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Original Article

Number of eyespots and their intimidating effect on naïve predators in the peacock butterfly

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Predation experiments have shown that the large eyespots (concentric rings of contrasting colors) found on the wings of several lepidopteran species intimidate passerine predators. According to the eye mimicry hypotheses, the intimidation is caused by predators associating the eyespots with the presence (of the eyes) of their own enemy. The conspicuousness hypothesis suggests, instead, that it is simply the conspicuousness of eyespot patterns that is intimidating, possibly due to a sensory bias. We studied how the number of eyespots, 2 or 4, influences intimidation. We predicted that if eye mimicry is important, the maximum response would be reached with a pair of eyespots. On the other hand, if conspicuousness is important, then more than 2 eyespots should result in an even stronger response. The peacock butterfly, *Inachis io,* has 4 large eyespots on its wings. We presented naïve insectivorous birds (pied flycatcher, *Ficedula hypoleuca*) 2 different prey items made from wings of dead peacock butterflies and a mealworm between the wings. One group of birds received prey that had no or only 2 eyespots visible and the other group received prey that had no or all 4 eyespots visible. Eyespots clearly increased hesitation before attacks. Because the birds were naïve, this difference in response to the eyespots was innate. Importantly, there was no difference in attack latency between 2 and 4 eyespots. We conclude that it is unlikely that conspicuousness as such has selected for eyespots in the peacock butterfly.
Key words: antipredator adaptation, intimidation, pied flycatcher, predation, prey coloration. *[Behav Ecol 22:1326–1331 (2011)]*

INTRODUCTION

Although camouflage provides for many animals an effective way to avoid predation (Stevens and Merilaita 2009), highly conspicuous body coloration or markings in prey animals are not uncommon either. One well-known example of such conspicuous prey coloration is eyespots (i.e., patterns consisting of concentric circles of contrasting colors). As the name implies, these patterns resemble, to a varying degree, the vertebrate eye. Eyespots have been most intensely studied in insects, especially in butterflies, but are found in many other taxa too, such as molluscs, fish, and birds (Poulton 1890; Blest 1957; Ruxton et al. 2004; Stevens 2005). The occurrence of eyespots in a wide range of taxa is interesting per se and suggests that these patterns are important and effective signals in the animal kingdom. However, our understanding of the possible adaptive functions of eyespots is still far from complete and is complicated by the fact that eyespots vary in shape, size, number, and position among different prey taxa.

In the present study, we focus on the antipredator function of eyespots and more specifically on their use to intimidate predators. Some eyespotted butterflies have been demonstrated to intimidate or startle predators, to make them less willing to attack, or to buy time to be able to escape (Vallin et al. 2005, 2006, 2007; Kodandaramaiah et al. 2009). These eyespots are typically relatively large, whereas eyespots that are located at wing margins and have been hypothesized to have a deflective function are smaller. Historically, there have existed 2 different ideas for why large eyespots in butterflies repel predator attacks. Due to their conspicuousness, eyespots appeared to be a good candidate for aposematic signals that warn predators for secondary defenses, such as distastefulness or toxicity. However, that eyespots constitute an aposematic warning signal was rejected as a general explanation by Morton Jones (1932, 1934) who showed that dead specimens of *Automeris io* (Saturniidae) were readily eaten by birds even when they were presented with their eyespots exposed. Furthermore, most of the present evidence for an antipredator function of large eyespots in butterflies has come from studies on nontoxic butterfly species (e.g., Vallin et al. 2005; Kodandaramaiah et al. 2009).

Recent experiments have provided firm evidence for the second historical idea, namely that large eyespots repel predators through intimidation. They demonstrate that some visually oriented predators hesitate or interrupt attacks toward butterflies

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that have large conspicuous eyespots. This evidence comes from experiments where wild-caught passerine birds were presented living or dead edible butterflies. In these experiments, butterflies with concealed eyespots were attacked at a higher rate than butterflies with visible eyespots (Vallin et al. 2005, 2006, 2007; Kodandaramaiah et al. 2009).

Why some eyespots deter predators is currently debated. According to the classic eye mimicry hypothesis, eyespots are intimidating due to their resemblance to the vertebrate eyes, which the attacking predators associate with the presence of their own enemy (e.g., Poulton 1890). The eye mimicry hypothesis has been challenged with the conspicuous signal hypothesis according to which it is not the resemblance to the eye but simply the high contrast of the eyespot pattern that intimidates predators (e.g., Stevens 2005). Stevens et al. (2007, 2008) have presented results supporting the conspicuous signal hypothesis. Their results come from field studies in which artificial prey items consisting of a printed piece of paper and a mealworm as a reward were pinned on tree trunks. In these studies, they have monitored the effect of elegantly manipulated artificial eyespots on predation rates by wild birds. By conducting a series of experiments in which more and less eyelike patterns have been varied in shape, number, size, and contrast within the pattern, Stevens et al. (2007, 2008) have attempted to identify those visual features that make eyespots intimidating.

