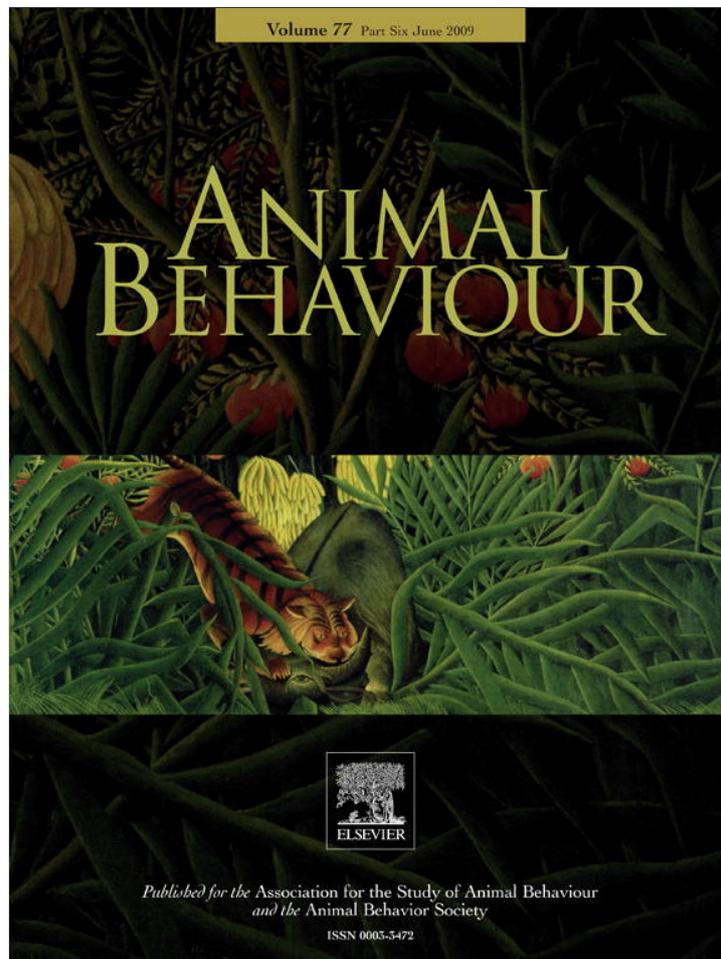


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## Fixed eyespot display in a butterfly thwarts attacking birds

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Eyespots have evolved in many lepidopteran insects, which suggests their adaptive value. One of their hypothesized functions is that predators are intimidated by prey with large and conspicuous eyespots and hence refrain from attacking them. Recent experiments have shown that a combination of eyespots and intimidating behaviour can increase survival. We tested whether the mere presence of conspicuous eyespots can thwart attacking birds, that is, when the eyespots are displayed constantly, without any intimidating behaviour. We used prey that consisted of wings of the peacock pansy butterfly, *Junonia almana*, glued onto a piece of cardboard so as to resemble a butterfly with its wings open. A mealworm was placed between the wings in place of the body. Great tits, *Parus major*, were used as the predator in the study and were offered a choice between two model prey, one with intact eyespots and one without. Prey with eyespots were attacked significantly fewer times than those without. The time between the first and second attack was longer when the prey without eyespots was attacked first. These results support the hypothesis that naturally occurring butterfly eyespots can increase survival even when they are constantly displayed and motionless.

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Eyespots are circular patterns found on the wings of many lepidopteran insects. Seemingly simple decorative features, they come in a diverse array of colours, shapes and sizes, and occur in varying numbers and positions on the wing (Nijhout 1991). They have appeared in multiple lineages of the Lepidoptera during the course of evolution (e.g. superfamilies Bombycoidea, Drepanoidea, Geometroidea, Papilionoidea; Monteiro et al. 2006). Several studies have sought to understand the genetics and developmental underpinnings controlling their formation and we now have a good understanding of these aspects (e.g. Nijhout 1991; Beldade & Brakefield 2002; French & Brakefield 2004; Monteiro 2008). In contrast, far fewer studies have attempted to get a handle on their functional significance. Recent studies (Breuker & Brakefield 2002; Lyytinen et al. 2003, 2004; Robertson & Monteiro 2005; Vallin et al. 2005, 2007; Vliieger & Brakefield 2007; Stevens et al. 2007, 2008a) have provided some insights into their functional value. In this study we tested one hypothesis of their functional value as anti-predator devices.

