between Fixed and Final groups, we set human subject id as a random factor. We transformed the latency by Box-Cox power transformation (Sokal & Rohlf 1995) to satisfy the assumptions of GLMMs (λ = -0.70, -0.71, -0.36 for Initial-Final, Initial-Fixed and Final-Fixed, respectively). P-values were adjusted by Holm correction (Holm 1979) to give control of the family-wise error rate.

We defined a moth's orientation as the angle between the direction in which its head points out and the direction indicated by the main axis of the tree trunk (mostly standing upright). Hence, the orientation of each moth was measured as an angle from 0 to 360 degrees which runs in the clockwise direction. For example, 0 degrees indicates a moth headed upwards and 90 degrees indicates a moth headed towards the right side. Since the angular data did not follow the assumptions of parametric tests (von Mises circular normal distribution), we performed Watson's two sample test for homogeneity (non-parametric circular statistics) to compare three groups of angles (Zar 1999). We presented approximated P-values for Watson's two sample test because exact P-values cannot be calculated by currently existing critical U^2 statistic table (Zar 1999).

All the field experiments were conducted within a 2.5 hour period (1000-1230), and we assumed that the effect of the time of the test on moths' behaviour was negligible. All the statistical tests were two-tailed.

Relationship between initial crypticity and probability of repositioning

To test directly whether the occurrence of repositioning behavior is related to the crypticity in the initial position, we initially used the original two variables obtained from each test (the binary response variable indicating whether the moth was detected or not within 10 sec, and the latency to the detection of a moth in each photo if the moth was detected within 10 sec) to calculate two new variables for each moth image: the proportion of humans who detected the moth and the mean latency to detect the moth if detected in 10 sec. Because these two new variables measured only slightly different aspects of crypticity and were correlated with each other (the Pearson's product-moment correlation test: t_{30}=6.24, r=0.75, P<0.001) we used Principal Component Analysis (PCA) to create principal component (Principal Component 1; PC1) as a composite crypticity index. This initial crypticity index ($PC1_{10s}$) explained 88% of total variance of the data (*eigenvalue*=1.76), and positively correlated with the original two variables (Pearson product-moment correlation test: $PC1_{10s}$-mean latency, t_{30}=14.55, r=0.94, P<0.001; $PC1_{10s}$-proportion undetected, t_{30}=14.55, r=0.94, P<0.001). However, it did not differentiate the moths well.

Many moths were located in the lower range of values of crypticity and they differed little among each other with this respect: only 13% of data (4 out of 32) were located within the upper 50% of the range of the crypticity values (Fig. 2.S1c). This was due to the skewed distributions of the two contributing variables (Fig. 2.S1a, b), and especially the proportion of humans who detected the moth within 10 sec (only 3 out of 32 moths have above proportion of 0.4). Hence, the power of testing the relationship over the upper range of crypticity was expected to be low.

By shortening the time basis from 10 sec to 8 sec, followed by shortening it to 6 sec and then to 4 sec, we devised a new index of crypticity that used less skewed variables (Fig. 2.S1d, e) and resulted in a more even distribution over the whole range of crypticity and in a better differentiation among moths in their crypticity values (Fig. 2.S1f). We also tried a PC extracted from the proportion of detection during 4 sec and the original latency until detection (measured between 0-10 sec). Hence, we decided to use all latencies, including those larger than 4 sec. However, the resulting PC also had a skewed distribution (Fig. 2.S2; only five moths were in upper range of crypticity values).

All these analyses resulted in similar outcomes and we present them in Appendices (Fig. 2.S1 and S2). But only the $PC1_{4s}$ had low degree of skewness of the distribution. Hence, for the main analysis, we used the principal component from two variables: proportion of humans who detected a moth during 4 sec, and the average latency to detect for those subjects who detected a moth during 4 sec. The $PC1_{4s}$ explained 78% of total variance of the data (*eigenvalue*=1.58), and positively correlated with the two original variables (Pearson product-moment correlation test: $PC1_{4s}$-mean latency, $t_{30}=10.41$, $r=0.89$, $P<0.001$; $PC1_{4s}$-proportion undetected, $t_{30}=10.41$, $r=0.89$, $P<0.001$). Then, we used Generalized Linear Model (GLMs) with binomial errors and logit link to determine the effect of crypticity index (the $PC1_{4s}$) on the binary variable that indicates whether a moth repositioned itself or not (1= a moth repositioned itself after landing; 0 = a moth did not reposition itself after landing).

RESULTS

Comparison of crypticity of moths using humans as predators

The detection probability of the moths that did not reposition themselves and remained in their landing positions (Fixed group) was significantly lower than the detection probability at landing positions (Initial group) for those moths that later repositioned themselves (GLMMs: $\chi^2_1=9.60$, *adjusted* $P=0.004$), but significantly higher than the detection probability of these moths in their final positions (Final group), after they repositioned themselves (GLMMs: $\chi^2_1=5.53$, *adjusted* $P=0.02$, Fig. 2.1a). The latency to detection of the moths in the Fixed group was significantly longer than that of the moths in the Initial group (GLMMs: $\chi^2_1=13.62$, *adjusted* $P<0.001$) but shorter than that of the moths in the Final group (GLMMs: $\chi^2_1=10.75$, *adjusted* $P=0.001$, Fig. 2.1b). After repositioning, the detection probability decreased (comparison between Initial and Final; GLMMs: $\chi^2_1=42.31$, *adjusted* $P<0.001$, Fig. 2.1a), and the latency to detection increased (GLMMs: $\chi^2_1=81.96$, *adjusted* $P<0.001$, Fig. 2.1b). Orientations of moths did not differ among the groups (Watson's two sample test for homogeneity: Initial-Final, $N_1=N_2=17$, $U^2=0.07$, $P\approx0.50$; Initial-Fixed, $N_1=15$, $N_2=17$, $U^2=0.11$, $P\approx0.20$; Final-Fixed, $N_1=15$, $N_2=17$, $U^2=0.11$, $P\approx0.20$; Fig. 2.1c). These results suggest that the repositioning behaviour involves changing resting positions rather than changing body orientation, and that those moths that were already in relatively cryptic positions rarely repositioned themselves, even though repositioning would still increase their crypticity.

Relationship between initial crypticity and probability of repositioning

The probability of repositioning decreased when the crypticity at the initial landing spot increased (Fig. 2; GLMs: χ^2_1=4.42, *P*=0.04). This tendency was also present when we used different crypticity indices for the analysis (see Fig. 2.S1 and 2.S2). Seventy percent of moths (12 out of 17) that were at the lower crypticity landing positions ($PC1_{4s} < 0$) repositioned their bodies, whereas only 14% (one out of seven) of those who were at the higher crypticity positions ($PC1_{4s} > 1$) repositioned their bodies.

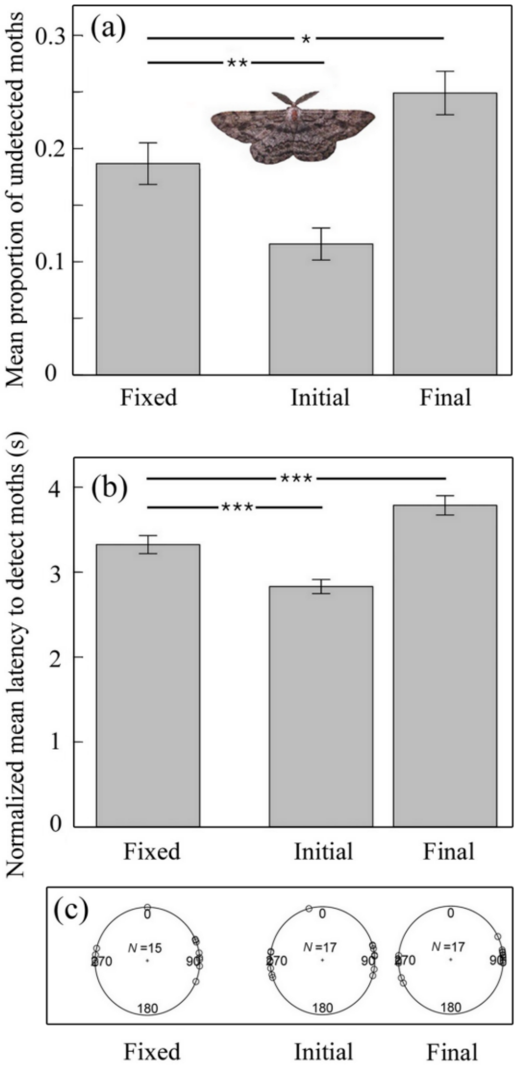


Figure 2.1. Comparisons between three types of moth, *H. roboraria,* locations with respect to the proportion of undetected moths (a), the latency to the detection (b), and the angular distributions of body orientations (c). Types of locations: “Fixed” - locations of moths that did not reposition themselves after landing; “Initial” – landing locations of moths that later repositioned themselves to the “Final” positions. The latency to the detection in (b) is transformed by Box-Cox transformation (see methods). The columns and error bars indicate mean and standard error of the mean, respectively. A photo of *H. roboraria* is inserted within (a). In (c), black circles indicate the orientation of the head relative to the vertical upward orientation. Almost all moths in the three treatment groups orient their heads towards the side (towards 90 and 270 degrees in the graphs).

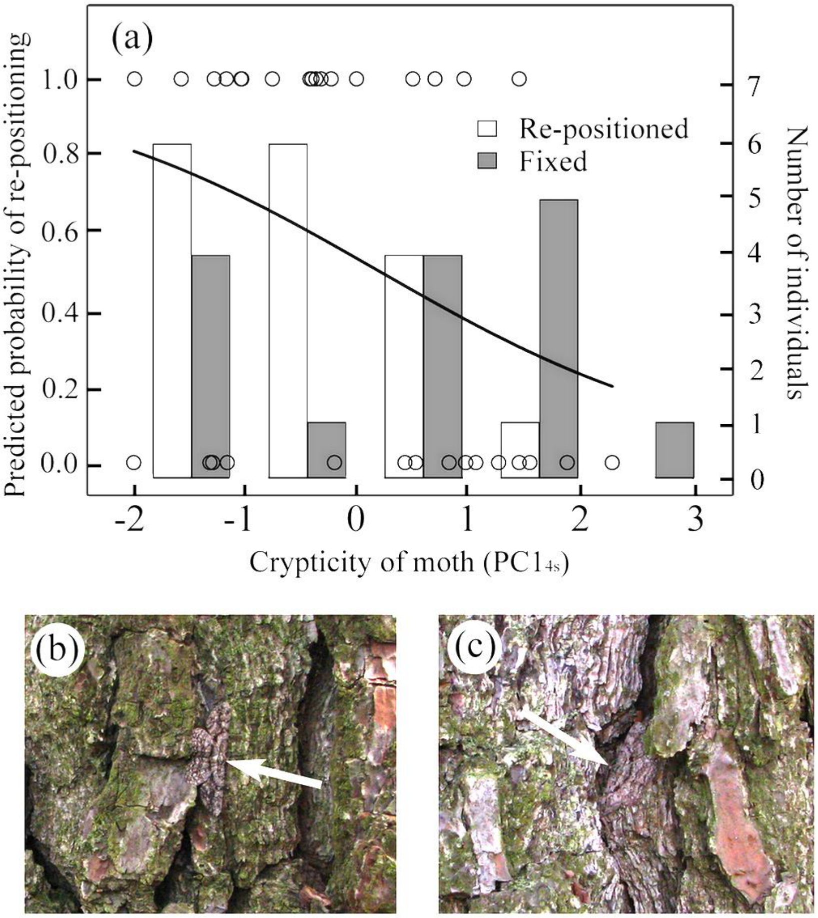


Figure 2.2. The effect of crypticity on the decision of moths to reposition. (a) The predicted probability of repositioning (the black line, left vertical axis) as a function of crypticity index. This relationship is derived from behaviour of *N*=32 moths (each circle represents one moth; 0 represents moths that did not reposition themselves, and 1 represents moths that repositioned themselves). Y-axis on the left shows whether a moth repositioned itself or not. The bar histogram, and the corresponding Y-axis on the right, shows the number of individuals who did (*N*=17) and those who did not (*N*=15) reposition their bodies in each of classes of crypticity values. (b) An example of a moth with low crypticity. (c) An example of a moth with high crypticity.

DISCUSSION

We have shown here that cryptically patterned moths adaptively adjust their behavior to their current level of crypticity: the moths that achieved substantial crypticity right after landing tended to stay put while the moths that landed on positions that offered low crypticity repositioned themselves and eventually achieved better camouflage at the new position. While our current findings do not provide information to determine the sensory mechanisms of these adaptive behavioural decisions by moths, together with our previous study (Kang et al. 2012) they indicate that moths not only are able to actively find a cryptic spot soon after landing (Kang et al. 2012), but they are more likely to do so when their current crypticity is low.

While the sensory mechanisms that underlie this adaptive behavioral decision by moths are unknown, moths need not be aware of their own pattern in relation to the pattern of the bark. Although moths perform subtle wing-lifting movements that may potentially allow for visual comparison between patterns on wings and on the nearby tree bark, it would be difficult or impossible for moths to visually recognize detailed visual patterns considering the location and resolution of moths' eyes (Land & Nilsson 2002). But previous experiments on moths indicated that vision is used to choose landing substrate according to its general reflectance (Kettlewell 1955a; Sargent 1966; Sargent 1968; Kettlewell & Conn 1977; Grant & Howlett 1988), and that some moths, once they have landed on the substrate, have a general preference for an edge between dark and light substrate (Lees 1975). However, our recent study on another geometrid indicates that these general visual stimuli are not crucial in choosing the resting spot via repositioning behavior (unpublished data).

The direct experimental evidence for the importance of tactile stimuli from the bark structure in finding a cryptic spot by geometrids (Kang et al. 2013) suggests that tactile stimuli may be important in triggering the decision to reposition and in finding the new resting position. For example, it is possible that a specific set of tactile stimuli, typically associated in this monomorphic species with poorer crypticity on the bark of their preferred tree species, may trigger the repositioning behavior while a different set of tactile stimuli, typically associated in this species with good crypticity, may promote staying put at the landing spot. Importance of tactile stimuli also has been suggested in experiments on artificial substrates (Sargent 1969b) and from observations of moths on natural substrates (Steward 1976). Therefore, we suspect that predation-mediated selection on monomorphic geometrid moths led to the evolution of moth sensitivity to a species-specific set of tactile cues from the bark structure and that the resulting decisions to reposition based on these tactile stimuli produce the species-specific resting positions that increase the species-specific visual crypticity of moths on their preferred tree species.

Specifically, we hypothesize that *H. roboraria* moths find a cryptic spot on a bark by finding a body position at which the frontal edge of forewings “touches” an edge of a furrow in the bark. This may lead to a concealment of the frontal outline of the moth body (which is ‘hidden” in the furrow or aligns with the furrow outline), and the degree of this “touching” may be also correlated, on average, with the degree of matching between the visual pattern on the wing and that on the bark. Whether this mechanism of repositioning evolved after or before the evolution of body patterns that match patterns on bark is currently unknown.

The performance of repositioning behavior does not seem to depend on individuality of moths because in an additional field test (repeated observations on the same individuals; see Table 2.S1; Fig. 2.S3), there was no apparent within-individual consistency in the occurrence of repositioning behavior. This may indicate that the occurrence of repositioning behavior depends on the circumstances that vary among resting spots. Although in this additional field test we did not measure the degree of crypticity at each spot, we believe that the degree of match between the bark and moth wings is one of the important characteristics that varies among consecutive landing spots of the same individual and affects moths' decision to reposition.

Our results suggest that if certain crypticity was already achieved at initial landing, the moths rarely repositioned themselves, even though repositioning would still improve their camouflage. We consider two adaptive hypotheses for this behavior. First, an increase in predation risk during conspicuous movements on the bark while repositioning their bodies can be more costly than the costs of remaining in this position at the intermediate level of crypticity for a day (maximally). Second, it is possible that above a certain threshold of match between the wing and bark pattern, the probability of a bird detecting a moth may be very low and further improvement in crypticity is not necessary. Saltatory search for prey by birds involves hops and pauses of species-specific durations (Anderson et al. 1997). Each hop brings a bird to a new spot where searching for prey lasts for the species-specific pause duration modeled as optimal giving up time (Anderson 1981). Only during the pauses, lasting for a half to a few seconds (0.54 sec for the painted redstart (Jablonski & Strausfeld 2000); 2.4 sec for wood gleaning warbler (Landres & MacMahon 1980)), the capacity of birds to scan their immediate surroundings is maximized. Thus, our second adaptive hypothesis is that the probability of not being detected by birds during these short scans already reaches near certainty for the intermediate levels of our crypticity index and therefore those moths that achieved this level do not decide to reposition themselves.

In summary, we showed that moths not only are able to find a new cryptic spot (Kang et al. 2012), but they also can change their behavior adaptively depending on their current crypticity, even though they are apparently not able to directly evaluate the degree of visual match between their patterns and the patterns on the background. Our studies on moths (Kang et al. 2012, these results), along with studies on several other organisms (Chiao & Hanlon 2001; Barbosa et al 2008; Allen et al. 2010; Barbosa 2011; Briffa & Twyman 2011), provide opportunities to explore how prey animals respond to their own degree of crypticity by seeking highly cryptic sites in complex visual environments.

SUPPORTING INFORMATION

The results of the further statistical analysis including 'presentation order' as a covariate

The order of the presentation significantly influenced the detection probability. GLMMs analysis revealed that human subjects became better to detect moth targets (Fixed-Final: χ^2_1=21.45, *adjusted P*<0.001; Fixed-Initial: χ^2_1=13.43, *adjusted P*<0.001; Initial-Final: χ^2_1=9.19, *adjusted P*=0.002) and spent less time (Fixed-Final: χ^2_1=30.55, *adjusted P*<0.001; Fixed-Initial: χ^2_1=23.97, *adjusted P*<0.001; Initial-Final: χ^2_1=35.27, *adjusted P*<0.001) to detect towards the end of the series of images.

After taking into account the order of the presentation into analysis, the results were congruent with the results in our main text. The detection probability of the moths that did not reposition themselves and remained in their landing positions (fixed positions) was significantly lower than the detection probability at landing positions (initial positions) for those moths that later repositioned themselves (GLMMs: χ^2_1=12.08, *adjusted P*=0.001), but significantly higher than the detection probability of these moths in their final positions, after they repositioned themselves (GLMMs: χ^2_1=4.59, *adjusted P*=0.03). The latency to detection of the moths in the Fixed group was significantly longer than that of the moths in the Initial group (GLMMs: χ^2_1=29.22, *adjusted P*<0.001) but shorter than that of the moths in the Final group (GLMMs: χ^2_1=13.69, *adjusted P*<0.001). After repositioning, the detection probability decreased (comparison between Initial and Final; GLMMs: χ^2_1=39.72, *adjusted P*<0.001), and the latency to detection increased (GLMMs: χ^2_1=107.12, *adjusted P*<0.001).

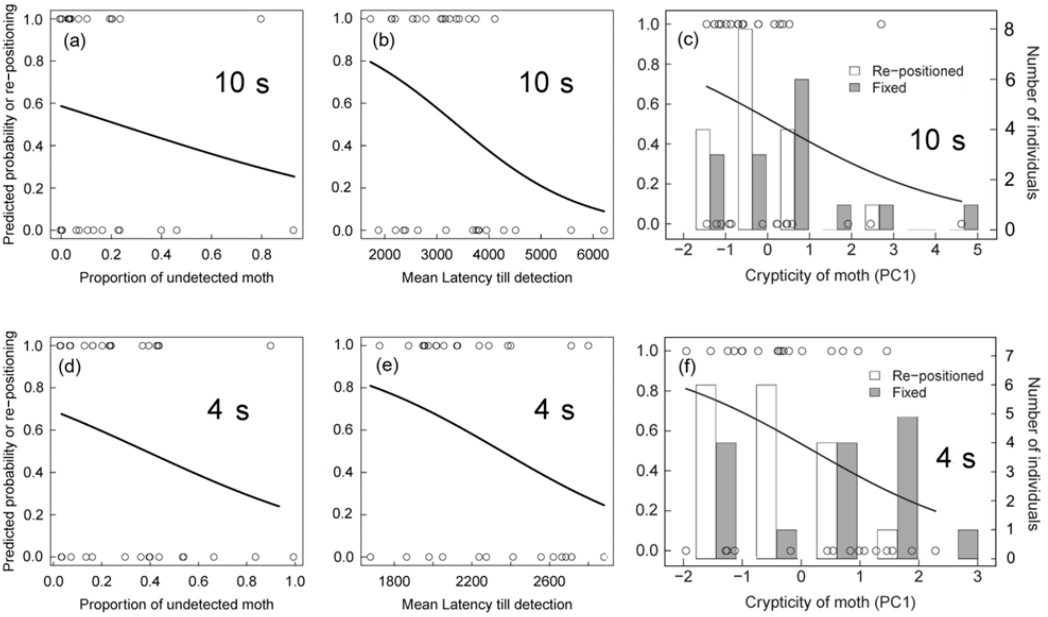


Figure 2.S1. The predicted probability of repositioning (the black line) as a function of variables related to crypticity: the proportion of humans who did not detect a moth during 10 s (**a**; [GLMs, χ^2_1=0.85, P=0.36]) or during 4 s (**d**; [GLMs, χ^2_1=2.05, P=0.15]), the average latency to detect for those human subjects who detected a moth during 10 s (**b**; GLMs, χ^2_1=4.23, P=0.04]) or during 4 s (**e**; GLMs, χ^2_1=3.61, P=0.06]), and the principal component derived from these two variables separately for 10 s (**c**; GLMs, χ^2_1=2.53, P=0.11) and 4 s (**f**; GLMs, χ^2_1=4.42, P=0.04) durations. Histogram in c,f shows the number of moths who repositioned (unfilled) or did not reposition (greyed) themselves within each of several classes on the x-axis.