Because vertebrate eyes come in pairs, one could expect that if eye mimicry explains the intimidating effect of eyespots, then 2 eyespots would have the strongest deterring effect. However, Stevens and colleagues showed that attack risk of artificial prey was not influenced by the number of eyespots (1, 2, or 3) when the total area of the eyespots was kept constant.

Interestingly, although some real butterflies only have 2 large eyespots, some others have 4. For example, the peacock butterfly (*Inachis io*) has one pair on its forewings and another pair on its hind wings. In accordance to the eye mimicry hypothesis, one could argue that a butterfly should expose 2 rather than 4 eyespots because having 4 eyes is not a characteristic of a vertebrate predator. On the other hand, if conspicuousness of the signal is more important, it should pay in terms of intimidation to double the number of exposed eyespots. Thus, if the conspicuous signal hypothesis explains why the peacock butterfly has evolved 4 eyespots instead of only 2, then we would expect the additional 2 eyes to markedly increase the intimidating effect to predators of the butterfly.

We used naïve insectivorous passerine birds (pied flycatcher, *Ficedula hypoleuca*) to test the response elicited by peacock butterflies with either 4, 2, or no eyespots. Because the birds had been reared in captivity, they were naïve both with respect to their natural prey and enemies. Thus, any hesitation to attack prey with eyespots should be innate and not based on prior learning or experience. The question whether the intimidation caused by eyespots is an innate (Blest 1957) or a learned response (Coppinger 1969) has been debated. Whether or not predators' aversive response toward eyespots is innate is an important step toward an understanding of their evolution because innate aversion would have substantially strengthened selection for eyespots. With the present study, we address 2 questions about the evolution of intimidating eyespots. First, we test whether 4 eyespots are more intimidating than 2 eyespots as would be predicted by the conspicuous signal hypothesis. Second, we want to find out if the intimidation is an innate response.

METHODS

The experiment was conducted in April 2009, at the Botanical Garden of the University of Turku, situated in south-western Finland. Pied flycatchers, *F. hypoleuca*, were used as predators in the experiment. Pied flycatchers are small insectivorous passerines that breed throughout a large range over Europe and Western Siberia and winter in Western Africa. They forage on the ground, on leaves and tree branches, and additionally through aerial attacks on flying insects (Lundberg and Alatalo 1992).

The birds used in this experiment had been collected from their nests as fledglings in June 2008 and reared in captivity over the winter. For half of the individuals, testosterone levels in the eggs had been elevated. This had been done because the birds were originally reared for another experiment (for further details, see Ruuskanen and Laaksonen 2010). Importantly, the testosterone treatment ($F_{1,35} = 0.33$, $P = 0.57$), the sex of the birds ($F_{1,35} = 1.46$, $P = 0.23$) or any interaction term, in which either of these 2 factors were involved, did not significantly influence the attack latency in this experiment, and therefore, we excluded these factors from further analyses. The birds were approximately 10 months old when they entered the present experiment.

Because the birds were raised in captivity, they were naïve with respect to prey eyespots and to natural enemies. Birds were kept in 5 indoor aviaries (5 × 3 × 2.5 m) in groups of 10–12 individuals. The aviaries were made of large white-washed wooden tiles and lit by daylight fluorescent tubes with a light regime following the local daylight hours. The inside of the wall containing the aviary door was covered with a large piece of black cloth to avoid birds escaping when we entered or left the room. Birds were free flying and had access to perches and shelter as well as ad libitum access to food and water. The food consisted of live mealworms (*Tenebrio molitor*) and dipteran larvae, dried insect patee (Orlux), egg food, supplementary vitamins, and calcium. In addition, approximately 2–3 times a week, the birds were offered live flies or crickets. For a full description of the rearing and housing conditions, see Ruuskanen and Laaksonen (2010).