For the current study we defined eyespots by the presence of concentric rings of contrasting colour surrounding a central pupil. The number of contrasting rings and their colours vary widely (Nijhout 1991). Given the diversity in structure and composition of

eyespots it is unlikely that eyespot evolution in the Lepidoptera has been mediated by a single evolutionary force. However, there is preliminary evidence indicating that some of the developmental pathways are shared between different types of eyespots across the Lepidoptera (Monteiro et al. 2006). Stevens (2005) and Ruxton et al. (2004) reviewed existing hypotheses about the adaptive value of different kinds of eyespots. Smaller serially arranged eyespots on the postdiscal region of the wing are thought to deflect attacks towards the wing margin and hence protect the body at the expense of a torn wing (Blest 1957; Wourms & Wasserman 1985; Cordero 2001). Individually occurring, large and conspicuous eyespots are thought to increase survival probability by being intimidating to predators either by their semblance to vertebrate eyes (the 'eye mimicry' hypothesis) or merely by being highly conspicuous (the 'conspicuous signal' hypothesis; Stevens et al. 2007, 2008a).

The peacock butterfly, *Inachis io*, is one species that has such intimidating eyespots. These butterflies hibernate as adults during winter (Wiklund et al. 2008), and when attacked during this period, they flick their wings open and close, sometimes repeatedly, thereby exposing their eyespots suddenly. This display is also accompanied by a hissing noise (Blest 1957). Vallin et al. (2005, 2007) showed that the eyespots on *I. io* increased the probability of survival from predation by blue tits, *Cyanistes caeruleus*, and great tits, *Parus major*. Vallin et al. (2007) also showed that eyespots on the hindwing of the eyed-hawkmoth, *Smerinthus ocellatus*, had a similar effect. As in the case of *I. io*, the hawkmoth keeps its eyespots hidden and, when approached by a predator, exposes

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them abruptly by protracting its forewings. The two studies demonstrate the adaptive value of the combined effect of conspicuous eyespots and the element of surprise in the sudden revealing of hidden eyespots. However, there are several moths and butterflies that do not have any wing-flicking behaviour, but still possess eyespots. This prompted us to hypothesize that such eyespots are effective despite the absence of a sudden appearance, that is, they are intimidating to predators even when they are continually visible. Although this hypothesis has been tested with artificial prey having simple geometric patterns (e.g. Forsman & Merilaita 1999; Forsman & Herrström 2004; Stevens et al. 2007, 2008a), the effect of naturally occurring eyespots has, to our knowledge, not been tested thus far. We addressed this question using the peacock pansy butterfly, *Junonia almana*, which has large and conspicuous eyespots on its upper wing surfaces (Fig. 1). We staged experiments in which we offered wild-caught great tits a choice between dead *J. almana* butterflies with either intact or covered eyespots.

## METHODS

The experiment was carried out between October and December 2007 at the Tovetorp Zoological Research Station



**Figure 1.** Eyespots on the dorsal surface of the fore- and hindwings of *J. almana*.

(affiliated to the Department of Zoology, Stockholm University) located about 100 km southeast of Stockholm, Sweden.

## The Study Organisms

*Junonia almana* is distributed in the tropical parts of Asia, having a pronounced seasonal polyphenism. The dry season form has cryptic undersides with reduced eyespots whereas the wet season form has prominent eyespots. Seasonal dimorphism is absent on the upper side; both forms have one large and two small eyespots on each forewing, and one large and one small one on each hindwing (Fig. 1). The ground coloration on the dorsal surface of the butterfly is tawny and its general appearance has been compared to an owl (Haribal 1992).

Pupae were ordered from Tropical Entomological House, Penang, Malaysia and allowed to eclose under laboratory conditions in Stockholm. All eclosed butterflies were of the wet season morph. They were pinch-killed when ready to fly and preserved for the trials.