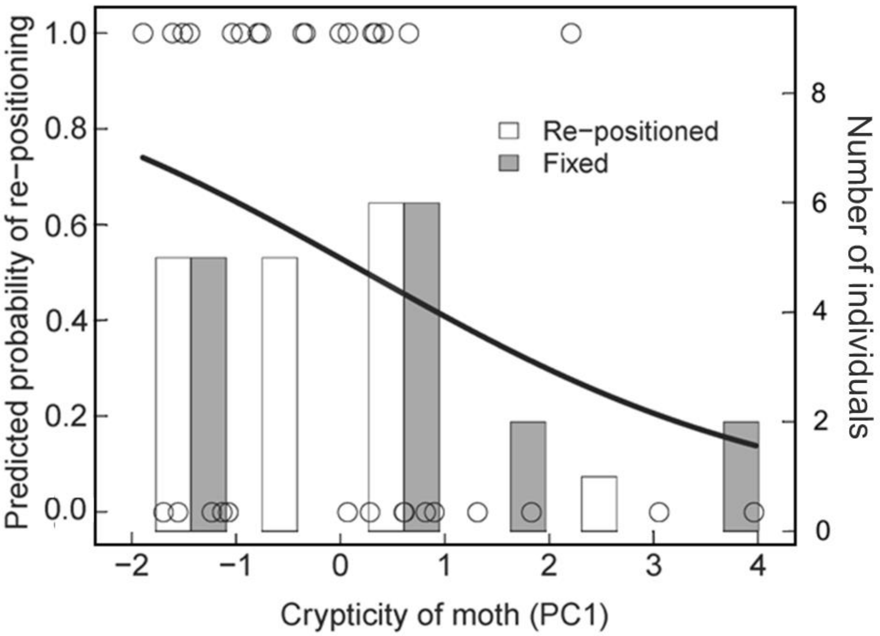


Figure 2.S2. The predicted probability of repositioning (the black line) as a function of variables related to crypticity: the principal component derived from the two variables (the proportion of humans who did not detect a moth during 4 s and the average latency to detect for those human subjects who detected a moth during 10 s. This PC1 value explained 96% of the variance (*eigenvalue*=1.93) and highly correlatedwith the original two variables (t_{30}=28.12,r=0.98, P<0.001 for both variables). GLM analysis revealed marginally significant effect of crypticity (PC1) on the binary response variable indicating whether a moth repositioned itself or not (χ^2_1=3.14, P=0.08). Histogram shows the number of moths who repositioned (unfilled) or did not reposition (grey) themselves within each of several classes of PC1 (on the x-axis).

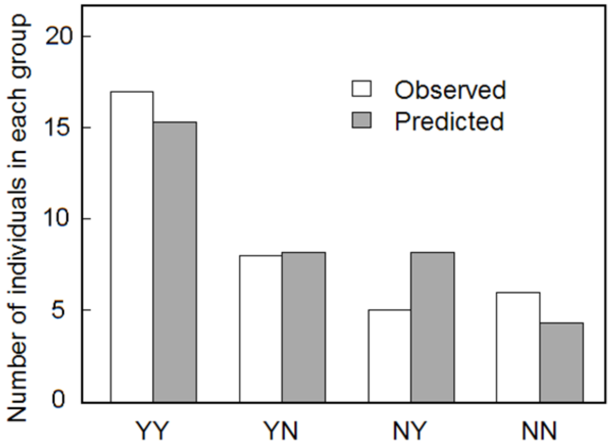


Figure 2.S3. Comparison of observed and predicted distributions of the results of pairs of two consecutive tests performed on 36 individual moths in 2011. “Y” indicates occurrence of repositioning behaviour; “N” indicates absence of repositioning behaviour within an hour from the initial landing on a trunk (i.e. YY indicates that a moth repositioned itself in two consecutive tests). The figure is based on three left-most columns in Table S1 (MothID, First, Second). We did not use the rest of the columns for analysis to avoid biases due to our data-collecting method; we tried to continue testing the same individual at least until one performs the repositioning behaviour twice, but we failed to recapture some individuals which resulted in fewer tests than we intended. But all the individuals were tested at least twice and the data from the first two consecutive tests are used here. The predicted probability p of repositioning per one trial is calculated as “the number trials with repositioning /total number of trials” in the two columns of the table S1: “First” and “Second”. The predicted probability of not repositioning is q=1-p. The predicted null probability of occurrence of each of the four pairs, YY, YN, NY and NN is calculated from p and q (p*p for YY; p*q for YN; q*p for NY; q*q for NN;) Chi-square test revealed no difference between the observed and the expected frequency (χ^2_3=2.041, *P*=0.56).

Table 2.S1. Results of consecutive tests on the same individual (*N*=36 individuals used) released and observed after landing on tree trunk; “Y” indicates that repositioning behaviour was performed and “N” indicates that the repositioning did not occur within an hour from landing on a trunk. This behavioural test was conducted in 2011 summer without determining the degree of crypticity. This table, and Fig. S2 based on the table, shows that the probability of repositioning does not depend on the individual identity. This indicates that the moths show no within-individual consistency of whether to reposition their bodies or not in the consecutive tests.

	The consecutive trials on the same moth				
Moth ID	First	Scecond	Third	Fourth	Fifth
1	Y	Y	Y	Y	Y
2	Y	Y	Y	Y	
3	N	Y	Y		
4	Y	Y			
5	Y	Y			
6	Y	Y			
7	Y	Y			
8	Y	N	N	Y	
9	Y	Y			
10	Y	Y			
11	Y	N	Y		
12	Y	Y			
13	Y	Y			
14	N	Y	Y		
15	N	Y	Y		
16	N	Y	Y		
17	Y	Y			
18	Y	Y			
19	Y	Y			
20	Y	Y			
21	Y	Y			
22	Y	N	Y		
23	Y	Y			
24	Y	Y			
25	N	Y	Y		
26	N	N	Y	Y	
27	N	Y	Y		
28	N	Y			
29	N	N	N	N	
30	N	N			
31	Y	N	N		
32	N	N			
33	N	Y			
34	N	N			
35	N	N	N		
36	Y	N			

Chapter 3. Cryptically patterned moths perceive bark structure when choosing body orientations that match wing color pattern to the bark pattern

ABSTRACT

Many moths have wing patterns that resemble bark of trees on which they rest. The wing patterns help moths to become camouflaged and to avoid predation because the moths are able to assume specific body orientations that produce a very good match between the pattern on the bark and the pattern on the wings. Furthermore, after landing on a bark moths are able to perceive stimuli that correlate with their crypticity and are able to re-position their bodies to new more cryptic locations and body orientations. However, the proximate mechanisms, i.e. how a moth finds an appropriate resting position and orientation, are poorly studied. Here, we used a geometrid moth *Jankowskia fuscaria* to examine i) whether a choice of resting orientation by moths depends on the properties of natural background, and ii) what sensory cues moths use. We studied moths' behavior on natural (a tree log) and artificial backgrounds, each of which was designed to mimic one of the hypothetical cues that moths may perceive on a tree trunk (visual pattern, directional furrow structure, and curvature). We found that moths mainly used structural cues from the background when choosing their resting position and orientation. Our findings highlight the possibility that moths use information from one type of sensory modality (structure of furrows is probably detected through tactile channel) to achieve crypticity in another sensory modality (visual). This study extends our knowledge of how behavior, sensory systems and morphology of animals interact to produce crypsis.

INTRODUCTION

Many prey animals have cryptic patterns that resemble background and reduce detection by predators (Poulton 1890; Thayer 1919; Cott 1940). Camouflage can be achieved through a range of mechanisms including background matching, disruptive patterns or countershading (Cott 1940; Ruxton et al. 2004). Many studies focus on the effectiveness of the morphological characteristics, colors or patterns in providing crypticity (Cuthill et al. 2005; Stevens 2007; Cuthill & Szekely 2009; Brechbühl et al. 2010), but behavioral mechanisms contributing to crypsis are less studied. In many cryptic prey animals, the color/pattern of animal body is fixed and the behavior of animals (e.g. choice of resting position or orientation) finally determines the degree of match between the animal body and the background (e.g. moths (Kang et al. 2012)). Therefore it is important to investigate behavior of animals to fully understand the crypsis of animals in natural environment.

Among many cryptic animals, moths have been extensively studied in terms of behavioral preferences for the background substrate. Most of the studies of moth preferences have explored two types of behavior: i) selection of a matching background in terms of color (Kettlewell 1995; Sargent 1966; Sargent & Keiper 1969; Boardman et al. 1974; Steward 1985) or ii) selection of an appropriate resting orientation (Kang et al. 2012; Sargent & Keiper 1969; Pietrewicz & Kamil 1977; Webster et al. 2009; Wang & Schaefer 2012) with regard to the orientation of the background pattern. These previous studies suggest that moths prefer to rest on the background of similar color to their own. This preference for certain color does not seem to result from direct comparison between their wing color/pattern and the current background, but rather seems to be a pre-determined (e.g. genetically) trait shaped by natural selection (Sargent 1968; Sargent 1969a). It has also been shown that some moths orient their bodies adaptively to maximize their crypticity by matching the pattern on their wings to that of the background (Kang et al. 2012; Sargent & Keiper 1969; Pietrewicz & Kamil 1977; Webster et al. 2009; Wang & Schaefer 2012).

On the other hand, we are aware of only one study of proximate behavioral mechanisms that result in such a match. Sargent (Sargent 1969b) presented experimental visual and tactile stimuli to see how two moth species (one geometrid and one noctuid) respond to simple directional elements of the background. He proposed that tactile cues and background-independent cues are used by the bark-like moths in finding resting positions and body orientations.

Our recent studies on two geometrid species have shown that these moths are not only able to find a position and a body orientation that provide good camouflage (Kang et al. 2012), but that they are also more likely to re-position their bodies to a more cryptic spot if their original landing positions do not provide good visual crypticity (Kang et al. 2013). Moth's vision is unlikely to be important here because visual recognition of fine bark pattern might be difficult for the moths due to low resolution of their compound eyes (Land & Nilsson 2002). Additionally, they cannot view their wings and the bark pattern from the view point of the visually hunting predators, which is crucial to determine the level of cryptic match between the wings and the bark. This indicates that they may perceive non-visual stimuli, such like the ones suggested by Sargent (1969b), that may be correlated with their current degree of visual crypticity at a resting position. We asked whether the non-visual cues proposed by Sargent (1969b) are sufficient to explain the re-positioning behavior of these species (Kang et al. 2012; Kang et al. 2013). Furthermore, we expanded the Sargent's classical experimental design by testing the additional hypothesis that moths could use the bark curvature to orient their bodies.

In this study, we use one of our previously studied geometrid moths, *Jankowskia fuscaria* (Leech 1891), to investigate the cues that these moths use to orient their bodies on a tree trunk. After landing on a tree trunk, *J. fuscaria* performs re-positioning behavior (Kang et al. 2012). *J. fuscaria* re-positions its body on the bark by repeatedly lifting and lowering its wings and changes its resting spot and body orientation to reinforce its crypticity. This suggests that *J. fuscaria* may use sensory cues from the background to re-position their bodies to a more cryptic position. The key characteristics of *J. fuscaria* that we utilize in this study are the following: i) the moths actively search for the resting positions and body orientations on the background substrates (Kang et al. 2012), and ii) they have a tendency to orient themselves towards either of two sides (head pointing towards left or right from the point of view of an observer) on tree trunk after the completion of this re-positioning behavior (Kang et al. 2012). If not disturbed during the day, they stay in this position until the following night. The position and body orientation at these chosen sites provide good crypticity because the patterns on the moth's wings match the main direction of visual patterns on the vertical tree trunk (Figure 3.1). This active preference for non-random resting orientation suggests that moths may be able to recognize some characteristics of the substrate and use those cues to orient their bodies. We asked what cues are used by *J. fuscaria* during re-postioning behavior, and tested whether *J. fuscaria* uses trunk curvature, visual pattern, or directional three-dimensional structure of crevices or furrows on a trunk.

MATERIALS AND METHODS

Collection of moths and experimental arena

Moths were collected just before dawn (about 4 AM) near the black lights that we setup in the forest (university forest which is preserved for research purpose; no permission is required for members). They were kept individually in small plastic containers with sugar-moistened tissue and used in experiments on the following day. Experiments were conducted under thick forest canopy between 0800 and 1800. To prevent escaping (flying away) of moths during the experiments, we set up a tent made of mosquito net and performed the experiments inside the mosquito net structure. The mosquito net was considerably transparent and visual patterns of a log and visually patterned experimental backgrounds were well visible. The mosquito net only slightly decreased the light intensity without changing the spectral properties of the light (Figure 3.S1), so that we considered the effect of mosquito net on moths' behavior (choice of a resting spot and body orientation on the backgrounds) negligible. After each experiment, some of the tested moths were frozen for another study and the others were released at a distant location (further than 2 km away) to avoid re-capturing of the same individual. All experiments were performed in Aug-Sep 2011 at Choosan field station, Mt. Baekwoon, South Korea (N35°,01',54.30"; E127°,36',22.30").

General scheme of experiments

We made several experimental backgrounds, each of which was designed to have a single characteristic that may be used by moths on tree trunks: visual pattern, directional furrow structure, and curvature. The backgrounds were presented in two treatments: vertical treatment which imitates a vertically standing trunk, and horizontal treatment for which we rotated the vertical treatment by 90 degrees. By exploring the resting orientation of moths on each background in both treatments, we asked i) whether moths orient themselves by using the cues from the background tree trunk, and ii) which sensory components of the background moths use to orient.

Each experiment comprised two treatments; vertical and horizontal. In the vertical treatment, we presented background cues in a natural manner, as they are present on a vertical tree trunk. In the horizontal treatment, we rotated the presentation of the background cues by 90°. For all the experiments, we assumed that i) if moths perceive the background cues provided in the vertical treatment and if they use the given cues to orient their bodies in a manner providing a cryptic match between the wings and the bark pattern, the resting orientation of moths would be the same as the orientation in nature (towards either of two sides), and ii) after we rotate the background, the resting orientation of moths should follow the orientation of background cues if the moths use the given background cues in each experiment (visual pattern, structure, and curvature). Curvature was tested in two conditions: thick trunk (with low curvature) and thin trunk (with high curvature). In each experiment, we measured the angle between the orientation of moth's head and the vertical line (0° if a moth's head points towards the top; the angle runs in the clockwise direction).

Experiment 1. Do moths recognize background cues on natural backgrounds?

We first tested whether moths recognize and rely on background cues to position and orient themselves on a natural tree trunk. As the natural substrate, we used a log of an oak *Quercus acutissima*, which is one of the common species at the study site and has prominent directional furrows. The directions of the main furrows were mostly vertical (Figure 3.1; see Figure 3.S2 for details of the furrow directions). The size of the log was 21 cm (radius) × 67 cm (length) so that it had sufficiently large area for moths to rest on. Two orientations of the log were used as the experimental treatments: vertical and horizontal. For the vertical treatment, we set the log standing upright; for the horizontal treatment, we laid the log down (90° rotated).

We released a moth on the log, and after one hour (one hour is sufficient time for *J. fuscaria* to re-position themselves to final resting spot and orientation (Kang et al. 2012)) we photographed the moth and captured it. Then, we rotated the log and repeated the same procedure with the same individual. The order of the testing was counterbalanced between moths: half of the moths were tested in the vertical treatment first (in the earlier part of a day) followed by horizontal treatment (in the later part of a day), and the other half were tested in the horizontal treatment first. Hence, the effect of testing time on moths' behaviors, if present, should not bias the results. From the photographs, we measured the head orientations of the moths. Although most of the moths stayed put once they settled down on the log, few moths flew away during the one hour of waiting time. This resulted in a slight difference in sample sizes between treatment groups (34 moths were tested in both treatments; one additional moth was tested in the vertical treatment).

For testing, it was essential to make the moth land on the background we provided. However it was difficult to let the moth fly to the log willingly and make it land on the background prepared for the experimental test. Therefore, we let the moths walk from the container box onto the log. Although this situation is not natural (usually, moths fly and land on a substrate), the moths sought new resting spots and orientations during walking on the log in a manner similar to normal re-positioning behavior (Kang et al. 2012). In order to validate this method of moth release, we compared the orientations of moths on the vertically standing log after released by this method with the orientations of moths on natural tree trunks at the study site after releasing them and letting them fly to the trunks (data collected in 2010 summer). If moths released in our experiments seek out resting orientations in a similar manner as they do in nature, their orientations in the vertical treatment should be similar to their orientations on natural tree trunks. The orientations of moths in the vertical treatment did not differ from the orientations of moths on the natural tree trunks in the forest (Watson test, U^2=0.05, N_1=29, N_2=35, P >0.1, Figure 3.S3). This demonstrates that our experimental procedure of letting a moth walk out of the box onto the substrate resulted in orientations similar to the orientations of moths in a more natural situation whereas a moth flies towards and lands on the substrate. Therefore we decided that our procedure is acceptable for exploring the resting orientation of moths.

Experiment 2. Do moths use visual pattern of the background?

To test whether moths solely use visual patterns of tree trunk to position and orient their bodies, we used a printed photo of the bark patterns of the log (the same log that was used in the experiment 1) in real size (YUPO synthetic paper, printed by Mimaki JV4-160 printer). The printed photo was carefully glued on a 66 × 98 cm flat cardboard (see Figure 3.2a). This background presents the pattern of the tree trunk, but does not have furrow structure or roundness. At a close examination of the printout, we found no directional microstructures or grooves made from paper manufacturing process, printing or gluing, which could affect moths' behaviors. Reflectance measurements of the real bark and the printout did not show much difference between these two types of background (see Figure 3.S4 for the reflectance data). Although we cannot exclude the possibility that the slight color difference between the printout and the real bark might have affected the choice of a resting site during the experiment (the difference in reflectance affects the background choice of moths (Boardman et al. 1974; Pietrewicz & Kamil 1977; Lees 1975)), it is unlikely that it affected the choice of a body orientation of the moths because the distribution of furrow directions, which would affect moths' orientations, did not differ between the real bark and the printout. The experimental treatments and moth releasing procedures were the same as in the experiment 1. We tested 32 moths in both treatments (each individual in both treatments), and two additional moths in the vertical treatment.

Experiment 3. Do moths use directional structure of the background?

To test whether moths use the three dimensional directional structure of the tree bark to orient their bodies, we made an array of artificial directional structures that correspond to simplified furrows and crevices of a tree bark. The artificial background consisted of rectangular (98 × 66 cm) flat cardboard paper with either horizontal or vertical narrow strips of cardboard papers (66 × 5 × 0.5 cm) glued parallel to one another with 5 cm spaces between them (Figure 3.2b). The 5 cm distance between the strips was chosen because it is wider than the wing span of *J. fuscaria* (which is about 3.75 cm for females) and therefore it did not physically restrict the moths from choosing resting orientations nor hinder the moths from walking around. The experimental treatments and moth releasing procedures were the same as the experiment 1. We tested 28 moths in both treatments (each individual in both treatments) and additional three moths in the vertical treatment.

Experiment 4. Do moths use curvature of the background?

To test whether moths use the curvature of trunk's outer surface to orient their bodies, we created the backgrounds of high and low curvature. Visual patterns and structural cues were absent in both types of backgrounds. Low curvature mimicked a tree trunk of thick girth (2 m in diameter) and high curvature mimicked a tree trunk of thin girth (40 cm in diameter). These are within the range the circumference of natural tree trunks. Moths are known to rest on both thick and thin trunks (Cook et al. 2012). For the low curvature background, we rolled a piece of cardboard paper (66 × 98 cm), made a semi-lunar shape in cross-section, and taped the corners of the paper together to maintain semi-lunar cross-section. Thus the background presented a round surface that is sufficiently broad for the moths to rest on (Figure 3.2c). For the high curvature background, we rolled a piece of paper (66 cm in length) and fixed its shape using a tape (Figure 3.2d). For both backgrounds, we covered the surface with brown (bark-colored) linen cloth which had no directional textures. The experimental treatments and moth releasing procedures were the same as the experiment 1. On the low curvature background, 37 moths were tested in both treatments, and additionally 3 moths were tested in the vertical treatment. On the high curvature background, 38 moths were tested in both treatments and additionally one moth was tested in the vertical treatment.

Statistical methods

For each of the four experiments, the prediction was straightforward: if the moths recognize the directional cue provided in the experiment, they should orient themselves towards either of two sides in the vertical treatment (like in natural situation, see above for the explanations) and upwards or downwards in the horizontal treatment (90° rotated from vertical treatment). To test this prediction, we compared the distributions of observed moth orientations with the predicted orientations in each treatment. This was done separately for each treatment, which allowed us to use the same individuals for different treatments without committing pseudo-replication at the level of a single statistical analysis (no moths contributed twice to the same statistical analysis) and without tracking the individual ID of moths (we decided to avoid handling the moths to minimize the disturbance by the experimenter). We did not use pairwise comparisons between the horizontal and vertical treatments (which can directly evaluate statistical significance of the observed differences in the orientations between the two treatments) because pairwise comparisons do not evaluate the type of hypothesis we aimed to test: "the moths orient themselves in a specific direction(s)", and also because this would require individually marking, and disturbing, the moths. Taking all the considerations into account, we think our statistical approach is sufficient for the main purpose of the study, the evaluation of whether moths' orientations follow the specific orientations predicted for horizontal and vertical treatments separately.