The peacock butterfly, *Inachis io*, is common throughout Europe and thus sympatric with pied flycatchers. It is a well-known species that is easily recognized because of the 4 conspicuous eyespots that adorn the dorsal side of the wings. The larvae feed on the stinging nettle, *Urtica dioica*, and in northern temperate areas, the adult butterfly hibernates in hollow trees, barns, and attics for up to 7 months from late autumn to early spring. The butterfly is not toxic and is consumed without hesitation by insect eating birds (Blest 1957, Vallin et al. 2007). Prior to the experiment, larvae of wild-caught females were raised on stinging nettles, and after eclosion, the adult butterflies were transferred to flight cages (0.8 × 0.8 × 0.5 m) where they were fed on a 25% sucrose solution for 1 week. They were then freeze killed and stored in dark, dry, and cold until shortly before the experiment.

Because we were interested in the flycatchers' unconditioned response to the eyespot pattern per se and not the combined effect of pattern and butterfly behavior, we used the wings from dead butterfly specimens. To ensure that this would constitute an attractive prey, the dry butterfly body was replaced by a mealworm (cf. Kodandaramaiah et al. 2009). Using tweezers, we separated the wings from the thorax of the dead butterflies. The wings were then glued, using paper glue (Casco RX, Akzo Nobel, Sweden) onto plates made of cardboard covered by gray paper (12 × 6 cm) and care was taken to place the wings in the position that can be observed when living peacock butterflies are exposing their eyespots. Using a black water-based permanent marker (Zig Posterman, Kuratake, Japan), we produced 3 different treatment groups of butterflies that had either all 4 eyespots covered (hereafter No Spots), the 2 forewing eyespots covered, and the 2 spots on the hind wings visible (2 Spots) or all 4

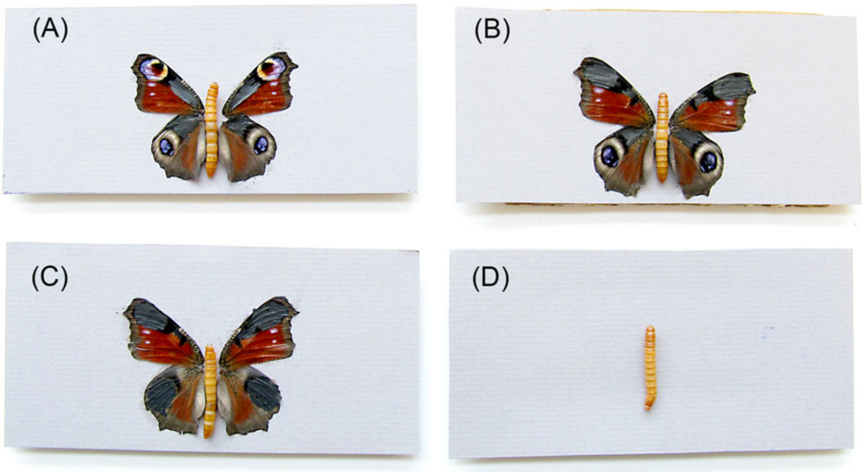

Figure 1
The 3 prey types used in the experiment: (A) all 4 eyespots visible, (B) only the 2 eyespots on the hind wings visible, and (C) all 2 eyespots covered. Notice that to control for the effect of the black felt tip pen that was used to cover eyespots, an equal area adjacent to all visible eyespots was also painted. The butterfly body was replaced with a mealworm to provide the birds with a reward. Prior to the experiment the birds were trained by presenting them with mealworm but no wings (D).

eyespots visible (4 Spots) (Figure 1). To control for the effect of the black marker that was used to cover eyespots, an equal area adjacent to all visible eyespots was also painted, so that an equal area of the wing was covered with the marker in all experimental butterflies (Figure 1). Each bird was subjected to either one of the 2 controlled treatments consisting of presentation of a random sequence of either 2 Spots and No Spots or alternatively 4 Spots and No Spots butterflies. Because the number of butterflies was limited, we used each plate in 2 randomly chosen trials, provided that it remained visually intact after the first trial.

The day before training, all birds in one aviary were captured and placed in individual home cages (63 × 55 × 35 cm) fitted with perches, food trays, and water containers. The following day birds were taken, 2 at the time and always from the same aviary, from their home cages and transported individually in small cloth bags to the experimental room (4 × 2 × 2.5 m) situated in an adjacent building. The experimental room was lit by 2 daylight fluorescent tubes and contained 2 experimental bird cages identical to the birds' home cages. These experimental cages contained a water container and 2 perches placed at 13 and 36 cm from the right cage wall. The floors of both experimental cages were covered with a sheet of beige dull paper. On 3 sides of the cages, black pieces of cloth, identical to the cloth in the aviaries, prevented the birds from seeing each other during training. The fourth wall of the experimental cage was left uncloaked to allow video recording of the training and experimental sessions. The door opening in the room was covered with a curtain in which a small hole allowed observation of the room without noticeably disturbing the birds. Both the training sessions and the experiment described below took place between 8.30 AM and 4.55 PM and were conducted in the experimental cages.