The great tit was used as the predator in the experiment because it has been used previously in similar experiments (Vallin et al. 2007) and has been observed to prey on butterflies in the wild (personal observation). It is a widely distributed species ranging from Northern Africa across Europe and Asia to Indonesia (Birdlife International 2004), and is sympatric with *J. almana* across the latter's range. Birds were caught with a mist net at the research station with a permit from the Swedish Bird Ringing Center. They were released in individual cages (80 × 60 cm and 40 cm high) which were housed in a room where the light regime was maintained at 7 h daylength to correspond approximately to the natural light conditions in Central Sweden during October–December. They were fed sunflower seeds, suet, water and mealworms. Housing conditions and general treatment of the birds during the experiment were scrutinized and approved by the regional ethical committee, Linköping. The birds were acclimatized to captive conditions for 2–5 days before being used in trials. Trials were begun 1 h after sunrise and were stopped at least 1 h before sunset. Birds were ringed and released immediately after the trials. No bird was kept in captivity for more than 6 days. All birds kept their condition and were healthy upon release.

*Junonia almana* feeds on members of Acanthaceae, Amaranthaceae, Fabaceae, Gesneriaceae, Melastomataceae, Onagraceae, Scrophulariaceae, Plantaginaceae and Verbenaceae (Igarashi & Fukada 1997; Vane-Wright & de Jong 2003), which are not known to be toxic. A few butterflies were saved for pilot studies to test whether great tits found them edible; they were consumed without signs of discomfort during these trials.

## The Experimental Set-up

Trials were conducted in an experimental chamber (2.3 × 2.4 m and 1.9 m high) constructed out of sound-proof material to minimize the effect of extraneous noise. High-frequency natural spectrum fluorescent tubes furnished the light for the trials and one-way windows on two of the walls allowed observers outside the room to monitor the proceedings. A log of a willow tree, *Salix caprea*, was laid down in the centre of the chamber and a perching place placed at its foot. The perching place consisted of a central vertical wooden pole, 1.8 m high, with smaller horizontal perches of 10 cm affixed to it at intervals of 10 cm, and was intended as a resting place for the bird during the trials. A petri dish with a white background was glued to the log close to the perching place, 120 cm from the prey. A fresh mealworm was placed in it before each trial so that the bird formed an association between the log and food. The contrasting background rendered the mealworm

readily discernible to a bird sitting on the perch. A bowl of water was always available to the bird during the trial, placed next to its perching place.

Wings of the dead butterflies were carefully separated from the body and glued to a small rectangular piece of cardboard measuring ca. 5 cm × 12 cm. They were positioned to resemble a butterfly resting with its wings wide open and the dorsal surface visible, albeit without the body. On half of the butterflies, the eyespots were painted over with a nontoxic water-soluble colour matching the ground colour of the specimen (henceforth eyespot-less prey). On the rest, regions on the wing other than the eyespots were painted over (henceforth eyespotted prey; Fig. 2). All model prey were allowed to dry for a few hours before trials to eliminate any potential odours.

Immediately prior to a trial, two equal-sized mealworms were decapitated and placed between the wings of two model prey (one eyespotted and one eyespot-less) where the body of a butterfly is normally present. The model prey pair were chosen such that the wing spans matched each other closely. The two model prey were placed on the log close to the edge away from the perching place and both were equidistant from the perching place. The prey pair was changed after trials such that the relative position of eyespotted and eyespot-less prey (left and right from the perspective of the bird's perch) alternated with each successful trial. A simple contraption made of two nails and rubber bands allowed us to affix the model prey at the same positions on the log for each of the trials (Fig. 2). The distance between the two prey was thus maintained at approximately 15 cm. An area of about 60 cm<sup>2</sup> surrounding the model prey was captured on a digital video camera to record the turn of events as the bird approached the model prey.

The trials began when a bird was released into the chamber through a hatch. The bird's movements were monitored throughout the trial through the one-way windows. A peck on one of the decapitated mealworms was scored as an attack. The bird was then allowed an additional period of 5 min to attack the second prey. The trial was terminated either after these 5 min or immediately after the second prey was attacked, in which case the time was noted. Trials were discontinued after 1 h if the bird did not attack either of the prey. Each bird was used only once.