The data was analyzed in several steps. For each experiment, at first, we tested whether the orientations of moths in each treatment are uniformly distributed on a circle (1-360^o) using Kuiper's one sample test of uniformity (nonparametric circular test) to test the null hypothesis that moths do not prefer any orientation. If the result of this test shows that data do not deviate significantly from null distribution, we can conclude that moths do not follow any specific body orientation. If the data did not follow the null uniform distribution, we transformed the original variables (body orientations of moths in each experiment, see below for the basis of the transformation and procedure) and set out to test whether their prevalent body orientations did not differ significantly from the predicted orientation.

If insects recognized the directional cues provided in the experiments, and if other factors did not bias the moths' orientations, we expected that the moths in the vertical treatments would orient their bodies horizontally in a bimodal fashion (heads pointed towards right or left; 90 and 270° respectively), and the moths in the horizontal treatments would orient their bodies vertically in a bimodal fashion (head pointed upwards or downwards; 0 and 180° respectively). Hence, for the tests of the hypotheses it did not matter whether a vertically (or horizontally) orienting moths head upward or downward (or leftward or rightward for horizontal treatment) as long as they show a significant tendency to orient their body axes along the vertical (or respectively horizontal) line. Therefore, we transformed the original orientation values to obtain a unimodal distribution.

The method of transformation followed Zar (1999); the angle was doubled if the doubled angle was less than 360°; if the doubled angle was more than 360° then we subtracted 360° from the doubled angle. By this transformation, both 90 and 270° (towards right or left) were transformed to 180°, and 0 and 180° (upwards or downwards) were transformed to 0°. Then, we performed the Kuiper's test of uniformity again on the transformed values (null hypothesis: the data are uniformly distributed on a circle). If the hypothesis of uniform distribution was again rejected (thus strongly indicating non-uniform distribution), we used Rayleigh's test to evaluate whether their body orientations (orientation angle) followed the predictions. Thus, Rayleigh's test evaluated the null hypothesis of the uniform distribution against the alternative hypothesis that the distribution had a specified mean direction (angle) of 180° (for vertical treatment) or 0° (for horizontal treatment). In the main figure we only show the distribution of the original circular variables but in supplementary material we also show the distribution of the transformed variables (Figure 3.S5).

All these tests were performed for each treatment and each experiment separately so that a single individual contributed a single datum to each analysis. Circular mean (θ_t) and circular variance (V_t) were calculated from the transformed angles. All the statistical tests were conducted using R 2.15.0, package "circular" (the function "kuiper.test", "rayleigh.test", "watson.two.test"). To analyze distributions of moth orientations between more and less shadowy edges in experiment 3 we used exact binomial test (“binom.test”).

Some moths were tested in two different types of experiments in order to maintain comparable sample size in each experiment/treatment. However, since we analyzed treatments separately no moths were included twice in the same single statistical test.

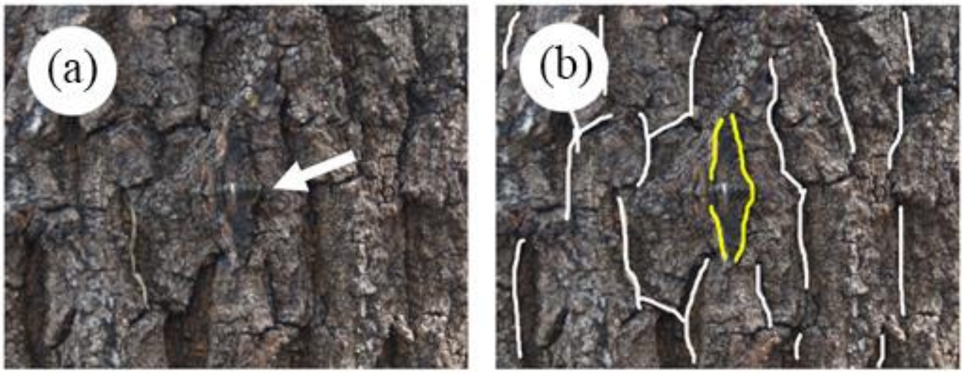


Figure 3.1. Photographs of a moth on a trunk of oak tree. Both (a) and (b) show the apparent match between the directions of the furrows in the bark and the patterns on moth’s wings. The arrow in (a) indicates the head of the moth. The lines in (b) show the direction of the main furrows in the bark (white lines) and the direction of main pattern on the moth (yellow lines).

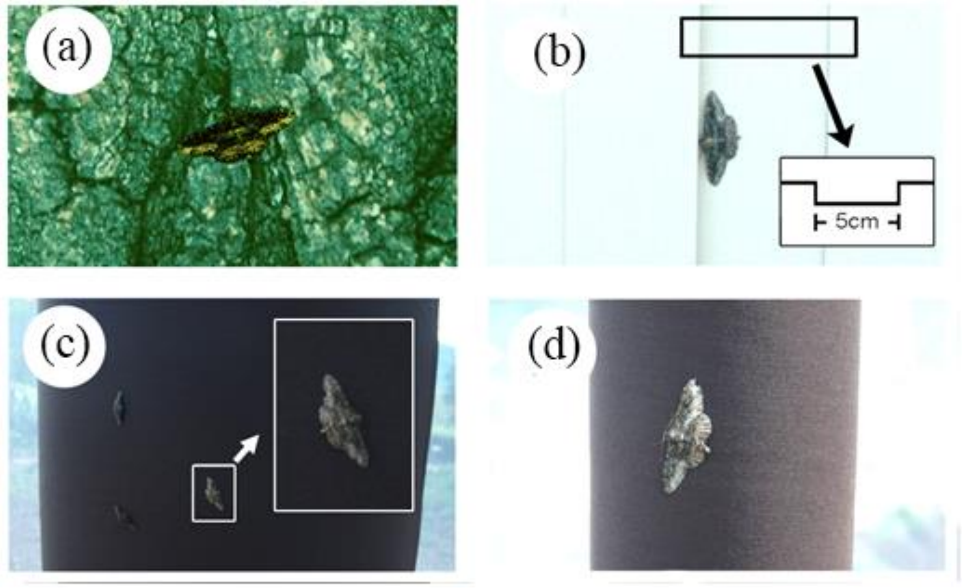


Figure 3.2. Photographs of moths on the backgrounds that were used in the experiments. These are examples of the moths on the log used in the experiment 1 (a), the printed photograph used in experiment 2 (a), the artificial flat background with with directional structure used in experiment 3 (b), the thin round (high curvature) background used in experiment 4 (c), and the thick round (low curvature) background used in experiment 4 (d). The inset in (c) shows a schematic cross-section of the background structure, and the inset in (d) shows the magnification of the moth.

RESULTS

Experiment 1. Do moths recognize background cues on natural backgrounds?

In the experiment with the natural log of an oak tree, moths' orientations were not uniform (Kuiper test, vertical, V=2.29, N=35, P<0.01; horizontal, V=2.29, N=34, P <0.01) in a manner that resulted in a match between the direction of the patterning on their wings and the direction of patterning on the bark regardless of the treatment (Figure 3.1). Their heads were directed towards either side in the vertical treatment (Rayleigh test, V=0.58, N=35, θ_t =181.49, V_t=0.42, P <0.001, Figure 3.3a), or either upwards or downwards in the horizontal treatment (Rayleigh test, V=0.47, N=34, θ_t =-12.35, V_t=0.51, P<0.001).

Additionally, we examined how moths oriented their bodies in relation to the orientation of the nearby furrow (i.e. the head orientation of a moth in relation to the orientation of the closest furrow). Rayleigh’s test with the specified mean direction showed that the moths oriented perpendicularly to the direction of the nearest furrow (see Figure 3.S6 for details). Because furrow orientations follow the orientation of the log (Figure 3.S3), this result is consistent with moths’ heads directed towards the sides in the vertical treatment and upwards or downwards in the horizontal treatment.

These results indicate that moths on the log used some kind of background cues to adjust their orientations according to the orientation of furrows in the bark.

Experiment 2. Do moths use visual pattern of the background?

On the visually patterned backgrounds, moths’ orientations had fairly uniform distribution; most angles were distributed in the upper half of the spherical region regardless of the treatment (Figure 3.3b; Table 1 for statistics). This suggests that the background visual patterns alone may not be crucial in choosing appropriate orientation, and that in the absence of structural cues (such like bark furrows) the moths have a general tendency to orient themselves upwards (Figure 3.3b).

Experiment 3. Do moths use directional structure of the background?

On the structural backgrounds, moths clearly showed a preference to orient themselves towards either of two sides in the vertical treatment (θ_t=184.24, V_t=0.35, Figure 3.3c; Table 1) and to orient upwards in the horizontal treatment (θ_t=-5.44, V_t=0.25). Many of the moths (50% in the vertical treatment and 74% in the horizontal treatment) positioned themselves similar to the moth in Figure 3.2b, with their heads and the frontal edge of forewings nearly touching the edge of the experimental structure. We further evaluated the possibility that moths might have responded to the differences between the shadows created by structures. The shadows made by the structure were slightly different between each side (example in Figure 3.2b), and this might have affected the orientation of moths. To determine if moths paid attention to the shadow differences in the vertical treatment, we categorized the moths as oriented (head direction) towards the more shady or less shady edge. The number of moths that headed towards the more shady edge (8 out of 14) was not statistically different from the moths heading towards less shady edge (6 out of 14; exact binomial test, P=0.79), indicating that this visual cue from a slight difference in shadows did not play a major role in moths' orientation. Additional tests in 2013 showed that moths did not show a preference for artificial vertical structural edges that did or did not create shadows, respectively (see Supporting information text). These results suggest that moths used directional structures to orient their bodies, but that the visually perceived shadows from the directional elements of the structure did not play a role in this behavior.

Experiment 4. Do moths use curvature of the background?

On the low curvature backgrounds, moths oriented themselves randomly in both treatments (Figure 3.3d; Table 1). On the high curvature backgrounds, moths showed a tendency to orient towards either of two sides in the vertical treatment (θ_t=163.18, V_t=0.71, Figure 3.3e), but oriented themselves randomly in the horizontal treatment (Figure 3.3e; Table 1).

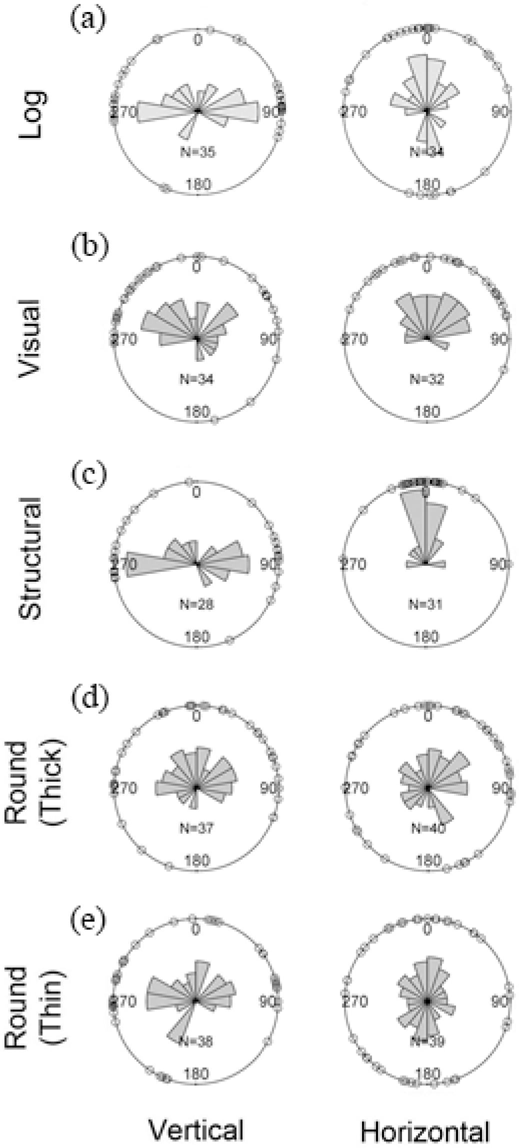


Figure 3.3. The angular distributions of transformed body orientations of moths in the experiments. The figures show the body orientation of moths on the log (A), and the background with visual cues (B; experiment 2), structural cues (C; experiment 3), low curvature cue (D; experiment 4), and high curvature cue (E; experiment 4). The rose diagrams (i.e. histogram of directions) show the relative frequency of moth’s head pointing

DISCUSSION

Our results indicate that moths use cues from the background to adopt the appropriate resting orientation. We have evidence that the details of the substrate's structure, such like the directionality of crevices and furrows in the bark, influence the resting orientation of moths. Moths tend to orient their heads towards the edge of a crevice/furrow, with the straight-lined frontal wing edges touching (or nearly touching) the wall created by the three-dimensional structures. This results in their body axis orientations roughly perpendicular to the prevalent direction of the furrows, which makes the orientation of wing patterns match the orientation of the bark pattern. The moths in our study had a bias to orient upwards in the absence of structural cues and regardless of the orientation of visual patterns of the background (Figure 3.3b). In the horizontal treatment of the structural background (Figure 3.3c), this tendency might have been amplified by the preference to face the edges (horizontal in this treatment). This suggests that the general tendency to orient upwards on the flat backgrounds (in experiment 2 and 3) might be due to factors unrelated to the background structure. We hypothesize that geotaxis or phototaxis may be responsible for the generally upward orientations on flat backgrounds.

One can argue that the single particular log used in the experiments (the properties of the log) might have caused the moths to rest in orientations that differed from the typical orientations in natural situations. However, the resting orientations of the moths on the vertically standing experimental log were very similar to those of the moths resting on natural backgrounds of many different trees (see Figure 3.S3). Additionally, although we used a single log to test all the moths, the furrow structures of the log can be regarded as those of typical oak trees (see Figure 3.S2) because most of the oak trees have similar and predictable furrow structures. Therefore, we do not think that the individual properties of that specific log caused the moths to rest in specific orientations, and we think that the results from the log experiment can be generalized to all oak trunks. One can also argue that, since we continuously used the same backgrounds (one or two backgrounds for experiments) to test all the moths, the initially tested moths might have left some cues (e.g. olfactory) that may have affected the behavior of moths tested later. However even if there was a cue left from the previously tested moth(s), it may affect the choice of the resting spot but not the orientation. Therefore, we think that the orientations of moths were not affected by the repeated use of the same log in the experiments.

What are the sensory mechanisms that moths use to detect the edges and furrows? Perception of background visual patterns to align the patterns on the wings with the patterns on the bark would require the viewpoint of the predators (i.e. from some distances above) rather than that of the moths. Additionally, visual recognition of fine bark pattern might be difficult for the moths because the resolution of their compound eyes is not sufficient to perceive fine-patterns of tree trunk or their wings (Land & Nilsson 2002). Although Sargent (1969b) suggested that geometrids may use tactile cues to adopt specific resting attitudes, we did not precisely determine how moths perceived the three-dimensional structural elements of the bark in experiment 1 or the artificial structure in experiment 3. It is possible that they used tactile cues, which probably were perceived through antennae (Schneider 1964) (and maybe through the frontal edge of the wings). They did not seem to pay attention to a difference between nearby shadows, and they did not seem to differentiate between the edges that created shadows and those that did not (Supplementary text S1). Hence, the results suggest that regardless of visual patterns of the background the three-dimensional structural cues were needed for the moths to choose their orientations that provide camouflage in natural situations. Therefore, we hypothesize that moths detect the structural cues using tactile, rather than visual, sensory channel.

Our results also suggest that curvature may play some additional role in moth orientation, but only when the curvature is prominent (thin trunks) and presented vertically (probably closer to natural situations). We noted that the distribution of moth orientations on high curvature background appears to be more dispersed (Figure 3.3e) than that on the background with artificial structures (Figure 3.3c). This suggests that any effect of curvature is weaker than the effect of directional structure of the background. In natural situations, curvature could be easily disrupted and masked by the existence of furrows especially when a moth is on a thick trunk with prominent furrows. Therefore, we believe that in many natural situations furrow structure is the cue that moths use because it reliably correlates with the visual patterns that match the patterns on moth wings.

In summary, we showed here that *J. fuscaria*'s behavior leads to the alignment between the direction of wing patterns of the moths and the direction of the furrow structures on natural tree trunks as well as on the artificial experimental backgrounds with directional structures. Many other species of geometrid moths perform similar re-positioning behavior (unpublished data, CKK), and the similarity between our results and those of Sargent (1969b) indicates that the use of structural cues from the background can be a general mechanism for the bark-like geometrid moths to adopt appropriate resting orientations that provide camouflage. Although it is clear that moths do not perform direct visual comparison between the patterns on their wings and those on the bark, the exact nature of the sensory mechanism responsible for achieving this visual crypticity is not well understood. Hence, our findings open the possibility to study how the moths use information from one type of sensory modality (hypothetically the tactile channel) to achieve crypticity in another sensory modality (visual) and extend our knowledge of how behavior, sensory systems and morphology of animals interact to produce crypsis.

Table 3.1. The results of the statistical analyses of moths' orientation. NA indicates 'Not Analyzed' because the null hypothesis (H_0: uniform distribution) was not rejected by the Kuiper's test. Significant results are marked bold. The significant *P* values in the right-most column (Rayleigh's test) indicate that moths orient towards either of two sides (left or right) in the vertical treatment, or that they orient towards either upwards or downwards in the horizontal treatment. The original distributions of the moths' orientations are shown in Figure 3.3. Bold texts indicate that moths oriented as we predicted (i.e. the moths perceived the cue that we presented; see main text for explanations).

Type of background	Treatment (sample size)	Kuiper's one sample test of uniformity on original orientation angles (H_0: Uniform distribution)		Kuiper's one sample test of uniformity on transformed orientation angles (H_0: Uniform distribution)		Rayleigh's test on transformed orientation angles (H_a: expected mean orientation angle)	
		V	*P*	*V*	*P*	*V*	*P*
Visual	Vertical (34)	2.52	<0.01	1.67	0.05-0.10	NA	
	Horizontal (32)	3.18	<0.01	1.27	>0.15	NA	
Structural	**Vertical** (28)	2.01	**<0.01**	2.74	**<0.01**	0.65	**<.001**
	Horizontal (31)	4.25	**<0.01**	3.73	**<0.01**	0.75	**<.001**
Round (thick)	Vertical (37)	2.20	<0.01	1.11	>0.15	NA	
	Horizontal (40)	1.70	0.05-0.10	0.98	>0.15	NA	
Round (thin)	**Vertical** (38)	1.84	**<0.05**	2.02	**<0.01**	0.28	**0.007**
	Horizontal (39)	1.48	>0.15	1.74	0.05-0.10	NA	

SUPPORTING INFORMATION

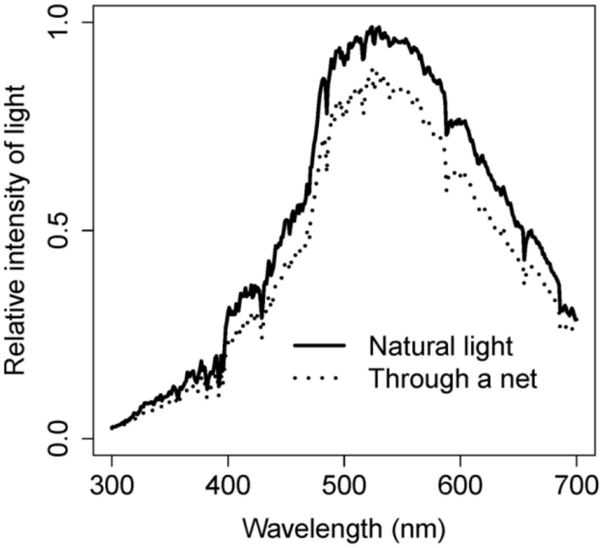


Figure 3.S1. The shape of spectra of natural light and the light through a mosquito net. This figure shows the relative intensity of light (max intensity = 1) measured by spectrometer (USB2000+, Ocean Optics). The light intensity was measured under forest canopy outside the mosquito net (straight line) and inside of the experimental tent made of the mosquito net (dotted line). The shapes of the light spectrum were similar between natural light and the light through a mosquito net.