According to our training protocol, the birds were left in the cloth bags for 30 min and thereafter transferred to one of the experimental cages. After an additional acclimatization period of 30 min, the lights in the room were switched off. A small cardboard plate covered with gray paper, identical to the plates on which the dead butterflies were mounted, was placed on the floor of each cage at 4 cm from the left cage wall. On top of each plate, we placed 3 dead mealworms. Before the lights were switched on, we began recording the experimental cages using 2 video cameras (Sony DCR-SR52). Birds were then allowed 45 min to forage in order to get habituated to eat from the cardboard plate without hesitation. After the training session was completed, birds were returned to their home cages in the aviary. Birds that ate 2 or 3 mealworms were considered to be adequately habituated to the situation and were used in the experiment the following day. Individuals that ate zero or only one mealworm were subjected to a new identical training session the following day. Birds that failed this second training session, that is, ate fewer than 2 mealworms were excluded from the experiment. In total 6 birds of 57 failed the training.

On the day of the experiment, one bird at a time was, as in the training procedure, transferred from the home cage and kept in a cloth bag for 30 min before being released into the experimental cage. Thus, during an experiment, only one bird was present in the room. After the additional 30 min of acclimatization in the cage, the lights were turned off. Then the video recording was started, a cardboard plate containing one of the 3 butterfly treatments was placed at the same spot on the cage floor as the training plate had been, and the lights were switched on again. In the empty space between the butterfly wings, a dead mealworm now replaced the original head, thorax, and abdomen of the butterfly (Figure 1). After 30 min or as soon as the bird had consumed the mealworm, the lights in the room were switched off, and the butterfly plate was removed from the experimental cage. In its place, we put a training plate containing one mealworm, and the bird again had 30 min to consume the offered mealworm. In order for the bird to build an appetite after this second mealworm, we waited an additional 10 min before switching off the lights. Now the third and final plate of the experimental session was placed in the cage. This plate again contained a mealworm surrounded by butterfly wings, and the 30 min limit was again applied. The 2 experimental plates, containing a mealworm and butterfly wings, always consisted of 1 eyespot treatment, 2 Spots, 4 Spots, or No Spots. Thus, every bird was exposed to one set of butterfly wings with eyespots and one set lacking eyespots. The order was randomized but was balanced in order to achieve treatment groups of approximately equal size. Of the 51 birds that completed the experiment, 25 received a 2-spotted prey and 26 received a 4-spotted prey as the prey item that displayed eyespots.

To estimate hesitation of the predators and deterring effect of the prey types, we measured the time until the flycatchers attacked the mealworm. In addition to the attack time, we also measured 3 supplementary behavioral variables as they might indicate frustration or agitation due to fear in the predator. These variables were 1) proportion of time spent on the first perch relative to the second, 2) number of "loops" per unit time (a loop was counted when a bird flew up from a perch into the air and returned to the same perch without landing elsewhere), and 3) number of perch shifts per unit time.

After the behavioral experiments had been conducted, the birds were released to the wild in May 2009 (after being reared for 11 months in captivity) at the normal breeding time of wild flycatchers in the natal population. The birds were in good body condition. Rearing the birds in large aviaries and providing them with diverse live food during captivity ensured that the birds could fly and forage normally. It is very difficult to recapture birds from the wild at this stage or in later years due to low return rates, and thus, recruitment of the captive birds could not be assessed. However, none of the birds was seen at the site of release later on, suggesting that they habituated quickly. Rearing of the birds in captivity, all the experiments and the subsequent release of the birds were conducted under license from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESLH-2008-03693) and the Environmental Center of Southwestern Finland (license number LOS-2007-L-264-254).

Statistical analyses

We used R 2.9.2 for Windows (R Development Core Team 2009) for analyzing the data. To analyze the times to attack, we used the function *aov* to conduct a repeated measurements analysis of variance (ANOVA). The data met the requirements for parametric analysis after the power transformation that was suggested by the Box–Cox analysis. We used paired *t*-tests and 2-sample *t*-tests with sequential Bonferroni correction for post-hoc analyses. The distributions of the data for the time that the birds spent on each of the 2 perches and for the numbers of loops and shifts between perches were skewed, and thus, these data were analyzed using the nonparametric Wilcoxon matched-pairs test.