#### Statistical Analyses

All analyses were performed in R 2.2.1 (R Development Core Team 2008). Exact binomial tests were performed to test whether the number of first attacks on the eyespotted prey was significantly different from that on the eyespot-less prey. Because data were heavily skewed, the Mann-Whitney *U* test was used to test

whether the average time taken to attack the second prey depended on which of the prey was attacked first. In trials where the first prey was attacked but not consumed completely, the remaining prey was not attacked, presumably because the bird lost interest in the mealworms. We did not include these trials when we tested whether the time taken to attack the second prey depended on what was attacked first. Values reported are mean ± SE.

#### RESULTS

A total of 37 trials were conducted. In a typical trial, the bird flew around the chamber before resting on one of the perches. It gradually calmed down on the perch and noticed the mealworm in the petri dish. Almost invariably after noticing it, the bird flew down to the mealworm and consumed it. Following this, the bird continued searching for more food, hopping along the log towards the prey. Once in the vicinity of the prey, the bird chose to peck on one of the mealworms and consumed it. Subsequently, the bird either consumed the remaining mealworm or left the scene.

The birds attacked at least one of the mealworms in 35 of the 37 trials, and consumed it in 32 instances. The eyespot-less prey was attacked first in 25 trials, in contrast to the eyespotted prey which was attacked first in 10 trials (binomial test:  $N = 35$ ,  $P = 0.017$ ).

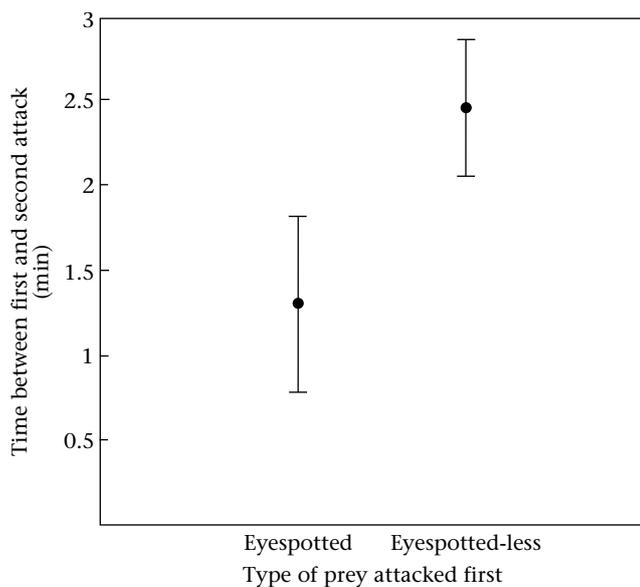
Among the 32 trials in which the first prey was consumed, the second prey was not attacked in eight instances. Seven of these instances were when the eyespotless-prey was attacked first. Since birds were only allowed 5 min after the first attack, we assigned a duration of 5 min for these eight trials. Birds that first attacked an eyespotted prey attacked the second prey after a significantly shorter period of time ( $1.3 \pm 0.52$  min) than birds that had the reverse attack order ( $2.45 \pm 0.40$  min), that is first eyespot-less then eyespotted (Mann-Whitney *U* test:  $U = 53$ ,  $N_1 = 10$ ,  $N_2 = 22$ ,  $P = 0.02$ ; Fig. 3).

#### DISCUSSION

The eyespotted prey were attacked fewer times than the eyespot-less prey. Birds that first attacked the eyespot-less prey took a longer time to attack the remaining prey than in the reverse order, that is, when the eyespotted prey was attacked first. These results support the hypothesis that eyespots such as those found on *J. almana* can intimidate predators and hence thwart attacks from them despite the absence of any wing-flicking behaviour. Although the experiments were carried out using dead prey, the general appearance of many prey in their natural conditions, for example when basking, is similar to the model prey used in the study and hence the results are relevant in nature.



Figure 2. One of the model prey pair used in the experiment, with the eyespotted prey to the left and the eyespot-less prey to the right.



**Figure 3.** Mean  $\pm$  SE times taken between the first and second attack when great tits were given two-choice tests involving prey with and without eyespots.