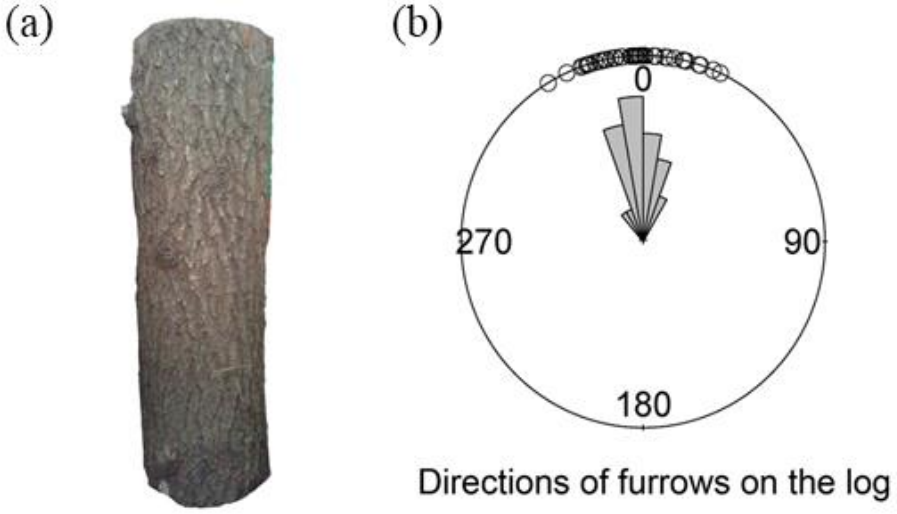


Figure 3.S2. The directionality of furrows on the log used in the experiment 1. We randomly selected 50 furrows on the log (a). For each furrow we measured the angle between the imaginary line running upward along a furrow and the vertical line (0° if the direction was vertical). Possible values were within the range 0-90 and 270-360 degrees. (b) shows the distribution of the furrow orientations. The result shows that the orientations of furrows were closely matching the general orientation of the log.

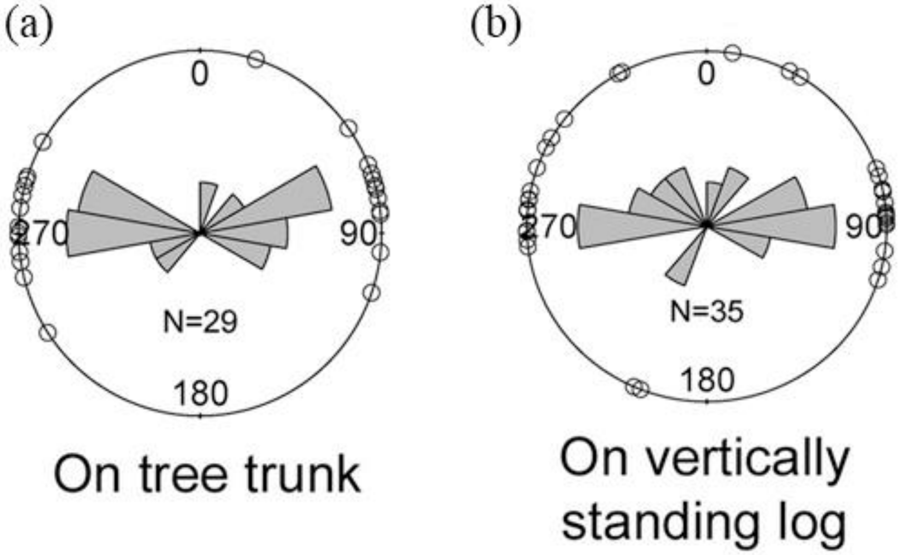


Figure 3.S3. Angular distributions of moth orientations on natural tree trunk and vertically standing log. (a) shows the distribution of moth orientations on tree trunks in natural situation (observed by releasing-following procedure). (b) shows the distribution of moth orientations on vertically standing log in the experiment 1. The two distributions did not differ from each other statistically (Watson test, U^2=0.05, N_1=29, N_2=35, P >0.1).

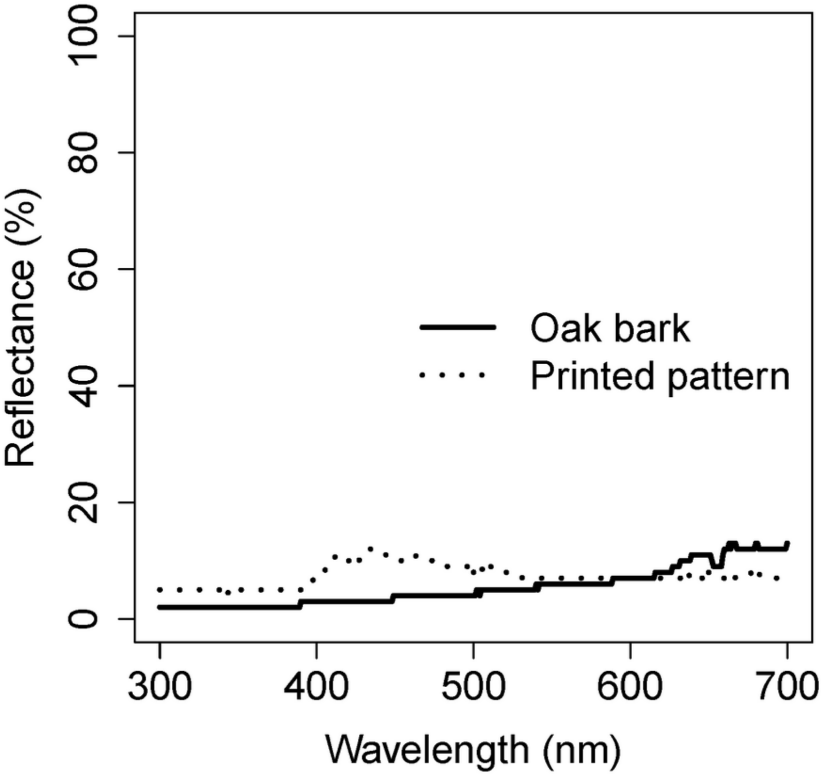


Figure 3.S4. The comparison of reflectance between the tree bark and the printed bark pattern. This figure shows the reflectance spectra of the tree bark (bold line) and the printed photo of the tree bark (used as the visually patterned background in experiment 2; dotted line). We used USB2000+ spectrometer (Ocean Optics), DT-Mini 2 tungsten light source (Ocean Optics), and Labsphere USRS-99-010 standard to measure reflectance of the oak bark and printed pattern.

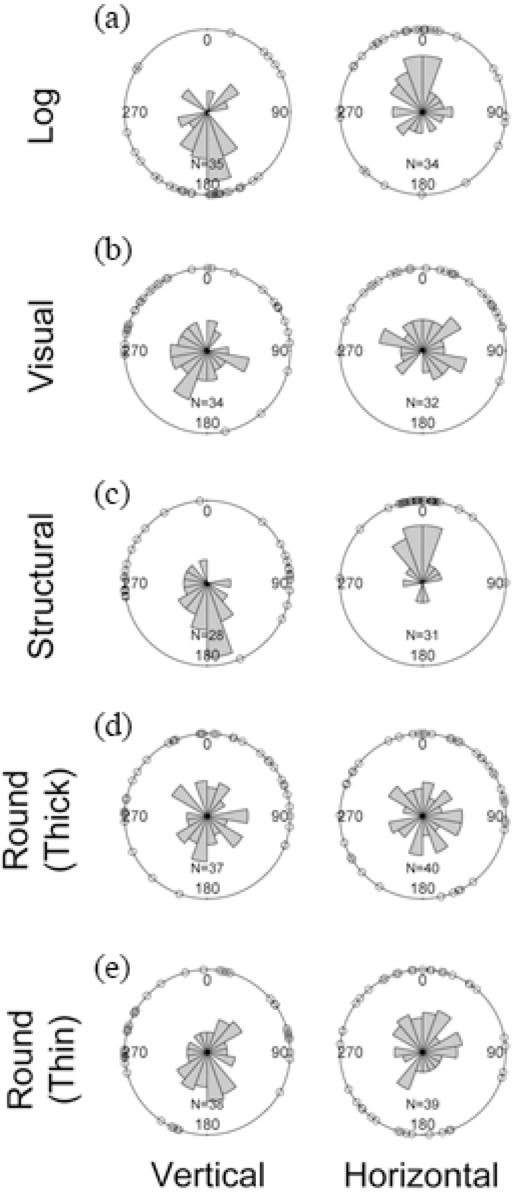


Figure 3.S5. The distributions of transformed body orientations of moths in the experiments. The figures show the body orientation of moths on the log (A), and the background with visual cues (B; experiment 2), structural cues (C; experiment 3), low curvature cue (D; experiment 4), and high curvature cue (E; experiment 4).

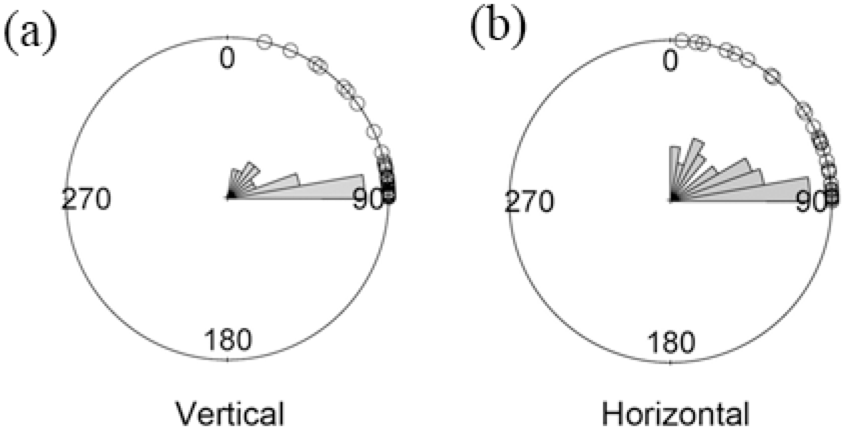


Figure 3.S6. The distribution of head orientations of moths relative to the orientation of the nearest furrow. These figures show the distribution of head orientations of moths on vertical (a), and horizontal (b) log. The angle between the head orientation of a moth and orientation of the closest furrow (closest to the moth's head) was defined as 0° if moth's body axis runs parallel to the closet furrow, and to 90° if moth's head pointed perpendicularly towards the furrow. Rayleigh's test with specified mean direction (towards 90 º) showed that the moths mostly oriented perpendicularly to the direction of the nearest furrow (vertical treatment: *N*=35, *V*=0.91, *P*<0.001; horizontal treatment: *N*=34, *V*=0.82, *P*<0.001).

Text S1. *Additional test for evaluating the effect of shadows on moths' behaviors in the experiment 3*

In the experiment 3 moths did not show preference for orienting towards stronger or weaker shadows. We additionally tested whether moths respond to shadows (or direction of light that comes from) created by directional structures in the vertical treatment of experiment 3. We followed the same procedures as the vertical treatment of the experiment 3, but additionally we put a commercial desk light on the corner of the background to generate shadows only in one side of the directional structure. After the moths settled on the background, we categorized the moths whether they oriented towards the shadowy part of the directional structure or not. This test was done in the warehouse where only dim light were coming through windows.

The number of moths headed towards the shadowy side (10 out of 16) did not significantly differ from the number of moths oriented towards the non-shadowy side (6 out of 16; exact binomial test, *P*=0.45). This suggests that moths' orientating behaviors were not affected by the shadows created by the directional structural elements of the background.

Chapter 4. Multimodal information use to adopt adaptive resting orientations in moths

ABSTRACT

Camouflage depends on the interplay between morphology and behavior of animals. Behavioral elements, such as a choice of a resting spot or posture, are important as well as the animal's cryptic appearance for effective camouflage. However, the type of sensory input that mediates the resting site choice has been poorly studied. Previously we showed that bark-like moths perceive and rely on bark structure to seek out cryptic resting positions and body orientations on tree trunk. However it is still unclear which sensory information type(s) they use to perceive bark structure. Here, we tested through which sensory organs (which are directly related to sensory information types) moths perceive bark structures to find adaptive resting orientations. We amputated (or blocked) one of the hypothetical sensory organs from moths (antennae, forelegs, wings, and eyes) and tested whether they were still able to perceive bark structures properly. We found that moths use visual information from eyes and tactile information from wings to perceive bark structure and to adopt cryptic resting orientations. This study reveals multimodal information use by moths to achieve visual camouflage and highlights sensory mechanism that responsible for the adaptive behavior of cryptic insects.

INTRODUCTION

Camouflage conceals animal body against background to avoid detection by predators or prey (Cott 1940; Stevens & Merilaita 2011). Although the term ‘camouflage’ has largely been understood as color patterns of animals, camouflage should be considered as a strategy that the interplay between multiple traits, such as morphological, behavioral, or in some cases physiological traits (Akino et al. 2004), makes the prey undetectable or unrecognizable to predators (Stevens & Merilaita 2009). To date, morphological mechanisms that provide camouflage have been investigated extensively, but behavioral elements are still poorly understood.

Behavioral background matching is a common process of cryptic animals. For example, many cryptic animals, including both terrestrial and aquatic animals, are known for their preferences to stay near the substrate which prevents the animals from detection by predators (Cooper Jr & Sherbrooke 2012; Kjernsmo & Merilaita 2012; Manríquez et al. 2008). Recent studies revealed that some animals can exquisitely seek out cryptic positions and postures in fine-scale. Quails know and choose locally most cryptic egg-laying sites and put their eggs on there (Lovell et al. 2013). It is likely that quails use visual cues using eyes to find cryptic spots for their eggs. Bark-like moths can also find better camouflaged spots and body orientations after landing on tree trunks (Kang et al. 2012; Kang et al. 2013b). However, it is still in question how moths seek out cryptic spots and body orientations in such a fine detail.

Previous study shows that a bark-like moth, *Jankowskia fuscaria* (Leech 1981), perceive and rely on bark structures to find cryptic resting orientations which align the direction of their wing patterns to the direction of bark patterns (Kang et al. 2013a). Here, we tested the follow-up question that through which sensory organs (which is directly related to the type of sensory information) moths perceive bark structure to adopt cryptic resting orientations. We hypothesized that moths may use the information from either (or a combinations of) their antennae, forelegs, wings, or eyes to perceive bark structure.

Insect antennae play a major role in chemoreception or flight control (Sane et al. 2007), but there also exist mechanoreceptors, such as Johnston's organs, that can perceive tactile information by direct contact (Gullan & Cranston 2009; Schneider 1964). Insect legs also have mechanoreceptors, such as Trichoform sensilla (bristle-like sensory organs) or Campaniform sensilla,that can perceive tactile stimulus (Gullan & Cranston 2009). It is uncertain that mechanoreceptors are present in the wings, but they are located on joints of wings where tactile information from wings can be perceived (Gullan & Cranston 2009). Insect eyes perceive visual information through photoreceptors (Land & Nilsson 2012). Regarding moths' resting posture, we hypothesized that one (or more than one) of these organs may be responsible for the perception of bark structure.

MATERIALS AND METHODS

Study species and experimental scheme

We used *J. fuscaria* to test our hypothesis. *J. fuscaria* is a bark-like species that its wing patterns resemble those of tree bark. Their natural resting orientations on vertically (naturally) standing tree trunks are towards either of two sides which are considered to be adaptive. We previously showed that moths perceive and rely on the background information (directional structure of tree bark) to adopt adaptive resting orientations (Kang et al. 2013a).

To investigate through which sensory channel(s) moths perceive structure of tree bark, we blocked (either by amputation or blocking information input, see below) one of hypothetical sensory organs that moths might use to perceive bark structure. Then we released the moths on natural/artificial backgrounds and observed whether the moths were able to perceive background structure properly (by adopting adaptive resting orientations). As forementioned, we hypothesized that the candidate sensory organs which may be responsible for the bark structure perception are antennae, wings, forelegs, and eyes.

Experimental procedure

We collected *J. fuscaria* at night near blacklights near field site. The moths were kept individually in small plastic containers till the next morning. We put sugar-moistened tissue with moths to provide water and energy. Next morning, we did experimental manipulation (amputation of sensory organs or blocking sensory inputs, see below) on moths and give moths at least one hour of resting time after the manipulation. Then we released the moths to natural tree trunks (or directionally structured cardboard box for visual treatment, see below), waited for one hour to give moths sufficient time to reposition themselves to final resting positions and body orientations (Kang et al. 2012), and photographed the moths. By watching some of the moths we ascertained that body positioning behavior was performed in all three treatments and therefore differences between treatments may be attributable to the sensory information perceived during the body positioning. When releasing, antennae/forelegs amputated individuals were able to fly and land on tree trunks, but it was impossible for the wing amputated ones. Therefore, for the wing amputated individuals, we let the moths walk from the container boxes to tree trunks. Previous study shows that moths are still able to perceive background structure and find adaptive resting orientations when released by this method (Kang et al. 2013a).

The tested individuals were re-collected and released at least 4 km away from the experimental site to avoid re-capturing of the same individual. All the experiments were conducted in the Seoul National Univ. forest at Mt. Baekwoon, South Korea during Aug-Sep, 2013.

Elimination of information inputs from each sensory organ

We predicted that if moths use the information from the sensory organs that we amputated (or blocked), the amputated moths would fail to adopt adaptive resting orientations (towards left or right side). We hypothesized that moths may use tactile information through antennae, forelegs or wings, and use visual information through eyes to perceive bark structure.

For antenna treatment, we amputated the basal part of both antennae using a surgical scissor. We left less than 1 mm of the both antennae to ensure that their antennae do not perceive bark structure by direct contact. For foreleg treatment, we pulled out both forelegs of the moths by hand. The forelegs were easily pulled out, and no leftovers or additional damages were found on moths' bodies. For wing treatment, we cut the basal part of all wings (both forewings and hindwings). We left less than 5 mm of the wings to ensure that no tactile information was transmitted by direct contact.

For visual channel treatment, we obviously were unable to block the visual information by amputation, and covering the eyes with dye might have also severely affected the moths. Therefore, we decided to entirely eliminate ambient light in the experimental container. This effectively cuts off visual sensory input entirely. For visual treatment, we used an experimental box into where no light can come and observed moths' resting behaviors in the box (40 cm × 33 cm × 26 cm). Previous study (Kang et al. 2013a) shows that moths respond to directional structure of background and adopt adaptive resting orientations (towards either left or right side on vertically structured background). We made 4 backgrounds with vertical directional structures (the same one that Kang et al. (2013a) used) by using cardboards. Then we attached these backgrounds inside the walls of the experimental cardboard box. We covered the opening of the box (the upper part) with transparent acrylic plate and used this plate as a 'door' to release moths.

At first, we checked whether the resting orientations of moths in the experimental box were similar to those that in natural situation (control experiment for visual treatment). We released the moths in the box with light present and waited one night. Then we checked the moths' resting orientations next morning. In the experimental box with light present, the orientations of moths were either towards left or right side (see Supplementary Information for the results) which ensured us that the moths performed body positioning that results in the match between the background structure and the wing pattern.

For visual treatment, we covered the whole box with three layers of thick black fabrics after moths were released into the box in order to block any light that can come into the box. Then we let the moths stay one night in there and photographed them next morning. This over-night procedure was essential because the light present during the releasing could affect the moths' choice of resting orientations of that day. Unlike in the other treatments, we were unable to watch the moths and we therefore did not ascertain that the positioning behavior was performed by the moths in this visual-treatment experiment. Therefore this treatment is only giving us general information if in total darkness moths will end up having adaptive body orientations. When photographing the moths next morning, we carefully uncover the fabrics and photographed the moths through the opening of the box. When a moth flew in response to the uncovering of the fabric, we discarded the moth from the sample. This visual treatment was conducted in the warehouse in where only dim light can penetrate through small windows which assured us that no light was present in the experimental box during testing. For analysis, we used only the moths that rested on the side walls of the boxes (where the structure were present) and excluded those moths that found on the ground of the experimental box.

Data analysis

From the photographs, we measured the head orientations of moths (by degrees) relative to the upright tree trunks (relative to the vertical directional structure in visual treatment). The angle was set 0 if moths oriented towards up and run clockwise. We predicted that moths orient towards either left or right side on the backgrounds if they perceived the background structure in each treatment. Therefore, our predicted distribution of head orientations of moths was symmetrically bimodal distribution (have two modes at 90° and 270°) which is difficult to incorporate currently existing hypothesis testing method. Therefore we transformed the angular data to have unimodal distribution (Zar 1999). Then, for each treatment, we tested whether the moths' head orientations were uniformly distributed or not using the the Kuiper's test of uniformity (non-parametric uniformity test). Then, we additionally performed the Rayleigh's test with specified mean angle to test whether moths oriented towards either of two sides or not. More details of the methods (mathematical formulae for transformation and null/alternative hypothesis of the test after transformation) can be found in Kang et al. (2013a). We used the functions ("kuiper.test", "rayleigh.test") in "circular" package in R (open-source program). Single moth contributed to single datum.

RESULTS

The resting orientations of antennae amputated moths were non-randomly distributed (N=30, V=2.455, P<0.001, Fig. 4.1A). These moths oriented towards either left or right side (N=30, *test statistic*=0.499, P<0.001). When forelegs were pulled out, moths oriented non-randomly when resting (N=27, V=3.356, P<0.001, Fig. 4.1B), but oriented towards either left or right side (N=27, *test statistic*=0.7491, P<0.001). These results suggest that moths were able to perceive bark structure and adopted adaptive body orientations without the sensory information from antennae or forelegs.

The resting orientations of wing-excised moths were randomly distributed (N=40, V=1.37, P>0.15, Fig. 4.1C). Therefore it is possible that moths use sensory information (probably tactile) from wings or from muscles that move wings, when adopting the resting orientations. However, the alternative hypothesis of specific resting orientations (towards either left or right side) was not rejected statistically in the wing treatment (N=40, *test statistic*=0.22, P=0.02).

In darkness, moths oriented non-randomly (N=37, V=3.10, P<0.001, Fig. 4.1D), but not towards either of the two sides (N=37, *test statistic*=-0.58, P=1.00).