RESULTS

The Order × Display interaction had a significant effect on attack times, indicating that presentation order influenced how the birds reacted to whether or not the prey displayed eyespots (repeated measurement ANOVA, Order × Display: $F_{1,47} = 8.63$, $P = 0.0051$; Figure 2A). Also, the factor Display was significant (Display: $F_{1,47} = 20.5$, $P < 0.001$; Figure 2A). However, because of the significant interaction, post-hoc tests (see below) were necessary for the interpretation of the effects of Order and Display on attack times. Interestingly, our analysis did not lend any support for the effect of the number of eyespots of the prey that displayed eyespots (2 Spots or 4 Spots) on attack times (Eyespot number: $F_{1,47} = 1.61$, $P = 0.21$; Eyespot number × Display: $F_{1,47} = 0.068$, $P = 0.79$; and Eyespot number × Display × Order: $F_{1,47} = 0.31$, $P = 0.58$). None of the remaining factors or interaction terms were found to be significant (Order: $F_{1,47} = 0.014$, $P = 0.90$ and Eyespot number × Order: $F_{1,47} = 0.030$, $P = 0.58$).

Due to the significant Order × Display interaction, we conducted post-hoc tests to more closely investigate the influences of the presence of eyespots and presentation order. Because the effect of the number of eyespots was not significant, we first pooled the birds that had been presented 2 eyespots and 4 eyespots. The attack times toward the prey with eyespots were significantly longer than toward the prey without eyespots, both when the prey with eyespots was presented first (paired *t*-test: $t = 4.77$, degrees of freedom [df] = 24, $P < 0.0001$; Figure 2B) and when the prey without eyespots was presented first (paired *t*-test: $t = 2.71$, df = 25, $P = 0.012$). In addition to the eyespot display, also the presentation order had an effect on attack times. Thus, the birds were more hesitant to attack both prey when the prey that had eyespots was presented first compared with when the spotless prey was presented first (prey that had eyespots, *t*-test: $t = 2.437$, df = 49, $P = 0.018$ and no eyespots: *t*-test: $t = -2.477$, df = 49, $P = 0.017$; Figure 2B). All these *t*-tests remained significant also after the sequential Bonferroni correction for multiple testing.

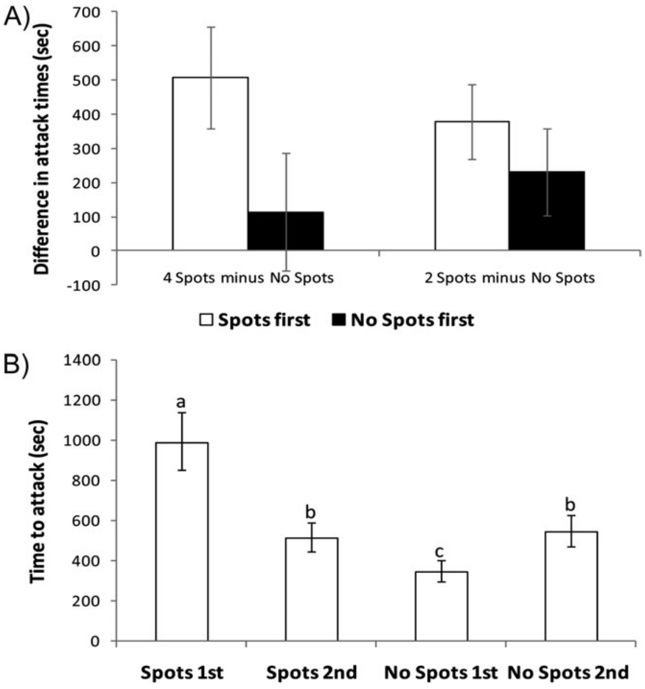

Figure 2
Half of the birds received a prey that had all 4 eyespots visible (4 Spots) and a prey with all eyespots covered (No Spots). The other half received a prey that had 2 eyespots visible (2 Spots) and a prey with all eyespots covered (No Spots). A bird received either the prey that had visible eyespots first (Spots first) or the prey that had all eyespots covered first (No Spots first). (A) Mean ± standard error (SE) of the difference in attack times between the spotted and the spotless prey. (B) Mean ± back-transformed SE of attack times. Because the number of eyespots did not significantly influence attack times, the data were pooled for the presentations in which the prey that had visible eyespots (Spots) and for the presentations in which the prey that had no eyespots visible (No Spots). First and second refer to presentation order. Different letters between 2 groups indicate that post-hoc test (6 paired and 2-sample *t*-tests corrected sequential Bonferroni method for multiple testing) was significant between those 2 groups.