Stevens et al. (2007, 2008a, b) performed a set of experiments using a set-up where dead mealworms were attached to uniform grey triangular pieces of card printed with specific patterns. Among other things, they showed that highly contrasting circular patterns of white and black were effective in decreasing predation from presumed avian predators on the model prey compared to uniform grey. Their model prey consisted of elementary patterns in the grey scale and the shape of the card was not intended to mimic any real species. Hence their model prey was unlike any real lepidopteran insect in many ways. Moreover, the nature of their experimental protocol precluded observations on the identity of predators and their reaction when they confronted the 'eyespots'. Together with the results of Stevens et al. (2007, 2008a, b), our study confirms that eyespots found in lepidopteran insects can intimidate predators without any intimidating behaviour.

Although difficult to quantify objectively, the trepidation in the bird when approaching the eyespotted prey for the first time was clearly visible to the observers. Video recordings also showed a few unambiguous instances where the bird jumped back or flew away immediately after noticing the eyespotted prey. In contrast, when the eyespot-less prey was approached there was little hesitation before the mealworm was consumed. This is reflected in the results where the average time taken to attack the second prey was longer when it had its eyespots intact. The above result could be influenced by the duration of 5 min assigned to the eight trials where the second prey was not attacked, because the eyespot-less prey was first in seven of these trials. However, we believe that the inclusion of these trials in the analysis is justified because it was obvious from the video recordings that the bird was frightened after looking at the eyespots in several trials (see [Supplementary material](#) for a video clip of one of the recordings). Moreover, the eyespotted prey was inspected before being rejected in all seven trials. In nature, a butterfly or moth can capitalize on the extra time to escape, potentially making the presence of eyespots the difference between life and death.

Blest (1957) argued that the most effective type of model for an intimidating eyespot is one that has a three-dimensional appearance, is circular and composed of concentric elements. He interpreted these as characteristics of eyes and hence argued in favour of the 'eye mimicry' hypothesis. Stevens et al. (2007, 2008a) showed that noncircular patterns with similar composition of black and

white were also effective. They argued in favour of the 'conspicuous signal' hypothesis. In this study we considered the combined effect of all eyespots in *J. almana*. The large eyespot on the forewing is a typical eyespot consisting of contrasting circular rings around a white pupil, whereas the large eyespot on the hindwing is composed of quasicircular rings (Fig. 1). To the human observer, the latter appears three-dimensional owing to displacement of pattern elements to the side and to 'highlights' (the central white pupils) which give the appearance of light being reflected from a spherical surface (Blest 1957). The advantage of such a pattern over a simple pattern of concentric circles, for example found in the forewing eyespots of *J. almana*, needs to be investigated in more detail. Blest's results suggest that the former may be better. Stevens et al. (2008b) could not find an increase in survival of patterns where the central black was displaced inwards. However, they themselves noted that the lack of a significant effect could have been because the differences were subtle. In the eyespot on the hindwing of *J. almana*, the three-dimensional appearance is much more striking and so is the difference compared to the eyespots on the forewing. We are unable to shed light on this issue in the current study because we did not present butterfly wings with either the 'two-dimensional' or alternatively the 'three-dimensional' eyespots covered. We believe further evidence is needed before concluding whether such three-dimensional eyespots are more effective or not.

Studies on another butterfly, the squinting bushbrown, *Bicyclus anynana* (Breuker & Brakefield 2002; Robertson & Monteiro 2005; Costanzo & Monteiro 2007), have shown that eyespots play a role in sexual selection. This implies that eyespots in the Lepidoptera have not evolved solely for protection against predators. Within the genus *Junonia*, large and conspicuous eyespots are found in a number of species (e.g. *coenia*, *everete*, *genoveva*, *lemonias*). The colour composition and size vary substantially across species. This suggests that these eyespots may also have some other function such as in sexual selection or species recognition.

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#### Supplementary Material

Supplementary Material associated with this article can be found, in the online version, at [doi:10.1016/j.anbehav.2009.02.018](https://doi.org/10.1016/j.anbehav.2009.02.018)

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