All the moths showed a tendency to face upwards when visual information was not available due to darkness. This suggests that presence of light is crucial for moths to orient their bodies in an adaptive manner (the manner they do in natural situation). This may be because visual information is used to perceive bark structure and to adopt adaptive resting orientations, or simply because presence of light triggers the natural positioning behavior.

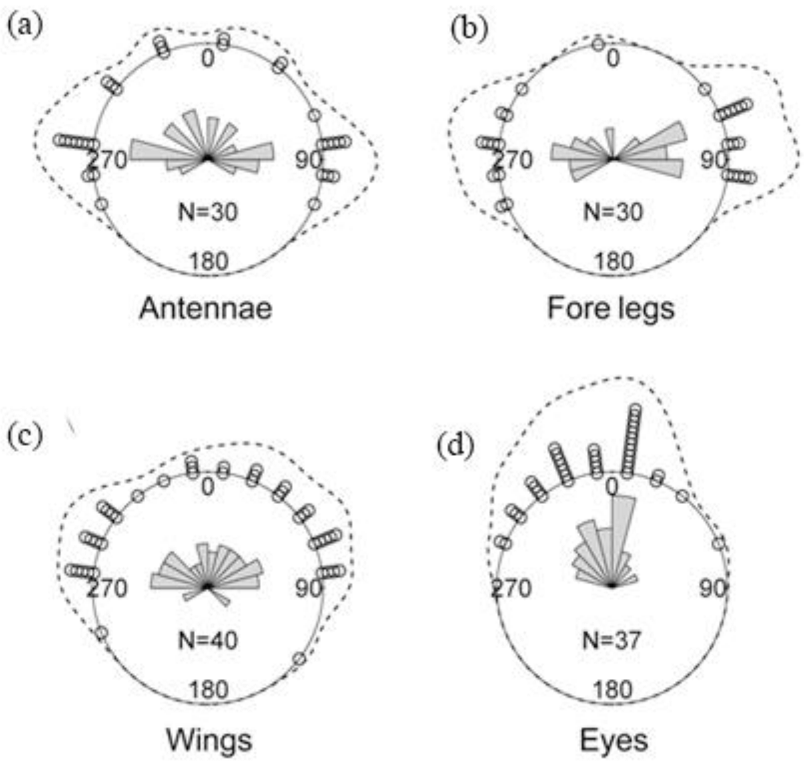


Figure 4.1. The distribution of moths' (*J. fuscaria*) head orientations (open circles) relative to vertically standing tree trunks (or directional structures for eye treatment). Each subplot shows the distribution of resting orientations of (a) antennae amputated moths, (b) forelegs amputated moths, (c) wing-excised moths, and (d) the moths when visual information was blocked, respectively. Moths were expected to orient towards either left or right side (as can be seen in A and B), if they perceived the background structure properly. We overlayed circular density plots (dashed lines) to visualize moths' distribution. We used binned data (24 bins) to draw circular plots and rose diagram (histogram of circular data).

DISCUSSION

Because cutting off the antennae or forelegs did not change the body orientation in comparison to the natural one (also shown in Fig. 1.2d), we suggest that tactile information from antennae and forelegs is not crucial for the perception of bark pattern and structure needed for the adaptive body positioning. However, cutting off the wings resulted in random distribution of body orientations (that looks different that the typical natural distribution of orientations in Fig. 1.2.d), suggesting that moths may use some sensory information from wings and their movements during body positioning behaviors. We hypothesize that this sensory information may originate from mechanoreceptors (at the base of wings, strain receptors in wing muscles, etc.) and future studies are needed to determine which mechanoreceptors may be involved.

Some wing-excised moths were able to orient their bodies towards sides, and some even re-positioned their bodies (Kang et al. 2012) and oriented towards sides (see Supplementary video to see the re-positioning of wing-excised moths). Nevertheless, without wings, the accuracy of adopting cryptic body orientations reduced considerably, and the overall distribution of the moths' orientations looks dispersed (Fig. 4.1D). These suggest that wings may play some role in accurately perceiving the direction of bark structures. Unlike our previous speculations (Kang et al. 2013a), tactile information from antennae or forelegs does not seem to be used when choosing body orientations.

One possible mechanism that mechanosensory inputs from wings may be used is that wings may physically restrict the posture of moths. Typically, when resting, geometridae moths put their wings flat on bark surface and their frontal wing parts of both left and right wings align well with each other (which makes a near straight line; see Fig. 4.S2). However, if the direction of frontal wings does not align well with the direction of furrows, wings of the moths would be disturbed by furrow structures and it may 'discomfort' moths to take the typical resting posture. This may lead to the adoption of specific resting orientations by restricting the orientation of wings. These hypothetical mechanisms need further investigations.

In the visual information treatment, the distribution of body orientations was almost identical to the typical distribution of *J. fuscaria* body orientations at their landing spots, before positioning behavior is performed (see Fig. 1.2. c). Because in this treatment we could not ascertain if moths indeed conducted the body positioning it is likely that in total darkness the positioning behavior was not performed and the resulting orientations represent the state before positioning behavior. This would mean that presence of light is needed to trigger the body positioning. However, if the moths did perform body positioning then the results suggest that moths use visual information to perceive the bark structure. Although we cannot reject this hypothesis, we think that it is not likely considering our previous results where visually perceived differences between shadows created by the background structures did not affect moth body orientations (Kang et al. 2013a)

In summary, we have demonstrated that sensory information from wing movements is likely to be important in perceiving bark structure during body positioning and that in total darkness moths do not end up in the typical adaptive orientations either because they do not perform body positioning or because visual information is important for the perception of the bark structure.

SUPPORTING INFORMATION

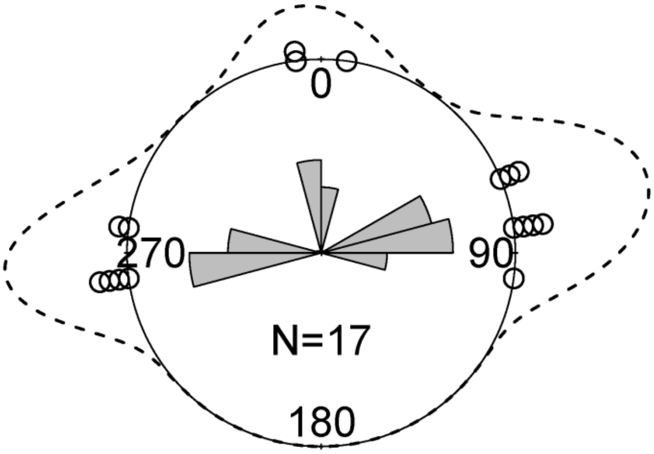


Figure 4.S1. The distribution of moths' (*J. fuscaria*) head orientations relative to vertical directional structures in control experiment for visual treatment. Moths oriented towards either left or right side (*N*=17, *test statistic*=0.58, *P*<0.001) which suggests that moths responded to directional structures when light was present.

Figure 4.S2. Typical resting posture of *J. fuscaria* on tree trunk.

Chapter 5. Effect of sex and bright coloration on survival and wing damage in an aposematic lantern fly with startle display

ABSTRACT

Aposematic coloration in prey promotes its survival by conspicuously advertising unpalatability to predators. Although classical examples of aposematic signals involve constant presentation of a signal at a distance, some animals suddenly display warning colors only when they are attacked. Characteristics of body parts suddenly displayed, such as conspicuous coloration or eyespot pattern, may increase the survival of the prey by startling the predator, and/or by signaling unpalatability to the predators at the moment of attack. The adaptive value of such color patterns suddenly displayed by unpalatable prey has not been studied. We experimentally blackened the red patch in the conspicuous red-white-black hindwing pattern displayed by an unpalatable insect *Lycorma delicatula* (Hemiptera: Fulgoridae) in response to predator's attack. There was no evidence that the presence of the red patch increased prey survival over several weeks. We hypothesize that predators generalized from the red-white-black patches on the hindwings of unpalatable *L. delicatula* to any similar wing display as a signal of unpalatability. Because higher proportion of males than females stay put at their resting sites, displaying their wings in response to repeated attacks by predators, wing damage was more frequent in males than in females. To our knowledge, this is the first experimental test of an adaptive role of aposematic signals presented by unpalatable prey during sudden displays triggered by direct predatory attack.

INTRODUCTION

Insects evolved a variety of adaptations to avoid predation (Cott 1940; Edmunds 1974a; Evans & Schmidt 1990). Some of these adaptations involve sudden movements of conspicuous body parts, which elicit startling response in the predator. Eyespots, novel patterns, and bright coloration on insect hindwings are potentially effective adaptations to avoid predators through such a “startle effect” (Schlenoff 1985; Ingalls 1993; Vallin et al. 2005). Displaying these parts may decrease the risk of predation when sudden nature of the “fearful” display causes predator to hesitate and to be more careful in handling the prey (Edmunds 1974a). The startle effect of such sudden displays has indeed been shown in palatable prey species (Blest 1957; Sargent 1973; Ruxton et al. 2004; Ruxton 2005; Vallin et al. 2005; Langridge et al. 2007; Edmunds 2008; Langridge 2009), and it does not assume any effect on predator’s learning to avoid certain prey. However, a possible aposematic function (signaling unpalatability to predators that learn to avoid unpalatable prey) of sudden displays of conspicuous coloration in prey has been largely neglected (Guilford & Cuthill 1989; Ruxton et al. 2004; but see Gamberale-Stille et el. 2009).

Aposematic coloration is another example of insect adaptations to avoid predation. It is well documented that bright, aposematic coloration, which indicates prey unpalatability, may increase prey survival because it aids predators in learning to avoid unpalatable prey (Poulton 1890; Edmunds 1974a; Alatalo & Mappes 1996; Gamberale & Tullberg 1996; Gamberale-Stille & Guilford 2003; Ruxton et al. 2004). Most studies on aposematic coloration have focused only on those prey species in which the bright signal is constantly displayed regardless of the presence or absence of the predatory threat. However, a number of unpalatable prey species remain cryptic unless they are attacked by a predator, to which they respond by displaying warning coloration. Although the role of this behavior in helping unpalatable prey to survive the attacks of predators has been largely ignored, some hypotheses have been proposed to explain this phenomenon (Ruxton et al. 2004).

For example, if cryptic coloration of prey decreases the risk of being detected by the predators that are resistant to the chemical defense of the prey (i.e. unpalatability or distastefulness), then it is beneficial for the prey to be cryptic and to display only at the moment of attack. This strategy would be effective against the predators that are susceptible to the prey defense, because the predators will be able to learn to avoid the prey based on the displays at the moment of predator's approach/attack. Another hypothesis proposes that the surprise effect due to sudden, unexpected display contributes to faster learning by predators to avoid unpalatable prey (Guilford & Cuthill 1989; Ruxton et al. 2004). Regardless of the hypothetical mechanism, the suddenly displayed conspicuous patterns in unpalatable prey should contribute to prey survival, and experimental color alteration (Blest 1957) can help to determine the survival value of the displayed pattern.

One of the chemically defended insects that perform sudden conspicuous wing display (deimatic display/startle display) in response to a predator's attack is a lantern fly *Lycorma delicatula* (Hemiptera: Fulgoridae; Fig. 5.1). Its cryptic forewings appear to be an adaptation to decrease detection by predators on the bark of host trees. In response to tactile stimuli, such like initial pecking or grabbing by birds, the insect either instantly jumps away or suddenly opens its hindwings that have conspicuous red-white-black markings (Fig. 5.1a). The insects' body contains chemical defence substance (Xue & Yuan 1996), and avian predators vomit after consumption of *L. delicatula* (unpublished observations on Magpies, *Pica pica*). Therefore, the sudden wing opening display appears to have an aposematic function. In this study, we manipulated hindwing coloration of *L. delicatula* to test whether coloration, presented during sudden aposematic displays of wing opening, affects survival of *L. delicatula* in a natural population. Predators have to learn and re-learn the association between the bright coloration and unpalatability, and this process causes observable wing damage in many aposematically colored unpalatable species (Smith 1979). The damage indicates that the prey was rejected by the attacking predator after being handled or that the prey managed to escape. If the damage in prey indicates the severity of non-lethal handling by the predators (Carpenter 1941; Edmunds 1974b; Shapiro 1974), then any association between the intensity of aposematic signal and wing damage can reflect the efficiency of the signal to deter severe handling by the predators during non-lethal attacks. Therefore, we were also interested whether there is an association between coloration, presented during sudden aposematic displays, and wing damage in the prey. Additionally, we determined if sex differences in antipredatory behavior, and in abdomen coloration, may contribute to prey survival and wing damage.

MATERIALS AND METHODS

Capture-Mark-Recapture procedure

We used the capture-mark-recapture method to compare survival of two classes of *L. delicatula* with different, experimentally altered, hindwing coloration. For the capture-mark-recapture experiment to be successful in indicating survival levels, the animals should show substantial level of sedentary rather than dispersal tendencies. We do not have direct data on when and how *L. delicatula* changes dispersal tendencies. However, typically in insects, dispersal, as well as noticeable sex differences in dispersal tendencies, occurs soon after emerging and before reproduction (Dingle, 1972; Harrison, 1980), and this corresponds to July/August in *L. delicatula*. Thus, we conducted the experiments in September and October, when the insects have already settled on the host trees. During this time the flight of the insects was only observed in response to human disturbance or predation attempts by birds (unpublished data).

The study plot (about 120 x 120 m) was located in a mixed forest in Mt. Soori, South Korea (N37° 21.95’, E126° 54.28’). We collected every *L. delicatula* that we could catch with insect nets at the study site. We intensively searched most of the tree trunk of host trees in our study plot and collected them by hand or using an insect net. Using this method we collected most of *L. delicatula* on the lower parts of the tree trunks (up to about 3 m). We collected and marked 506 individuals

(232 females and 274 males), similar numbers per day, over a period of five days: 22th, 23rd and 24th September, and on 1st and 2nd October, 2009. During the marking procedure, we avoided collecting from the same tree twice to prevent disturbance of the marked individuals.

We experimentally created two groups of insects with different coloration of the hindwings. For the treatment group (253 insects), the conspicuous red part of both hindwings was totally covered with a non-toxic black marker (MORRIS permanent marker) and the black part was untreated (Fig. 5.1c). For the control group (253 insects), the red part of each hindwing was untreated, but the black part was painted with the same black marker. Insects were assigned to either group in an alternating manner, which resulted in the balanced assignment of the two treatments on each marking day. Additionally, we applied non-toxic blue marker (MORRIS permanent marker) to the ventral surface of the abdomen in order to identify marked individuals during recapture. After marking, we held an insect by its body for at least 15 seconds. This caused an insect to keep its wings open, which facilitated drying out of the dye. Both, the black pigment of insect wings and the black coloration of the marker, similarly exhibited less than 3% of total reflectance from the white standard (measured with the Ocean Optics USB 2000 Portable Spectrophotmeter) over the range between 300 and 700 nm, suggesting that our blackening treatment properly matched the natural black coloration. Marked insects were released on the same host tree where they were collected.

Some proportion of the *L. delicatula* had wing damage at the moment of capturing (Fig. 5.1b). We separately marked these insects by putting a different type of blue marking on the ventral surface of their abdomens (Fig. 5.1d) and did not include these individuals in the wing damage analysis. Occasionally we found insects with wing damage which apparently occurred during development (such as rolled up wings), and they were not included in the study. A few insects with severe wing damage, to the extent that they could not effectively display hindwing coloration to the predators, were also excluded from the study. We did not observe any noticeable effect of the wing treatment on the insects' ability to display, jump away or fly away. Recapture procedure was conducted between the 20th and 23rd of October, 2009, which was three to four weeks after the initial capture. We used the same method described above to collect insects and did not release marked individuals once they were caught. Although the individuals captured at the earliest date were exposed to potential predation for at least 10 more days than the individuals captured at the last capture date (assuming that they might have been recaptured at the first recapture date) the marking procedure resulted in a balanced sampling scheme, where the same nr of control and treatment individuals were released on each capture date. Hence there was no difference between the control and treatment group in the duration of exposure to factors affecting survival (e.g. predators).

Comparing body length and yellow-striped area in each sex

In *L. delicatula*, sex difference in survival or the degree of wing damage could be related to differences between sexes in the body size or in the size of yellow coloration on the abdomen, which may serve as an additional warning signal. Therefore body lengths (tip of head to tip of genitalia) of 274 males and 232 females were measured. In order to compare males and females with respect to the surface area of the yellow stripes visible on the abdomen from above (the typical view by a predator), we randomly chose 10 individuals of each sex. Forewings and hindwings were removed and photo was taken from a dorsal view of each insect put on a paper with 1 × 1 cm grid (using Cannon PowerShot S5 IS). The surface area of the yellow color in each picture was measured using ImageJ 1.43u. The difference between sexes in the size of the red patch was not considered because the presence of red patch did not affect survival or wing damage (see results).

Sex difference in responses towards simulated predatory attacks

Sex difference in the display or jumping behavior may lead to sex difference in the degree of wing damage and differential survival. Therefore, it is important to examine sex differences in antipredatory behavior in response to predatory attacks. Experiments were conducted in October 2008 at Olympic park, Seoul, South Korea. To standardize experimental conditions, *L. delicatula* were collected from the host-trees (*Ailanthus altissima*) and released on the ground. After an insect settled on the ground in a non-displaying posture, an experimenter provided a tactile stimulus which mimics pecking by a predatory bird. Since *L. delicatula* is insensitive to visual or vibratory stimuli, but it is sensitive to tactile stimuli (unpublished data), we mimicked predatory attack by pecking the insects once with the tip of tweezers, observed their reaction and repeated this procedure once more. The antipredatory responses of the *L. delicatula* were categorized as two types: instantly jumping (without any preceding startle display) or staying put and displaying. Total 40 females and 11 males were collected and tested.

Statistical analysis

We used generalized linear models (GLMs) in order to identify the effect of sex, the treatment, and the presence of wing damage at initial capture on the probability of the insect being recaptured (binary variable: insect being recaptured or not). We also used GLMs to identify the effect of sex and the treatment on the presence of wing damage in the recaptured animals among those individuals that did not have wing damage at the initial capture (binary variable: wing damage present or absent). Binomial error distribution and logit link function were used. We conducted backward elimination starting from the full model containing sex, treatment, the presence of wing damage and their two-way interactions using “step” function in R. We used two types of statistical modeling. First, we used the Akaike information criterion (AIC) to choose the most acceptable model (Quinn & Keough 2002). Additionally, we also used the P value as the criterion of significance of a given effect (Sokal & Rolhf 1995). Some of the models chosen by the AIC contained all non-significant effects and the second approach removed the non-significant effects. The variables and model selection procedure are summarized in Table 1. Because body length and yellow stripe area did not match any of the distributions offered in the GLM analyses, we used Wilcoxon’s rank sum test to compare between sexes. Continuity correction was applied to Wilcoxon’s rank sum test and chi-square test to compensate for small sample size and to get conservative results. All statistical analyses were conducted with software R 2.12.1.

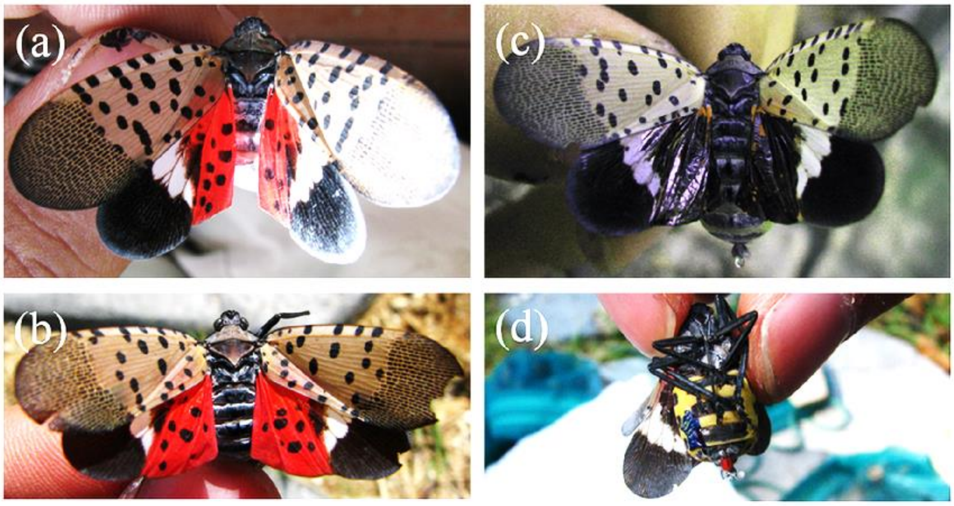


Figure 5.1. Photographs of a female *L. delicatula* with no wing damage (a), a male with wing damage (b), a black painted (treatment group) *L. delicatula* (c), and abdominal part of *L. delicatula* with blue marking on one side used to indicate wing damage (d).

Table 5.1. The explanatory variables, response variables and model selection methods used for each GLMs analysis.