Apart from attack latency, we did not find any effect of the eyespot display on any of the other behaviors that we recorded. Hence, the eyespots did not influence the proportion of time spent on the perch located closer to the prey (Eyespots: median = 96.2%, interquartile range (IQR) = 14.9 and No Spots: median = 94.7%, IQR = 13.2; Wilcoxon: $W = 1241.5$, $N = 50$, $P = 0.96$), the frequency of switching perch (Eyespots: median = 0.013 s^{-1}, IQR = 0.046 and No Spots: median = 0.006, IQR = 0.040; Wilcoxon: $W = 1391$, $N = 50$, $P = 0.32$), or the frequency of flying "loops" (Eyespots: median = 0.007 s^{-1}, IQR = 0.032 and No Spots: median = 0.001, IQR = 0.036; Wilcoxon: $W = 1391.5$, $N = 50$, $P = 0.31$). The results regarding these 3 behaviors would not change qualitatively, even if the birds that were presented the 2-spotted prey and the birds that were presented the 4-spotted prey were analyzed separately.

DISCUSSION

Our experiment demonstrated a deterring effect of eyespots. The flycatchers took a markedly longer time to attack the peacock butterflies that had eyespots than those that had no visible eyespots. This result confirms the findings of some previous experiments that have also shown a deterring effect of large eyespots of butterflies toward passerine predators (e.g., Vallin et al. 2005; Kodandaramaiah et al. 2009). We found, in addition, that when the prey item with visible eyespots was presented first, it also made the birds hesitate longer before attacking the prey item that had no visible eyespots when compared with attack times when the order of presentation was reversed. Importantly, our experiment did not show any noteworthy difference in the deterring effect between the prey displays consisting of 2 and 4 eyespots.

Our experiment differs from the previous experiments on intimidating effect of eyespots in that we used naïve birds as predators (see also Blest 1957). The use of naïve birds enabled us to exclude the effect of any previous experiences, such as, for example, encounters with their enemies or prey that has eyespots, which might have influenced the response of the predator in our experiment. Although the birds were not naïve to the eyes of their own species, it is very unlikely that this would have had any effect on our results as the eye of the pied flycatcher is plain black and small compared with the tested eyespots. Naïve pied flycatchers have also been used in an earlier study on butterfly eyespots, but the aim of that study was to investigate if small eyespots located at the wing margin of butterflies could deflect predators (Lyytinen et al. 2004). In the present study, focusing on the deterring effect of eyespots, the birds hesitated markedly longer before attacking the spotted prey than the spotless prey despite the fact that the birds were naïve. This clearly shows that the response is innate rather than induced by any experiences, for example, of enemies. Furthermore, the spotted prey also increased the hesitation before attack in the following prey presentation. Interestingly, this suggests that the deterring effect of the eyespots is not only limited to the moment when the predator views the signal, but it increases the cautiousness of the predator also for some time after the signal is no longer present. Although the predators in this experiment attacked the prey after hesitation, this prolonged influence is indicative for the power of the deterring effect caused by eyespot patterns. We also note that although the behavioral aspect of the eyespot display in the peacock butterfly may play an important role in intimidation (Blest 1957; Vallin et al. 2005, 2007), the effect that we found in the present experiment was caused solely by the eyespot pattern, independently of butterfly behavior.

The reason for the intimidation experienced by predators that encounter eyespots is still unclear and debated. According to the classic explanation, eyespots intimidate predators due to eye mimicry, that is, the association of eyespots with the presence of a predator's own enemy (Poulton 1890; Cott 1940; Blest 1957; Janzen et al. 2010). Because humans tend to experience a resemblance between many eyespots and eyes, this idea has been widely accepted. However, it has shown to be difficult to prove or to falsify. The eye mimicry hypothesis has been challenged with the conspicuous signal hypothesis according to which it is not the resemblance to the eye but a feature such as conspicuousness or contrasts of the eyespots that exploits predators' innate biases or avoidance responses (Blest 1957; Stevens 2005; Stevens et al. 2008). Importantly, in our experiment, the deterring effect was not markedly stronger when all 4 eyespots were displayed than when only the 2 hind wing eyespots were displayed. Hence, our results based on real butterfly color patterns do not support the conspicuous signal hypothesis, according to which a larger or more conspicuous signal would be expected to induce a stronger response (Stevens et al. 2008). Also, we think that the fact that the sexual signals of many passerine species (e.g., the pied flycatcher: Siitari et al. 2002; Sirkiä and Laaksonen 2009) employ similar features (brightness and contrast) may on a more general level question the conspicuous signal hypothesis and the suggestion that such features could as such function as antagonistic signals that would induce a hard-wired fright response in passerine birds.