Response variables	Explanatory variables in full model	Model selection methods
recapture rate (binary response)	sex, treatment, presence of wing damage, their two-way interactions	backward elimination based on both AIC and p-value
presence of wing damage (binary response)	sex, treatment, their interaction	backward elimination based on both AIC and p-value

RESULTS

Recapture probability

Among 506 individuals that were captured, 71 individuals (14%; 34 out of 253 individuals in the treatment group, and 37 out of 253 individuals in the control group) were recaptured. We did not find any effect of the experimental treatment (blackening of the hindwing red patch), sex, the presence or absence of wing damage at initial capture or any of the two-way interactions between these three factors, on the probability of insect recapture. In the best model based on AIC value, three explanatory variables remained with non-significant effects: treatment (GLMs, χ^2 =0.16, d.f.=1, p=0.69), presence of wing damage at initial capture (GLMs, χ^2 =0.94, d.f.=1, p=0.33) and their interaction (GLMs, χ^2 =2.36, d.f.= 1, p=0.12). Similar result was found from an additional model selection based on P values where all the explanatory variables, as they were not significant, were deleted. These results suggest that presence of red patch in hindwings did not affect survival of the insects over the experimental period of three to four weeks.

Wing damage

At the stage of initial capture, more males had wing damage than females (185 among 274 males versus 124 among 232 females; GLMs, χ^2 =4.56, d.f.=1, p=0.03, Fig. 5.2a). At the stage of recapture, wing damage was also found significantly more often in males than in females among the individuals which had no wing damage at the initial capture (n=24; GLMs, χ^2 =6.17, d.f.=1, p=0.01, Fig. 5.2b). The effects of treatment and the interaction between sex and treatment on the wing damage were eliminated by model selection procedure (all p>0.5), regardless of the model selection criteria (AIC or P value).

Comparing body length and yellow-striped area in each sex

On average, females were 26% larger than males (19.9 ± 0.13 mm (mean ± SE), n=228 for females; 15.8 ± 0.06 mm, n=269 for males; Wilcoxon's rank sum test with continuity correction, W=59,462, p<0.001). Females had larger yellow-striped area than males on dorsal part of their abdomen in terms of absolute area (n=20, W=100, p<0.001, Fig. 5.3) or relative area (i.e. proportion of total abdominal surface area; n=20, W=100, p<0.001).

Sex difference in responses towards simulated predatory attacks

There was no sex difference in the probability of jumping away in response to the first peck that imitates the very initial attack by the predator (Chi-square test with continuity correction, $\chi^2=0.01$, d.f.=1, p=0.94; Fig. 5.4). However, in response to the second peck, imitating a continued attack of the predator, females jumped away significantly more often than males (Chi-square test with continuity correction, $\chi^2=4.48$, d.f.=1, p=0.03; Fig. 5.4).

Evidence of predation on L. delicatula

After marking the insects at the study site, we did not make attempts to detect predation on the marked insects. However during experiments at the study site we found several dead *L. delicatula* under tree trunks. Their wings were still attached to the remains but abdominal and thorax parts were destroyed or missing. Based on the appearance of the dead insects, we suspected that they were depredated by birds. Several species of insectivorous and omnivorous birds can attack *L. delicatula* in our study area: magpies (*Pica pica*), jays (*Garrulus glandarius*), bulbuls (*Ixos amaurotis*), great tits (*Parus major*), marsh tits (*Parus palustris*), varied tits (*Parus varius*), Rufous turtle dove (*Streptopelia orientalis*). In summer 2007, one of the authors (PGJ) observed a great tit (*Parus major*) and a jay (*Garrulus glandarius*) attempting to attack *L. delicatula* at Ewha Womans University campus in Seoul (insects were pecked at, but not consumed).

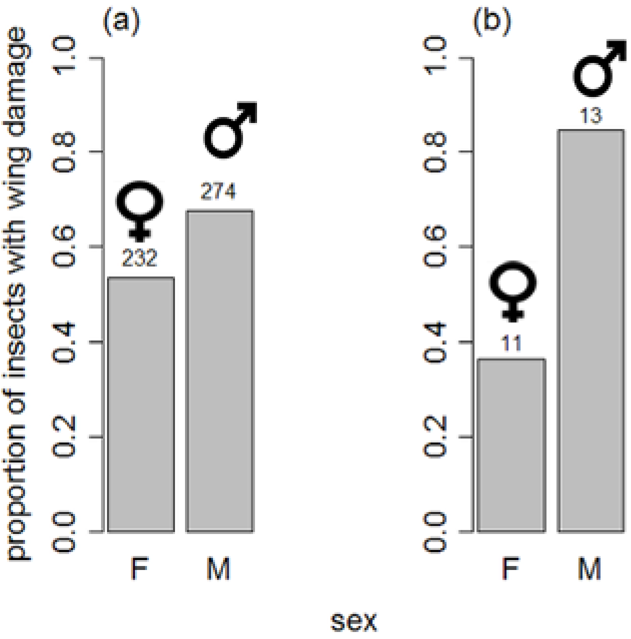


Figure 5.2. Comparison of proportion of wing-damaged individuals between male and female *L. delicatula* at the marking stage (a; *P*=0.03), and at the recapture stage for individuals who carried no wing damage at the marking stage (b; *P*=0.01). The number above each bar represents the total sample size (i.e. the number that corresponds to 100%).

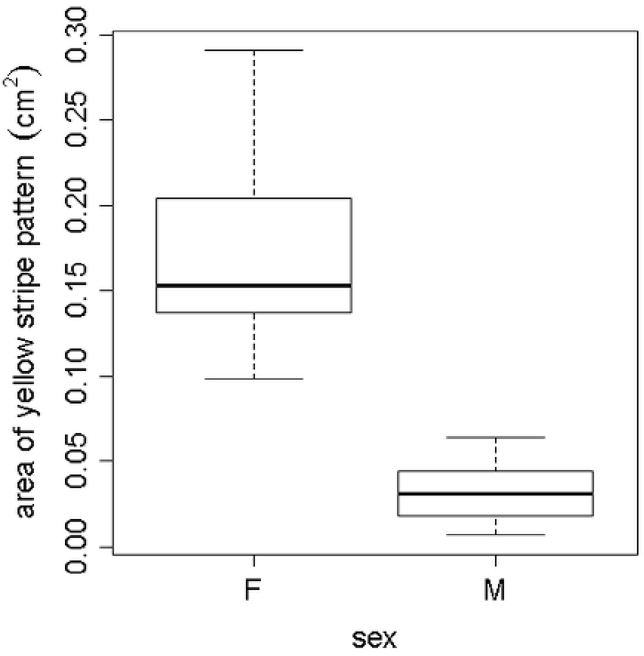


Figure 5.3. Comparison of yellow-striped area between male and female *L. delicatula* (n=10 for each sex; *P*<0.001). The thick black line indicates the median, the box indicates the first and third quartiles, the dotted line shows the minimum and maximum values.

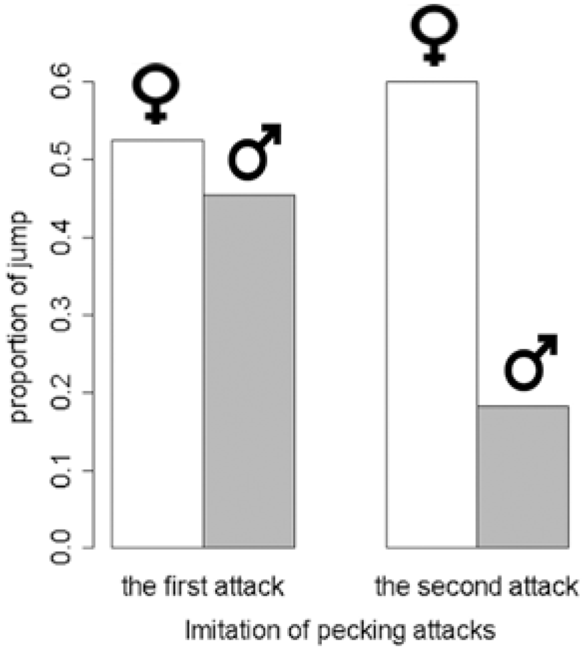


Figure 5.4. Effect of sex (33 females and 11 males) on the response of *L. delicatula* to tactile stimuli imitating pecks by predators. The bars (open bars for female, filled bars for male) represent the proportions (among males and females separately) of individuals who jumped in response to the first peck (difference between sexes, *P*=0.93) and in response to the subsequent second peck (*P*=0.03).

DISCUSSION

From classical studies on aposematism (Cott 1940; Edmunds 1974a; Guilford & Dawkins 1991) we predicted that conspicuous traits in *L. delicatula* may facilitate learning by the predators to avoid this unpalatable prey. Additionally, we expected that the sudden display of bright colors may simply startle the predator contributing to the survival of the prey in accordance with the classical startle effect (Edmunds 1974a; Vaughan 1983). Since *L. delicatula* presents an aposematic signal, which is displayed in a startling manner, we predicted that *L. delicatula* enjoys the benefit of both strategies of aposematism and startling for deterring predators. Furthermore, one of the hypothetical explanations of sudden conspicuous displays by chemically defended animals proposes that the surprise effect due to sudden, unexpected display contributes to faster learning of prey avoidance by predators (Guilford & Cuthill 1989; Ruxton et al. 2004). However, in contrast to these predictions and what has been found in laboratory studies (Vaughan 1983; Schlenoff 1985; Ingalls 1993), our results suggest no effect of conspicuous red coloration on survival of *Lycorma delicatula* – an unpalatable prey that performs startle display.

Why haven't we observed the predicted effects? Possible, mutually non-exclusive, reasons include the following; (i) the predators are not startled by the wing display of *L. delicatula*; (ii) the startle effect might not have depended on the hindwings' red coloration (for example the black-and-white wings maybe as efficient as the black-white-and-red wings in their effect on the predators) or (iii) after two months of learning and memorizing the association between prey unpalatability and sudden display of red-white-black patches on prey hindwings, the predators generalized to any conspicuous wing-display, including white-black patches that lacked the red color. Among these possibilities, we think that the third one is more plausible than the others because, to our knowledge, no unpalatable insects of similar size performing similar wing displays are common at the study site. *Catocala* sp. moths in Korea bear red-white-black hindwing pattern, albeit different than the pattern on *L. delicatula* hindwings (Shin 2001). These moths also produce startle displays, but they are palatable, larger in size and far less common than *L. delicatula* which occurs in high densities. We did not observe any *Catocala* moth disturbed by our activities during the capturing, marking and recapturing at the study site. Thus, it is possible that predators may generalize and treat any wing display by an insect of the size similar to *L. delicatula* (regardless of the detailed hindwing pattern) as a signal specific for chemically defended prey. The experiments were conducted about two months after the emergence of adult *L. delicatula,* which approximately coincides with two months after fledging of young insectivorous birds from their nests (June and July). Hence, at the time of the experiments (October), even the young birds were no longer naïve and might have learned to generalize the sudden wing display of *L. delicatula* as an indicator of the prey to avoid.

Based on our results, we cannot exclude the possibility that red coloration on the hindwings is an effective antipredatory adaptation only earlier in the season. For example, it is possible that sudden display of the red patch may enhance only the initial learning process of the naïve predators to recognize unpalatable *L. delicatula*. Since aposematic coloration accelerates learning (Gittleman & Harvey 1980; Roper & Wistow 1986; Johnston & Burne 2008) and delays forgetting (Speed 2000) in predators, hindwing coloration of *L. delicatula* may reinforce the learning processes in naïve young birds present in the habitat around the time when the adult insects emerge (June/July). Since none of the birds in October is likely to be naïve, this “reinforcement of learning” effect of hindwing coloration, which could have occurred earlier, may not be detected at this time any longer. *L. delicatula* may cause digestive problems in birds, and we have seen wild birds rejecting *L. delicatula* after initial pecking. Therefore, we believe that the display of *L. delicatula* does not simply function to promote startle reaction in the predator, but also to signal unpalatability. If this were the case, then the two hypothetical reasons above (i and ii) could not have fully explained why *L. delicatula* have red hindwing patches. However, even in those cases when wing display does startle the predators (the first and the second possibilities explained above), it may cease to be effective if the predators quickly habituate to startle displays (Ruxton et al. 2004). It is known that sometimes only naïve predators are startled by the display (Schlenoff 1985; Vaughan 1983). Because even the young birds in the wild were no longer naïve at the time of our experiments, we cannot exclude possible presence of habituation to the pure startle effect of the display (regardless of any association with unpalatability) by most predators present at our study site.

Although there was no sex difference in the probability of being recaptured, higher proportion of males than females suffered wing damage regardless of the presence or absence of the red patch,. The wing display in *L. delicatula* is activated in response to tactile, rather than visual, stimuli associated with pecking or grabbing an insect by a predator (in preparation). Additionally, the shape of the missing wing surface often matched avian beak shape (pers. obs.). Therefore, wing damage indicates bird attempt(s) at predatory attacks that did not lead to consumption of an insect. One can predict that wing damage occurs mostly in individuals who have a low tendency to jump in response to the predatory attacks, and therefore are subsequently pecked more times and handled more (at least by those predators who are not immediately deterred by the wing display) before the predator abandons them (due to aposematism). Because females have higher tendency to escape by jump than males in response to repeated pecking (Fig. 5.4), they are less likely to be handled by the predators, which may contribute to less wing damage in females than in males. Additionally, because females have larger yellow patches on their abdomen, which are visible to the predator during handling of the displaying prey, they may trigger predators' avoidance sooner than males do owing to stronger intensity of this possibly aposematic signal (Johnston & Burne 2008; Aronsson & Gamberale-Stille 2009). This would also contribute to less wing damage. Although nothing is known about the sex difference in the content of the defensive chemical (Xue & Yuan 1996) in the body of *L. delicatula*, we cannot exclude the possibility that females may be chemically better defended than males. If predators, while handling the prey, taste-detected such a chemical defence (Skelhorn & Rowe 2006a, b, 2007, 2010) earlier in females than in males, then less wing damage would have been observed in females than in males. Some or all of these effects may be sufficiently strong to counterbalance the hypothetical higher initial attack rates on females owing to female's larger and noticeable body size.

The two alternative antipredator strategies of *L. delicatula* (staying put with startle and jumping away without startle) cause different types of costs. Staying put with startle is energetically almost non-costly but bears a risk because insect survival depends on predator's experience and status (e.g. hunger). Jumping away without startle is beneficial when not followed by the predator, but includes energetic costs to prey such as direct cost of muscle use or indirect cost of loss of feeding time (Ydenberg & Dill 1986). At the time of our experiments, females *L. deilcatula* were getting ready to oviposit. If a female dies before opviposition she does not leave offspring (her fitness is be zero). This may explain why females jumped away more often than the males did. Males, on the other hand, have probably already mated many times, depositing their sperm in the bodies of the females. Therefore, we hypothesize, that males can risk being attacked as a consequence of staying put rather than jumping away.

In summary, the probability of being recaptured did not depend on the presence or absence of the hindwing red patch in an aposematic insect *L. delicatula,* at least during the time of our experiments. Although this indicates that conspicuous hindwing coloration did not noticeably affect the predation risk, we hypothesized that this could be due to the learning and generalization by the predators to avoid *L. delicatula*. Further studies are desirable earlier in the season when many predators are naive and inexperienced. Based on the larger area with yellow stripes on the abdomen in females and their tendency to jump away, rather than displaying at the site of attack, yellow stripe patterns in abdomen could also be effective as an aposematic signal that may contribute to less handling of females by the predators during non-lethal attacks.

Chapter 6. Hidden conspicuous signals augment predators' learning to avoid non-conspicuously colored defended prey

ABSTRACT

Defended prey are often characterized by conspicuous coloration and this phenomenon is called "aposematism". The main advantage of aposematism is that it enhances learning by predators to avoid the prey. Some defended prey species use a different strategy; they remain cryptic in the normal state, but display conspicuous aposematic signal (which is normally hidden) in response to a predator's approach/attack. This anti-predator strategy of a defended prey has not been well studied yet although it can theoretically give the benefits of both crypsis and aposematism. Using wild birds (*Parus minor*) and novel prey models, we tested whether hidden conspicuous display of defended prey accelerates the avoidance learning rate of predators and how does it compare with the typical conspicuous/non-conspicuous signal. We found the evidence that hidden aposematic signal indeed accelerates the avoidance learning rate of predators by two stages: i) enhancing the learning rate of the association between non-conspicuous (normal) state of the prey and prey defense, ii) promoting rejection after an attack has occurred by showing hidden conspicuous coloration. We show here the unique defensive coloration of prey which may provide dual benefits of crypsis and aposematism and highlight the specific mechanisms that hidden-aposematic signal provide the prey with survival benefits.

INTRODUCTION

Defended prey advertises its unprofitable properties (e.g. toxicity, distastefulness) by being noticeable and conspicuous (Poulton 1890; Fisher 1930; Cott 1940; Ruxton et al. 2004). Being conspicuous confers several benefits (Ruxton et al. 2004; Speed et al. 2009). Mainly, predators learn to avoid defended prey faster and forget slower when associated with conspicuous rather than non-conspicuous coloration (Gittleman & Harvey 1980; Riipi et al. 2001; Speed 2000; Guilford 1990). Typical aposematic colors are either red, orange or yellow often combined with black pattern, and these colors enhance avoidance learning rate of predators (or delay forgetting rate). The properties of these colors that give the enhanced learning effects to predators are their conspicuousness (Speed 2000; Osorio et al. 1999; Stevens & Ruxton 2012; Speed 2001), novelty (Roper 1993; Mappes & Alatalo 1997), distinctiveness relative to cryptic prey (Riipi et al. 2001; Gagliardo 1993), or high contrast against background (Roper & Redston 1987; Gamberalle-Stille 2001; Prudic et al. 2007). These colors are also known to induce neophobia (Coppinger 1969) and to decrease a chance of false recognition ((Gamberalle-Stille 2000); but see (Gamberalle-Stille et al. 2009)). These benefits have made a strategy of being constantly conspicuous a succesful antipredatory defense in chemically defended species.

However, defense of the conspicuous prey does not always guarantee protection; there usually are predators that can attack and prey upon the prey. Therefore one of the main problems of being conspicuous is that the conspicuous color not only advertises their defense to susceptible (and educated/experienced) predators), but also reveals the prey to the predators that can prey upon the prey. The defended prey can be attacked by the predators that are resistant to prey defense (Brodii & Brodii 1999; Exnerová et al. 2003), naïve or adventurous predators (Exnerová et al. 2010) or in some circumstances depending on the individual predator’s current body condition (Skelhorn & Rowe 2007). Because of this inter/intra-species variation in predator susceptibility to prey defenses, conspicuous prey cannot entirely avoid the risk of being attacked (and being killed). Therefore, although defended prey can gain many advantages from being constantly conspicuous, they rarely maximize their conspicuousness but rather maintain it at the intermediate level (Stevens & Ruxton 2012). The diversity in the form and strength of aposematic signal is considered to have arisen from the variation in predator community (i.e. variation in prey susceptibility of predators) in local area (Endler & Mappes 2004; Valkonen et al. 2012) or from the trade-offs between a range of costs and benefits (Speed & Ruxton 2007; Lindstedt et al. 2011) e.g. physiological costs of producing bright pigments versus costs of producing chemical defenses of being conspicuous (Darst et al. 2006).

While the strategy of continuous aposematism has been studied in details (Ruxton et al. 2004; Stevens & Ruxton 2012; Mappes et al. 2005), the other aposematic signaling strategies and the role of behavioral elements of animals for effective signaling have been largely ignored until recently (Marek et al. 2011; Willink et al. 2013).

To cope with the variable predator community, some prey have adopted strategies that result in different degree of conspicuousness depending on the context. For example, the swallowtail butterfly larva, *Papilio machaon*, appears cryptic from a larger distance, but appears more conspicuous (thus warning its distastefulness) at a close distance (Tullberg et al. 2005). Such a strategy may yield better protection than the strategy of constant distant-independent conspicuousness because benefits of being cryptic at a distance and being aposematic at a close range are put together.

Another strategy involves chemically defended prey species that are non-conspicuous or even cryptic, but in response to predator attack they show hidden conspicuous body parts (Cott 1940; Grober 1988; Kang et al. 2011; Cable & Nocke 1975; Matthews & Matthews 2009; Williams et al. 2000). The mechanism by which the chemically defended prey benefit from such a hidden-conspicuous signal has not been empirically studied yet. The benefit of being non-conspicuous (when the hidden signal is not displayed) is apparent due to the decrease in the probability of detection by predators, including those that may be dangerous to the prey. However, we still know very little about the mechanisms in which the sudden display of the hidden signal benefits the chemically defended prey. Current hypothetical explanations (Guilford & Cuthill 1989) propose that, in addition to the the benefits of the classical startle display of undefended prey (Evans & Schmidt 1990), the defended prey may benefit from sudden show of a bright signal at the moment of predator attack because predators may learn to stop attacks when seeing the signal of distastefulness ("*aposematic signaling*" hypothesis). This phenomenon is well discussed in Cott (1940) and has not been explored empirically for the last 70 years.

If this mechanism is the sole process that operates, then the frequency of initial attacks on, or approaches to, the defended prey in normal non-conspicuous state is not expected to differ from the frequency of attacks on other non-conspicuous prey. Thus the frequency of attacks is not expected to change as the predator is learning the association between the prey defense and the display of hidden signal at the moment of attack. Based on the classical psychological literature concerning the effect of potentiation (Mackintosh 1983) in learning we see another possibility that has not been considered yet: the sudden display of bright color may augment the speed of learning to treat the non-conspicuous color as aposematic signal. This reinforcing/potentiation effect may be in some aspects similar to the well documented potentiation in odor, flavor, visual cues paradigms in psychology (Rusiniak 1979; Lett 1985).

Hence, we may expect that sudden displays at the moment of approach or handling of the defended prey will help the predators to learn faster the association between the prey defense and the species-specific features of the defended prey in the non-signaling (non-conspicuous) state ("*enhanced learnin*g" hypothesis).

Here we tested these two hypotheses by comparing avoidance learning of wild-caught Oriental tits (*Parus minor*) between two treatments: bitter tasting non-conspicuous prey that reveals a conspicuous color at the moment of bird attack (H-CONS) and bitter tasting non-conspicuous prey (N-CONS) that lacks any additional display at attack. The *aposematic signaling* hypothesis predicts that after learning the association a predator will stop an attack at the moment of seeing the sudden conspicuous signal of prey defense in the H-CONS prey because, unlike for N-CONS prey, the predator is reminded at this moment of seeing the signal that the prey is defended. This hypothesis also predicts that the frequency of stopping an attack at seeing the hidden signal will increase with time as the predator learns the association between the hidden signal and the distastefulness of prey. If *enhanced learning* mechanism operates then the frequency of attacks on the H-CONS prey is expected to decrease faster in than for N-CONS prey as the predators learn the association between non-conspicuous color and distastefulness. Therefore, the final frequency of attacking the H-CONS prey by educated predators is expected to be lower than the frequency of attacks on the N-CONS prey.

Additionally, in order to put our results in the perspective of previous studies on continuous aposematic displays, we compared the avoidance learning of tits foraging on H-CONS prey with the classical well studied continuously conspicuous aposematic prey (CONS). This will give us idea if hidden-conspicuous signals are less or more efficient than the continuously displayed conspicuous signals in promoting avoidance learning of predators.

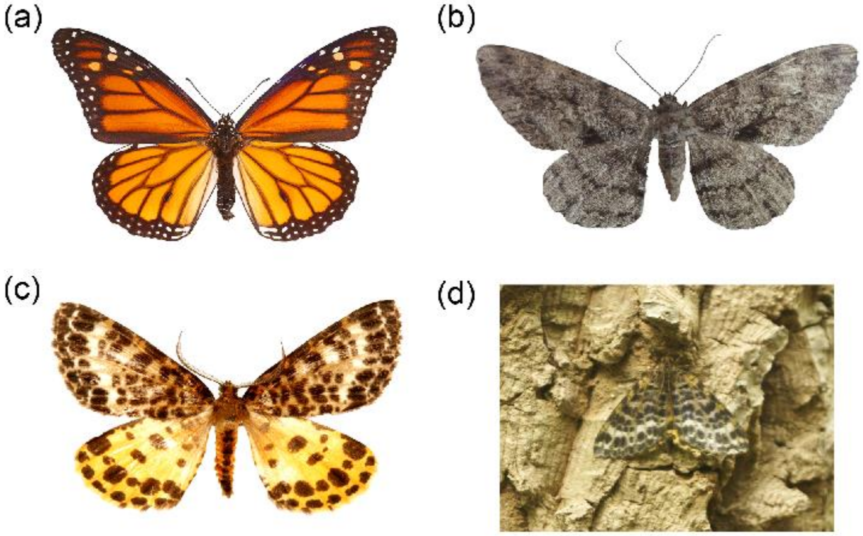


Figure 6.1. Examples of lepidopteran species that each has different types of defensive coloration. The names of each species are (a) *Danaus plexippus* (conspicuous), (b) *Hypomecis roboraria* (non-conspicuous), (c) *Arichanna melanaria* (hidden-conspicuous), (d) *A. melanaria* on a tree trunk. *A. melanaria* is considered to have cryptic forewings and conspicuous hindwings which are normally hidden under their forewings.

MATERIALS AND METHODS

Predators and experimental arena

We used oriental tit (*Parus minor*; ecological equivalent to great tit) as a general avian predator. During Dec 2011 – Feb 2012, wild oriental tits were caught and housed individually in outdoor compartment (80 × 80 × 150 cm, covered by wire mesh walls) in which all experiments were carried out (IACUC permission number: SNU-130621-6). They were raised under natural lighting condition (but through transparent plastic sheets on the roof) and temperature. We covered the walls between each compartment with white curtains to prevent visual interactions between the birds and set several perches in each compartment to give the birds places to sit on. However, during testing, we left only one perch near ground (to monitor prey handling of birds after taking a prey item from an experimental feeder placed on the ground) and few perches at high up (above 1 m). We provided sunflower seeds, butter, mealworms ad libitum with fresh water except during training and testing. On day 1-2 (day 1 indicates the day that birds were caught), we acclimatized the birds to the compartments (some seeds were scattered on the ground to induce the birds to forage on the ground). On day 3-4, we trained the birds to forage on the experimental feeder (see training section). On day 5-7, we performed the learning trials and on day 8, we released the birds back to the wild. Total 36 birds were tested.

Artificial prey models and experimental feeder

Prey items were divided into two classes: treatment (defended) prey and background (non-defended) prey. As treatment prey, we developed artificial prey models to mimic three kinds of insect prey: non-conspicuously colored (or winged) prey (N-CONS), conspicuously colored prey (CONS), prey with non-conspicuous visible wing and conspicuous hidden wing (H-CONS). All three types of prey have a single forewing part (a colored isosceles triangular paper, 2.5 cm height × 4.8 cm base) that a half-cut sunflower seed was glued on one side. H-CONS prey additionally has a hindwing part (a conspicuously colored trapezoid paper, 2.5 cm height × 4.8 cm base) that attached at the bottom of the forewing part. The sunflower seeds (that were attached to each prey) for the treatment prey were prepared by soaking the seeds in 2% Bitrex solution for 24 hours and drying them. Bitrex is a non-toxic bitter tasting chemical with no perceptible odor which have been known to induce aversive reaction of birds after tasting (25). The background prey were prepared as the same as N-CONS prey except that the sunflower seeds were soaked in distilled water instead of Bitrex solution, thus induce no aversive behavior of birds. The background prey provided variability in prey items and gave the birds a choice between palatable and unpalatable prey in each feeder. Some of the background prey also had a cryptically colored (the same color as the forewing) hindwing part.

To make the experimental feeder on which prey items were presented, we used a flat square Styrofoam plate (50 cm × 50 cm). We covered the plate with a kraft paper (which has light brown tone) and made sword cuts (about 5 cm for each) on the kraft paper to make a cutting plane. We fixed each prey by inserting hindwing part of the prey into each cutting plane. When the prey were fixed to the feeder, each prey only presented its forewing part, and hindwing part (if present) was hidden beneath the kraft paper. By this mechanism, we mimicked the situation where the hindwing of the prey was only shown when a bird attacks (in our case, pulls) the prey (see Fig. S1 for the shape of prey and the detailed mechanism of displaying the hidden-conspicuous hindwing part). The birds were trained to pull the prey item to eat sunflower seeds beneath each prey (see training section). Each feeder presented 16 prey items by 4 × 4 design. For each feeder, we randomly assigned 8 treatment prey and 8 background prey (see learning trials section).

Conspicuousness of colors is not absolute condition, but rather determined based on relative properties against background (such as relative contrast against background). Thus when we chose the colors that considered either ‘conspicuous’ or ‘non-conspicuous’, we considered conspicuous colors for those that generally considered aposematic (yellow, orange, or red), non-conspicuous colors for those that generally considered as cryptic (green, brown, or grey). For the generalization of the conspicuous and non-conspicuous colors (and to avoid biases which could be induced by using only one specific color), we made a color 'pool' which consists of a variety of colors (5 colors for non-conspicuous: green, light brown, dark brown, light grey, dark grey; 3 colors for conspicuous: yellow, orange, red) and assigned randomly chosen colors to each tit. When presented on the experimental feeder, both the conspicuous and non-conspicuous colors were well distinguishable from the background such that all prey items were easily visible to the birds, thus preventing camouflage-mediated variation in the encounter rate between the birds and the prey. Spectrometry of each color is provided in Fig. S2.

Training

On day 3-4, we trained the birds to pull out the prey items on a feeder to consume the sunflower seed beneath each prey. Prior to the training, we starved each bird 1 hour to motivate foraging. Then, to each bird, we presented an experimental feeder with prey items on which an edible sunflower seed was visibly glued. After birds learned to pick up a prey item to gain the sunflower seed, we presented another experimental feeder with prey items beneath which a sunflower seed was glued. The birds quickly learned and associated prey items with sunflower seeds and pulled out the prey items to get seeds. We continued this procedure until all the birds were able to associate the prey items with a sunflower seed. Since we used wild birds for testing, we had no priori information about their pre-existing experience against aposematically colored prey, which may affect aversive behavior of the birds. Therefore, during the training, we used all the 8 colors from our color pool (5 non-conspicuous + 3 conspicuous) to make prey items and observed whether the birds show a tendency to avoid specifically colored prey (especially aposematic ones) because of their prior experience. During training we followed the behavior of the birds. All birds attacked most of the prey items on the feeder and we found no observable avoidance behavior against specific color in all birds except for one bird which did not attack both orange and yellow color during training. For this bird, we did not use neither orange nor yellow prey model).

Learning trials

To test our hypotheses, we compared the learning rate of predators between three treatment groups which correspond to the three prey types: N-CONS, CONS and H-CONS. For each trial, we presented an experimental feeder, which consists of randomly assigned 8 background prey and 8 treatment prey according to the treatment type, to each tit. We used three different non-conspicuous colors to make 8 background prey in order to provide variation in prey items on each feeder: 4 prey with the same colored hindwing part, 4 prey without hindwing part (two prey for one color, two prey for another color). This variation in background prey was considered to make the learning task more difficult, but should reflect what happens in nature better (a choice among many other prey options). We randomly selected three non-conspicuous colors among the color pool for background prey for each tit. Likewise, when we chose the color for treatment prey, we randomly assigned one of the colors (not overlapped with background prey) among the color pool.

Before the trial, we carefully scrutinized any food items in the compartments and took out them to remove the alternative source of food. For each trial, we put the experimental feeder on the ground of each compartment and recorded each tit's foraging behavior until they finished exploring prey items on the feeder up to one hour. We conducted two times of the trial consecutively each day and continued for three days (i.e. there were total six learning trials for each tit). We always re-randomized the position of the prey each trial to avoid positional memory formation about treatment prey. Before the initiation of the trial in each day, we starved each tit for one hour to motivate them to forage on the feeder and no other food (except water) was given during the testing. All the learning trials were done between 1000-1600. After testing, we provided sufficient food to make sure that birds can preserve enough fats in body to endure night-time. We randomly assigned each tit to one of the three treatment groups, and 12 tits were tested in each treatment group.

Data analysis

To focus on our experimental purpose, we chose the first 8 prey items (half of the prey items in each feeder and also the same number as the number of treatment/background prey in each feeder) that attacked by each bird and analyzed it. These variables are robust to individual variations in handling time of the prey and other potential factor that might influence the behavior of birds (e.g. the degree of satiation). Most of the attacks were performed within 20 minutes (451 out of 464 observations) and we only analyzed the first 8 attacks within this duration because, after this duration, the birds' decision to attack were likely to be affected by (restricted to) less available choice among remained prey items (mostly distasteful prey). From the video, among the first 8 attacked prey, we extracted two variables, the number of attacked treatment (defended) prey and the number of eaten treatment prey. We considered a prey was attacked when a bird pulled the prey item by its beak (thus showed hindwing part if present), and a prey was eaten when the bird did subsequent handling towards the seed by beak after the attack. Although we set up a lower perch on where we intended to make the birds handling the prey, some birds sometimes took the prey to higher perches where we could not monitor its behavior through video. However, the birds almost always ate the sunflower seed when they took the prey to lower perch and we considered that when a bird took a prey to a higher perch, the prey was eaten.

In addition, to directly test whether the birds associated prey unpalatability with attack-induced conspicuous display of prey (i.e. whether the probability of rejection for those prey that have been attacked increased as trial goes), we recorded the binary response variable whether each attacked treatment prey was rejected (now we call this as ‘rejection’ for simplicity) or subsequently handled (thus tasted and consumed) by the bird. Thus, a prey was considered to be eaten based on the combination of two behavioral events; attacked and then not rejected by birds.

Previous studies on animal learning curve have claimed that learning curve does not follow linear relationship, but follows power function on average, so called “power law of learning” (Ritter & Schooler 2001; Newell & Rosenbloom 1983). We adapted power function for analysis and assumed that avoidance learning process of birds follow simple power law function ($y = a \times x^b$) with negative b (thus decreasing as trial goes), where a determines the range of y (i.e. the initial y value at $x = 1$), b determines the slope (the degree of deceleration) of y and. In our study, the parameter a for each treatment group was compared to test whether the behavior of birds at the first trial differ between treatment groups, b was compared to test whether the learning rate differ between treatment groups.

To estimate parameters for the learning curve function in the context of modern statistical tools which can take into account individual difference between each subjects, we log transformed both the explanatory variable trial and continuous response variables (i.e. analyzed in log-log space which makes the relationship linear) (Ritter & Schooler 2001) and fitted linear mixed models (LMMs). In this model, the estimates of the interaction between treatment group and log (trial) was equivalent to the parameter b in power function for each treatment group, the estimates for treatment effect have equivalent meaning as the parameter a (for the underlying logic, see Supplementary Information). In LMMs models, we identified the effect of treatment groups and interaction between treatment groups and log (trial) on the number of attacked/eaten treatment prey. We excluded log (trial) from the fixed effects to appropriately determine the estimate of a and b for learning curve model (see Supplementary Information). As random effects in LMMs analysis, we set log (trial) to have random slope within each bird with correlated intercept.

For the analysis of rejection among those that have been attacked, we could not employ mixed model scheme since only few prey items were attacked especially during trial 4-6 which made it difficult to incorporate random effects. Instead we used generalized linear models (GLMs, binomial family with logit link) to identify the effect of treatment group, trial as well as their interaction on the binary response whether the prey was rejected or not. In addition, we used Chi-square test of independence to determine whether the overall frequencies of rejection were associated with treatment groups.

To directly compare the behavior of birds at the first trial (before learning has occurred) and at the sixth trial (when learning process has progressed) separately, we employed analysis of variance (ANOVA; the effect of treatment groups on the number of attacked/eaten treatment prey) and GLMs (the effect of treatment groups on the probability of rejection).

The analysis were conducted in R environment (http://www.r-project.org). All test were two-tailed and P values for LMMs and GLMs were obtained using likelihood ratio test. For LMMs analysis we used ‘glmer’ function (package ‘lme4’) which can deal with unbalanced design (Bates et al. 2012) and ‘glht’ function (‘multcomp’ package) for post-hoc analysis. For post-hoc multiple comparisons, we used either planned comparison or Tukey’s all-pairwise test. We adjusted P-values to control the false discovery rate whenever multiple comparison was performed (Benjamin & Hochberg 1995). For other analysis, we used the given function in R (‘glm’, ‘aov’, ‘chisq.test’). Two birds (H-CONS and CONS group respectively) were excluded from the analysis since they attacked only a few prey items every trial. We lost several videos because of recording errors, and total 193 videos from 34 tits were analyzed.

RESULTS

Testing the enhanced learning hypothesis

The birds learned not to attack defended prey as trial goes in all treatment, but the speed of learning (i.e. the slope of avoidance learning curve) differed between treatment groups (Fig. 1A-B; LMMs; N-CONS learning rate = -0.160 ± 0.110 (SE); H-CONS learning rate = -0.551 ± 0.115;CONS learning rate = -0.577 ± 0.118; Chisq2=8.462, P=0.015). Post hoc multiple comparison showed that birds learned faster for both H-CONS and CONS prey than N-CONS prey (H-CONS vs N-CONS, Z=-2.459, P_{adj}=0.021; CONS vs N-CONS, Z=-2.593, P_{adj}=0.021). There were no difference between H-CONS and CONS prey (Z=-0.160, P_{adj}=0.873). We found no difference in the starting point (intercept) of learning curve (i.e. the values at the first trial) between treatment groups (Chisq2=0.614, P=0.736).

When we focused only on what happened at the first trial, we found no difference between treatment groups on the number of attacks (grey area in Fig. A; ANOVA; F2,30=0.138, P=0.872). However, at the sixth trial, we found significant difference between treatment groups (grey area in Fig. 1A; average attacks to H-CONS = 2.190 ± 0.227 (SE); to N-CONS = 3.118 ± 0.163; to CONS = 1.581 ± 0.189; F2,25=6.842, P=0.004). Post-hoc comparison revealed that both H-CONS and CONS prey were less attacked than N-CONS prey at the sixth trial (H-CONS vs N-CONS, t1=2.732, adj.P=0.017; CONS vs N-CONS, t=3.491, adj.P=0.005). We found no difference on the number of attacked defended prey between H-CONS and CONS prey at the sixth trial (t1=0.740, adj.P=0.466). These results suggest that predators associated the cryptic forewing with distastefulness faster for H-CONS prey than N-CONS prey which supports our enhanced learning hypothesis. The speed of avoidance learning rate for H-CONS prey was nearly as fast as CONS prey.

Testing the aposematic signaling hypothesis

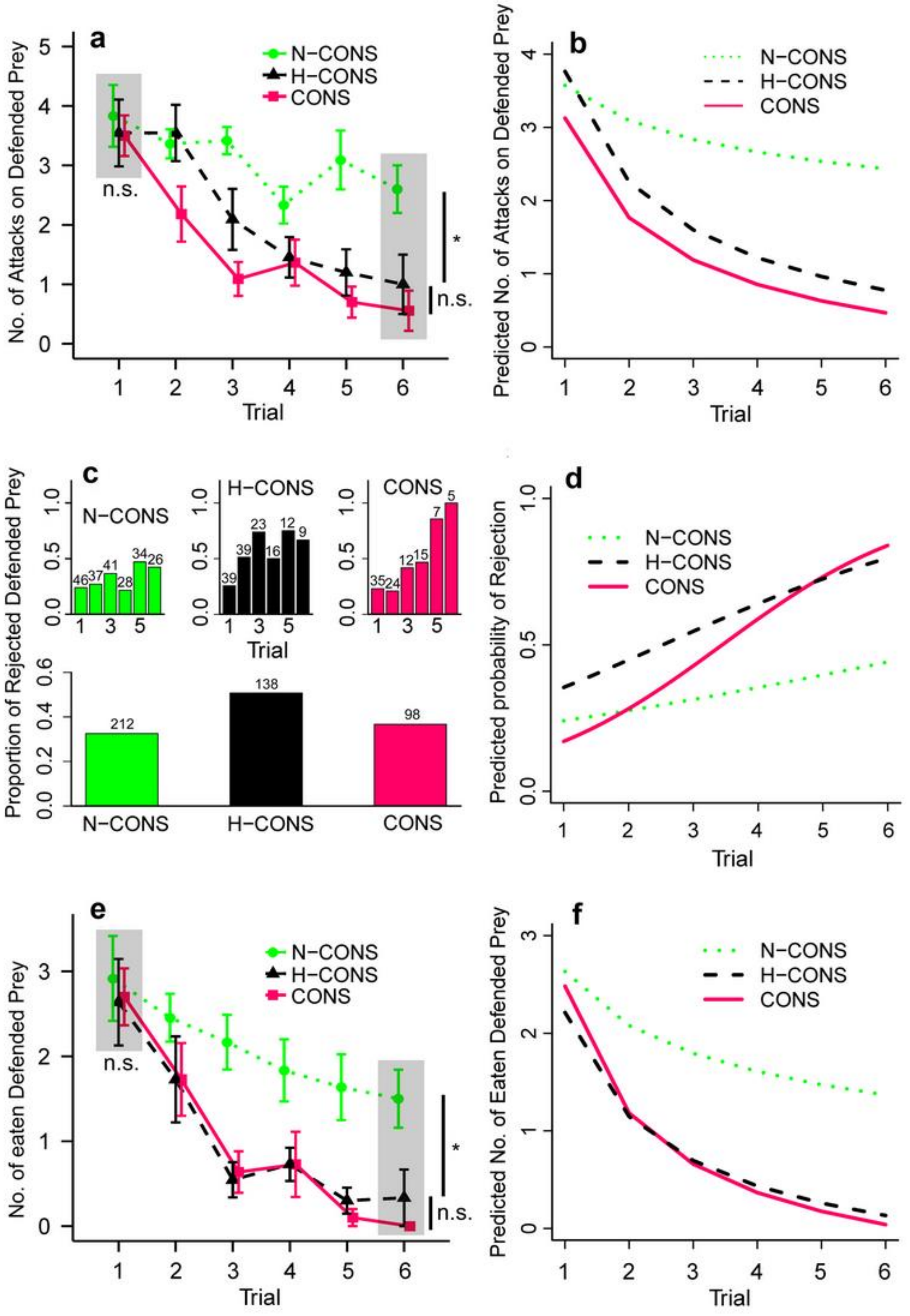
Among those prey that attacked, the birds rejected defended prey more often as trial goes (Fig. 1C-D; GLMs,Chisq1=19.163, P<0.001) and the increment in the rejection probability differ between treatment groups (Fig. 1D; GLMs, Chisq2=7.267, P=0.026). As trial goes, the rejection probability increased faster for CONS prey than N-CONS prey (z=2.512, adj.P=0.036). However we found no significant difference neither between H-CONS and N-CONS prey (z=1.381, adj.P=0.213) nor H-CONS and CONS prey (z=1.246, adj.P=0.213).