In a previous field experiment that used artificial prey made of triangular piece of printed paper and a mealworm as the food item, the mealworm disappeared faster due to predation when the prey had no eyespots compared with prey that had eyespots. That the prey "survival time" was further increased with increasing number and area of the eyespots was suggested to support the conspicuous signal hypothesis as the underlying reason for predator intimidation (Stevens et al. 2008). However, it is also possible that instead of intimidation, this result was caused by a distractive effect: The predators had not been trained to look for the food item or to associate the piece of paper with the food item, and the eyespots may have attracted the attention of the predators away from the food item, making it more difficult to detect it rather than making the prey item intimidating (Stevens et al. 2008; Dimitrova et al. 2009). Another possible reason for the different results is that in our experiment, only a single predator species was used, whereas the field experiments are likely to reflect combined predation pressure involving a large number of disparate predators.

The intimidation that eyespots cause could either be an innate response of predators or a response based on learning or experience (Blest 1957; Coppinger 1969; Stevens 2005). If the reason for intimidation is eye mimicry, for it to be a learned response would require that a substantial proportion of the predator population has experienced and survived close encounters with its enemies. Conversely, a learned intimidation response caused by conspicuousness of the eyespot signal would require that the conspicuousness would be associated with an actual cost or threat to the predator for the learning to be meaningful. However, there is no evidence for such a cost or threat. Hence, both these scenarios based on learning seem unlikely to us. Furthermore, the fact that our inexperienced birds were more hesitant to attack the prey that had eyespots than the prey that had no eyespots, although both the prey types were equally novel to the birds, argues for the importance of innate response toward eyespots rather than for avoidance of eyespots due to prey novelty (cf. Coppinger 1969).

Our experiment suggests that 4 large eyespots do not give any additional protection compared with 2 large eyespots. Then why do some butterflies such as the peacock butterfly have 4 eyespots? Our experiment does not provide an answer for this question, but we speculate that the eyespots may also have been favored by selection for some other function in addition to predator intimidation, such as predator deflection or signalling in mate choice (Robertson and Monteiro 2005; Vallin et al. 2011). Hence, it is possible that the "additional" pair of eyespots has been selected for by some other factor than predation. Also, in the peacock butterfly, the eyespots in the forewings and hind wings are roughly the same size, but they differ to some extent in color and spatial arrangement. The central area ("pupil") of the forewing eyespot appears black and red to human viewers, whereas in the hind wing, it is black and blue. Because the number of the naïve flycatchers was limited to ensure a reasonable number of replicates in each treatment group of our experiment, we could not include an additional treatment in which only the forewing eyespots would have been visible. Therefore, although we find it unlikely, we cannot fully exclude the possibility that the differences in the details of appearance between the forewing and the hind wing eyespots may also have had some influence in our results. However, we would expect that the intimidating function of eyespots would have selected for each eyespot to contribute maximally to the scariness of the prey. Hence, considering the conspicuous signal hypothesis, differences in the appearance of eyespots that would reflect differences in the level of intimidation would not be expected. On the other hand, if the impression of 2 different enemies staring at the predator would increase the intimidating effect, eye mimicry could have selected for a difference in appearance between the 2 pairs of eyespots, but our results do not support such benefit from a second pair of eyespots. Future experiments investigating the intimidating effect of forewing eyespots and hind wing eyespots separately and together both in species with 2 different and 2 similar pairs of eyespots could shed more light on the selection pressure that has shaped the appearance of species with 4 eyespots.

In our experiment, half of the birds received the prey with eyespots first and the other half received it after the spotless prey, allowing us to statistically control for any effect of presentation order on the birds responses. Presentation order influenced both prey types such that attack time was shorter when the prey type was presented as second, suggesting some habituation to the experimental situation. However, because our birds were not exposed to eyespots repeatedly, this result does not necessarily mean that the effect of the eyespots would weaken with repeated encounters. We know that both naïve (this study) and experienced birds (Kodandaramaiah et al. 2009) are intimidated by eyespots, and it seems that when eyespots are combined with a display behavior that, for example, *I. io* shows, the intimidating effect does not disappear very quickly as a consequence of repeated encounters (Vallin et al. 2007; but see Blest 1957). Nevertheless, questions related to the permanence of the intimidating effect have so far not received very much attention. A more systematic study of how particular visual features of eyespots and display behaviors of butterflies influence predators' habituation to eyespots and if, for example, predators' encounters with their predators might strengthen the intimidation would provide interesting directions for future studies.

To summaries, our experiment confirms that large eyespots of butterflies deter passerine birds. However, our result that 4 eyespots are not more deterring than 2 eyespots contradicts the hypothesis that conspicuousness of the eyespots could alone explain the intimidating effect. It suggests that eye mimicry or some other reason explains the intimidating effect of eyespots on the wings of *Inachis io*. It also challenges the validity of the conspicuous signal hypothesis as a general explanation for the intimidation caused by eyespots.

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REFERENCES

Blest AD. 1957. The function of eyespot patterns in Lepidoptera. Behaviour. 11:209–256.

Coppinger RP. 1969. The effect of experience and novelty on avian feeding behavior with reference to the evolution of warning coloration in butterflies part I: reactions of wild-caught adult blue jays to novel insects. Behaviour. 35:45–60.

Cott HB. 1940. Adaptive coloration in animals. London: Methuen & Co.

Dimitrova M, Stobbe N, Schaefer HM, Merilaita S. 2009. Concealed by conspicuousness: distractive prey markings and backgrounds. Proc R Soc B Biol Sci. 276:1905–1910.

Janzen DH, Hallwachs W, Burns JM. 2010. A tropical horde of counterfeit predator eyes. Proc Natl Acad Sci U S A. 107:11659–11665.

Kodandaramaiah U, Vallin A, Wiklund C. 2009. Fixed eyespot display in a butterfly thwarts attacking birds. Anim Behav. 77:1415–1419.

Lundberg A, Alatalo R. 1992. The pied flycatcher. London: Poyser.

Lyytinen A, Brakefield PM, Lindström L, Mappes J. 2004. Does predation maintain eyespot plasticity in Bicyclus anynana? Proc R Soc Lond B Biol Sci. 271:279–283.

Morton Jones F. 1932. Insect coloration and the relative acceptability of insects to birds. Trans Roy Ent Soc. 80:345–371.

Morton Jones F. 1934. Further experiments on coloration and the relative acceptability of insects to birds. Trans Roy Ent Soc. 82: 443–453.

Poulton EB. 1890. The colours of animals. London: Paul, Trench, Trübner & Co.

Robertson KA, Monteiro A. 2005. Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. Proc R Soc B Biol Sci. 272:1541–1546.

Ruuskanen S, Laaksonen T. 2010. Yolk hormones have sex-specific long-term effects on behavior in the pied flycatcher (*Ficedula hypoleuca*). Horm Behav. 57:119–127.

Ruxton GD, Sherratt TM, Speed MP. 2004. Avoiding attack: the evolutionary of crypsis, warning signals and mimicry. Oxford: Oxford University Press.

Siitari H, Honkavaara J, Huhta E, Viitala J. 2002. Ultraviolet reflection and female mate choice in the pied flycatcher, Ficedula hypoleuca. Anim Behav. 63:97–102.

Sirkiä PM, Laaksonen T. 2009. Distinguishing between male and territory quality: females choose multiple traits in the pied flycatcher. Anim Behav. 78:1051–1060.

Stevens M. 2005. The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. Biol Rev. 80:573–588.

Stevens M, Hardman CJ, Stubbins CL. 2008. Conspicuousness, not eye mimicry, makes "eyespots" effective antipredator signals. Behav Ecol. 19:525–531.

Stevens M, Hopkins E, Hinde W, Adcock A, Connelly Y, Troscianko T, Cuthill IC. 2007. Field experiments on the effectiveness of "eyespots" as predator deterrents. Anim Behav. 74:1215–1227.

Stevens M, Merilaita S. 2009. Animal camouflage: current issues and new perspectives. Philos Trans R Soc B Biol Sci. 364:423–427.

Vallin A, Dimitrova M, Kodandaramaiah U, Merilaita S. 2011. Deflective effect and the effect of prey detectability on anti-predator function of eyespots. Behav Ecol Sociobiol. 65:1629–1636.

Vallin A, Jakobsson S, Lind J, Wiklund C. 2005. Prey survival by predator intimidation: an experimental study of peacock butterfly defence against blue tits. Proc R Soc Lond B Biol Sci. 272:1203–1207.

Vallin A, Jakobsson S, Lind J, Wiklund C. 2006. Crypsis versus intimidation—anti-predation defence in three closely related butterflies. Behav Ecol Sociobiol. 59:455–459.

Vallin A, Jakobsson S, Wiklund C. 2007. An eye for an eye"—on the generality of the intimidating quality of eyespots in a butterfly and a hawkmoth. Behav Ecol Sociobiol. 61:1419–1424.