When focused on comparison within the first and within the sixth trial, the rejection probability did not differ between treatment groups neither at the first trial (GLMs, Chisq2=0.080, P=0.961) nor at the sixth trial (GLMs, Chisq2=4.329, P=0.115). However, generally, we found significant association between treatment groups and overall rejection frequency (Fig. 1C; Chi-square test, Chisq2=11.889, P=0.003). Specific comparison between two groups revealed that H-CONS prey were more rejected than both N-CONS prey (Fig. 1C; H-CONS: 51%, 68 rejections/138 attacks; N-CONS: 33%, 69/212; Chisq1=10.789, adj.P=0.003) and CONS prey (CONS: 37%, 36/98; Chisq1=3.985, adj.P=0.069). We found no association between CONS prey and N-CONS prey in overall rejection frequency (Chisq1=0.354, adj.P=0.552). These results indicate that, although the degree of increase in rejection rate of H-CONS prey was not differ with the other prey types, birds were more likely to reject H-CONS prey.

The number of consumed prey as a result of an attack/non-rejection

The birds learned not to eat the defended prey as trial goes and there were significant difference in the speed of learning between treatment (Fig. 1E-F; LMMs; H-CONS learning rate = -0.582 ± 0.104 (SE); N-CONS learning rate = -0.239 ± 0.100; CONS learning rate = -0.675 ± 0.106; Chisq2=9.738, P=0.008). Post hoc multiple comparison revealed that birds learned to not to consume both H-CONS and CONS prey faster than N-CONS prey (H-CONS vs N-CONS, z=-2.386, adj. P=0.026; CONS vs N-CONS, z=2.994, adj.P=0.008). There were no difference between H-CONS and CONS prey (z=-0.627, adj.P=0.531). We also found no difference in the starting point of the learning curve between treatment groups (Chisq2=0.412, P=0.814).

When focused on the comparison within first trial, we found no difference between treatment groups on the number of eaten prey (grey area in Fig. 1E; ANOVA; F2,30=0.105, P=0.901). On the other hand, at sixth trial, we found significant difference between treatment groups (average eaten H-CONS prey = 0.333 ± 0.333 (SE); N-CONS prey = 1.500 ± 0.342; CONS prey = 0; F2,25=8.060, P=0.002). both H-CONS and CONS prey were less eaten than N-CONS prey at the sixth trial (H-CONS vs N-CONS, t1=2.952, adj. P=0.010; CONS vs N-CONS, t1=3.795, adj.P=0.003). We found no difference between H-CONS and CONS prey (t1=0.822, adj.P=0.419).These results suggest that H-CONS prey were as effective as CONS prey to prevent consumption by predators in terms of predator avoidance learning speed.

a
N−CONS
H−CONS
CONS
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n.s.
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6
Trial
b
N−CONS
H−CONS
CONS
Predicted No. of Attacks on Defended Prey
0
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2
3
4
1
2
3
4
5
6
Trial
c
Proportion of Rejected Defended Prey
N−CONS
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41
28
34
26
H−CONS
39
39
23
16
12
9
CONS
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24
12
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0.5
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Trial
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N−CONS
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CONS
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CONS
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Figure 6.2. Comparison of the avoidance learning rate of predators in three treatment group: non-conspicuous (green dotted lines; N=12 birds; N-CONS), hidden-conspicuous (black dashed lines; N=11; H-CONS), conspicuous (red straight lines; N=11; CONS). (a-b) As trial proceeded, birds learned to not to attack H-CONS and CONS prey faster than N-CONS prey. (c-d) The frequency rejection of defended prey among those that have been attacked increased as trial proceeded. H-CONS prey were generally more rejected than the other prey types during learning stages. (e-f) As trial proceeded, birds learned to not to eat H-CONS and CONS prey faster than N-CONS prey. (b,f) These sub-figures show the number of attacked defended prey for each trail predicted by learning curve function (see methods). (d) This sub-figure shows the relationship between trial and probability of rejection of defended prey for each treatment. The speed of increase only differed between N-CONS and H-CONS prey (d). The grey areas and statistics in (a,e) show the comparison between treatment at the first and at the last (sixth) trial, separately. The bars (points) and error bars indicates mean ± SEM. The symbol n.s. indicates non-significant difference, * indicates $P<0.05$.

DISCUSSION

Our results support the enhanced learning hypothesis. The birds learned to avoid the prey with hidden conspicuous display faster than non-conspicuous prey only judging by the non-conspicuous appearance of the prey. Interestingly, the speed of learning was similar between hidden-conspicuous prey and conspicuous prey. This indicates that, in terms of avoidance learning, the hidden-conspicuous signal can be as effective as continuously conspicuous signals.

On the other hand, our results only provide restricted support for the aposematic signaling hypothesis. Although the rejection rate increased at the later trials, the speed of increase in rejection probability does not differ between hidden-conspicuous prey and non-conspicuous prey. However, the probability of rejection was always higher for the hidden-conspicuous signal than non-conspicuous signal throughout the trials. We propose two possibilities for this result. First, the overall higher rejection rate of hidden conspicuous prey raises the possibility that hidden-conspicuous prey might enjoy "startling" effects (Schlenoff 1985) by displaying conspicuous hidden part and prevented further handling even when associative learning process has not been fully formed yet. Second, it is also possible, and more plausible, that birds actually associated the hidden-conspicuous display to the unpalatability in part. If the rejection process was solely induced by startling effects, the rejection rate at the first trial (before learning has occurred) should be higher for the hidden-conspicuous prey. However, the rejection rates at the first trial were similar between the two treatments (24% for non-conspicuous prey, 26% for hidden-conspicuous prey). However, unlike non-conspicuous prey, the rejection rate for hidden-conspicuous prey rapidly increased at 2-3 trials and maintained higher rate.

These results are intriguing since hypothetical expectations from literatures, as well as our naïve expectations, predicted the aposematic signaling hypothesis as a main advantage of the hidden-conspicuous prey. However, our study suggests that benefits of hidden-conspicuous display are not only warning to predators when attacked, but facilitating/potentiating the association between their cryptic appearance and unpalatability.

According to the classical conditioning process, learning occurs through an association between conditioned stimulus (CS) and unconditioned stimulus (US) to elicit a conditioned response (CR) (Mackintosh 1983). In our study, the birds associated unprofitability (US) with color of the prey (cryptic forewing as CS1; conspicuous hindwing as CS2 if present) to avoid the prey (CR). Conspicuous colors are stronger signal than cryptic colors in terms of learning. Interestingly, our study suggest that sequential display of stronger signal (CS2) just after the weak signal (CS1) augment the association between CS1-CR.

In addition to the benefits of hidden conspicuous display, additional benefit conferred by hidden-aposematic prey is that it is cryptic normally: the cryptic form decreases the frequency of encounter with predators. However decreasing the encounter rate might diminish the effect of accelerated learning of predators and may cause the predators forget about the prey (Speed 2000). Because the display of conspicuous colors are mostly sudden, one potential benefit of the attack-induced display of hidden-aposematic prey could be it may function as a cue to predators to remind of the past experience with the prey. It would be intriguing to investigate whether the display of hidden conspicuous signal actually helps in forming long-term memory and delays forgetting of predators.

One can argue that the use of experienced birds can be problematic in learning trial experiments since we do not know about their pre-existing experience. Since we were not aware of the prior experience of individual birds formed in the wild, we could not rule out the possibility that the experience of individual birds might have affected our results. However, we found no sign of pre-existing avoidance behavior against specific color during training. In addition the results of the comparison within the first trial show no difference between the treatment groups in the number of attacks. Therefore we consider our novel prey models and colors of the models did not elicit pre-existing avoidance behavior of the birds.

Furthermore we argue that experienced birds may be more realistic predators of most of the prey than naïve ones since naïve birds quickly learn about aposematic colors and become experienced (Skelhorn & Rowe 2005) so that experienced predators are the most common types that prey encounter in nature. Thus we think that the use of wild predators has biological relevance in our study.

Because of the scarcity of the research, which might be attributed to the restricted knowledge about the presence of chemical defense in prey (mostly focused on the apparently aposematic species), little is known about characteristics of the prey with hidden-conspicuous signal. Behaviorally, we particularly concern how and when these prey display the hidden conspicuous signal. Especially, the moment of display might be crucial. When a predator is in certain distance, by displaying, the prey will increase a chance of preventing attack by warning before the predator approaches nearby. But it also increases the chance of being detected when the actual detection by the predator is in uncertainty. On the other hand, if the prey remains cryptic until the moment of attack, it might be safe from the 'false display' that revealing itself to the ignorant predator. However, if detected, it bears a risk of an initial attack (because of its non-conspicuous form) and may lose a chance to warn predator. It is predicted that prey should follow optimal decision of displaying moment which may vary between species (Parker & Smith 1990). Since the hidden-conspicuous signal facilitates avoidance learning of predators, aggregating behavior could be evolved as many aposematic prey since aggregation of prey facilitates avoidance learning.

Morphologically, prey with hidden-aposematic signal would also gain benefits by having thick, durable cuticle, as predicted in aposematic species (Cott 1940) since cryptic form is less likely to prevent initial attack from predators once detected. Thick cuticle would prevent fatal injury by an initial attack and would give a chance to display warning signal during prey handling of the predator.

Two species with hidden-aposematic signal, *A. melanaria* and *L. delicatula*, would provide good insights (among those have hidden-aposematic signal, as far as we know, two of few insect species that presence of defensive chemical has been identified (Nishida 194; Xue & Yuan 1996). *A. melanaria* is solitary species and sensitive to approaching (either vibratory or visually). In response to the approaching, it usually escapes to a nearby tree before being attacked. After landing on a new tree trunk, it often delays closing its forewings and displays its conspicuous hindwings for certain time which might function as a warning signal to the proximate predator (personal observation, Kang). Although *A. melanaria* has been described as a potential mimic of *A. gaschkevitchii* (Nishida 1994)*, A. gaschkevitchii* is native to Japan while *A. melanaria* can be found over most of the Eurasia. Thus we argue that the color patterns of the wings of *A. melanaria* (Fig. C-D) provide protection mainly by crypsis (by dull colored forewings) with hidden-aposematic signal (yellowish hindwings), not mimicry. *L. delicatula*, on the other hand, is insensitive to visual stimulus but displays its reddish hindwings (or escapes by instantaneous jumping) in response to tactile stimulus (Kang et al. 2011). The cuticle of *L. delicatula* is leathery so that it manages to survive predator's initial attack well (unpublished data, Kang; 93 among 111 survived young chick's initial attack). They often are found as an aggregated form.

In conclusion, our study provides empirical evidence for the effectiveness of hidden-conspicuous signals as antipredatory strategy. We show here that hidden-conspicuous signals facilitates avoidance learning of predators and provide the mechanisms by which the prey with hidden-conspicuous signal get survival benefits. We discuss the adaptive function of hidden-conspicuous signals in terms of predator-prey relationship. Further investigations no other types of trade-offs of prey such as a physiological cost of bearing chemical defense (Dobler & Rowell-Rahier 1994; Bowers 1992; Longson & Joss 2006) should expand our understanding of this unique defensive strategy of prey. We particularly encourage the optimality modeling approach since it can provide insights about the condition that hidden-aposematic signal can be favorable than other types of signals. We infer that the prey with hidden-aposematic signal would gain maximum fitness with a moderate level of secondary defense, like *A. melanaria* (Nishida 1994), in which there exists a relatively high risk of being attacked after detected by predators.

General Conclusion

Animal coloration has attracted attention of many people for a long time. Artists were mesmerized by beautifully colored animals such as peacocks or butterflies, and military services have been interested in concealing mechanisms by means of which cryptic animals hide their bodies in order to utilize them during wars. However, only recently researchers started to empirically explore the function of animal coloration and the processes that provide animals with crypticity in nature.

During my Ph.D., I tried to add novel findings and intriguing results into the field of animal coloration research using insects as model organisms. To sum up my studies shortly, through chapter 1 to 4, I revealed that bark-like moths are able to find locally most cryptic spots and body orientations by perceiving bark structure, and I proposed hypotheses on proximate mechanisms of this behavior. I also found that they are more likely to perform positioning behavior when their initial landing positions were poorly cryptic (i.e. when further improvement of their own crypticity was necessary). Through chapter 5-6, I investigated the understudied areas of animal coloration research: hidden-aposematic coloration. I tried to reveal the function of hindwing colors of *L. delicatula* and examined the effectiveness of conspicuous hindwing colors in natural population. In addition, I explored the mechanism by which conspicuous hindwing colors gain benefits in hidden-aposematic prey. I found that the hidden-aposematic coloration facilitates avoidance learning of predators as fast as continuously displaying aposematic coloration.

Apart from my thesis, still there are lots of interesting questions remained. For example, in relation to camouflage of moths, there are many other moth species that perform similar positioning behavior as *H. roboraria* and *J. fuscaria*. It will be interesting to investigate the evolutionary origin of this behavior and how this behavior co-evolved with morphology using comparative methods. This again raises another question whether positioning behavior evolved first and cryptic wing pattern evolved next or vice versa. It would be also interesting to see what specific elements of furrow structure moths visually recognize to adopt resting orientations. In relation to hidden-aposematic coloration, the studies on this issue are surprisingly scarce and many questions are still remained unanswered. For example, it will be interesting to investigate the toxic levels (such as chemical profiles and the amount of toxic chemicals) of hidden-aposematic prey and how it is compared with typical aposematic prey.

In conclusion, animal coloration is one of the most evident examples of natural and sexual selection. Each species evolved its own specific color patterns which are undoubtedly related to their ecological/evolutionary position and their life-history. There are still many interesting questions that remained to be investigated. My studies are just a fraction of the huge number of studies in the study of animal coloration, more widely in the field of behavioral ecology. I hope my thesis can contribute to expanding our knowledge on animals and encourage further studies in this field.

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Chapter 1. - Kang, CK., Moon, JY., Lee, SI., Jabłoński, P.G. 2012. Camouflage through an active choice of a resting spot and body orientation in moths. *Journal of Evolutionary Biology*, 25: 1695-1702

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국문초록

동물의 보호색 (defensive coloration)은 포식자로부터 자신을 보호하기 위해 진화한 피식자의 대표적인 방어전략이다. 생물의 보호색은 동물들이 1 차적으로 보내는 신호 (signal)로써 반포식 적응(anti-predator adaptation)의 기능을 하는 생물의 색채 패턴을 포괄적으로 일컫는다. 보호색의 대표적인 예로는 주변환경과 비슷한 색채와 무늬를 가짐으로써 포식자가 자신을 발견하지 못하게 하는 위장술 (camouflage)과 방어믈질의 존재를 (독 혹은 역겨운 성분) 밝고 눈에 띄는 색채를 통해 포식자에게 광고함으로써 포식을 피하는 경고색 (aposematism)이 있다. 본 박사학위 논문에서는 효과적인 위장을 위해 진화한 생물의 색채 패턴과 이러한 보호색과 맞물려 공진화한 행동을 연구하였으며, 방어물질을 지닌 생물들이 자신의 경고색을 선택적으로 광고(facultative aposematism)하는 ‘숨겨진 경고색’ (hidden warning coloration) 의 효용성에 대해서 연구하였다. 위장술 연구를 위해서는 자나방과의 나방을 이용하였으며, 숨겨진 경고색 연구를 위해서는 꽃매미과의 꽃매미와 인공먹이 모형 (artificial prey model) 을 이용하여 이러한 전략의 효용성을 연구하였다.

위장술은 많은 생물들이 1 차 방어수단으로 사용하는 전략이다. 전세계적으로 많은 연구들이 효과적인 위장을 위해 진화한 동물들의 형태학적인 특징과 그 효용성을 탐구해왔다. 하지만, 실제 자연상태에서 생물이 효과적으로 위장하기 위해서는 형태와 무늬 뿐 아니라, 그 생물의 행동적인 측면들도 매우 중요하다. 예를 들면, 위장을 위해 주변환경과

비슷한 색채와 무늬를 가지기 위해서는 자신과 비슷한 색채와 무늬를 가지는 배경 주변에 머물러야 한다. 실제 자연상태에서는 잎, 나무껍질 및 죽은 잎 등 다양한 배경요소들이 존재하는 것을 고려하였을 때, 자신과 비슷한 배경주변에 머무르는 행동은 효과적인 위장을 위해 매우 중요하다.

본 박사논문에서는 효과적인 위장을 위해 어떠한 행동들이 진화하였으며, 행동이 위장정도에 미치는 영향을 연구하기 위하여 자나방과의 나방 2 종 (세줄무늬가지나방, 줄구름무늬가지나방)을 선택하여 연구하였다. 나방은 피식자로서의 생태학적 위치로 인해 포식으로 인한 선택압이 강하여 다양한 보호색을 진화시켜 왔으며, 많은 포식자들(조류나 포유류, 곤충 등)이 활동하는 주간에는 대개 위장을 통해 자신을 보호하고 있다. 본 박사과정 연구에서는 나방들이 주변환경에 자신의 모습을 숨기기 위해 어떠한 행동을 보이는지 연구하였으며, 그 결과 나무껍질에서 쉬는 나방들이 ‘자리잡기 행동’ (positioning behavior) 을 통해서 위장정도가 높은 자리를 스스로 찾아간다는 사실을 밝혀내었다. 이후의 후속연구들을 통해 나방들이 자신의 위장정도가 낮을수록 이런 행동을 보이는 경우가 많았으며, 나무껍질의 3 차원적인 구조를 인지하여 좀 더 위장이 잘되는 자리를 찾아간다는 사실을 규명하였다. 또한 눈을 통한 시각정보와 날개에서 오는 촉각정보에 의존하여 구조를 인지한다는 사실도 규명하였다. 이 연구결과들은 나방의 행동과 형태가 위장이라는 목표를 위해 어떻게 공진화(co-evolution) 혹은 공전문화(co-specialization) 해왔는지를 보여주며, 이를 위해 쓰이는 감각기관에 대한 증거를 제시하고 있다.

박사학위논문의 2 번째 주제에서는 ‘숨겨진 경고색’ (hidden warning coloration) 이라는 이제껏 연구되지 않은 피식자의 방어전략에 대해 연구하였다. 많은 생물들이 포식자를 내쫓기 위한 방어물질 (독 또는 역겨운 물질)이나 방어기작을 가지고 있으며, 이런 생물들은 대개 눈에 띄는 색채를 가짐으로써 자신의 방어물질을 주변 포식자들에게 광고한다. 방어물질을 가진 피식자가 경고색을 가짐으로써 누릴 수 있는 대표적인 이점 중의 하나는 많은 포식자들이 피식자의 독 혹은 방어물질에 대해 좀 더 빨리 배우고, 회피반응을 보인다는 것이다. 그렇기에 방어물질을 가진 많은 피식자는 화려하고 눈에 띄는 경고색을 진화해 왔다. 하지만, 자연계에는 다양한 포식자들이 있으며, 어떠한 포식자들은 피식자의 방어물질을 무시하고 피식자를 포식하기도 한다. 그렇기에 눈에 띄는 색채를 가지는 경고색 전략은 자신을 회피하는 동물 뿐 아니라 자신을 포식할 수 있는 동물에게도 자신의 위치를 쉽게 드러내게 된다. 이를 극복하기 위해 어떤 피식자들의 경우에는 평상시에는 위장을 통해 자신을 보호하고 있으면서, 포식자에게 발각당하고 공격당했을때에만 경고색을 보임는 전략을 쓰기도 한다. 이러한 전략은 이론적으로 보호색과 경고색의 이점을 둘 다 가질 수도 있지만, 실제 이러한 전략의 효용성에 대한 연구는 진행된 바가 없다. 본 박사학위 논문에서는 이런 형태와 행동의 기능 및 효용성을 규명하기 위해 1) 자연상태에서 ‘숨겨진 경고색’이 피식자(본 연구에서는 꽃매미)의 생존에 미치는 영향을 실험하였으며, 2) 이러한 숨겨진 경고색을 가지는 피식자의 방어행동에 대한 조류 포식자(박새)의 반응을 연구하였다. 연구 결과 실제 자연상태에서

꽃매미의 밝은 색 속날개가 생존에 주는 이점은 발견하지 못하였다. 하지만 인공 피식자 모형을 이용한 실험에서는 숨겨진 경고색을 가지는 먹이에 대해 조류 포식자가 좀 더 빨리 배우고 회피행동을 보인다는 사실을 규명하였다. 이 연구 결과는 실제로 숨겨진 경고색이 일반적인 경고색과 유사하게 포식자의 회피반응을 유도할 수 있으며, 위장술 및 경고색의 이득을 둘다 취할 수 있는 전략이 될 수 있을 것이라는 이론을 뒷받침하고 있다.

본 박사학위 논문의 결과를 종합하면, 위장술 연구에서는 나방들이 자연상태에서 효과적인 위장을 위해 ‘자리잡기 행동’을 통해 위장효과가 높은 자리를 능동적으로 찾아간다는 사실을 밝혀내었으며, 효과적인 자리를 찾아가기 위해 이용하는 주변배경요소 (나무껍질의 3 차원적 구조)와 이 요소를 인지하는 감각정보/기관 (눈을 이용한 시각정보 및 날개에서 오는 촉각정보)의 종류를 규명하였다. 경고색 연구에서는 ‘숨겨진 경고색’이 가지는 효용성 및 자연상태에서의 효과에 대해서 탐구하였으며 이러한 전략이 경고색의 측면에서 가지는 이점에 대해서 최초로 밝혀내었다.

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