Eyespots divert attacks by fish

Karin Kjernsmo and Sami Merilaita

Behavioural and Evolutionary Ecology Group, Environmental Biology, Department of Biosciences, Åbo Akademi University, Turku, Finland

Eyespots (colour patterns consisting of concentric rings) are found in a wide range of animal taxa and are often assumed to have an anti-predator function. Previous experiments have found strong evidence for an intimidating effect of eyespots against passerine birds. Some eyespots have been suggested to increase prey survival by diverting attacks towards less vital body parts or a direction that would facilitate escape. While eyespots in aquatic environments are widespread, their function is extremely understudied. Therefore, we investigated the protective function of eyespots against attacking fish. We used artificial prey and predator-naive three-spined sticklebacks (Gasterosteus aculeatus) as predators to test both the diversion (deflection) and the intimidating hypothesis. Interestingly, our results showed that eyespots smaller than the fish’s own eye very effectively draw the attacks of the fish towards them. Furthermore, our experiment also showed that this was not due to the conspicuousness of the eyespot, because attack latency did not differ between prey items with and without eyespots. We found little support for an intimidating effect by larger eyespots. Even though also other markings might misdirect attacks, we can conclude that the misdirecting function may have played an important role in the evolution of eyespots in aquatic environments.

1. Introduction

There are several ways an animal may use its body coloration to avoid predation [1]. One such anti-predator adaptation is eyespots [2,3]. Eyespots (previously also called ocelli) are colour patterns which consist of roughly concentric rings of contrasting colours and have received their name because they, at least to humans, more or less resemble the vertebrate eye. Eyespots are found in many terrestrial animals such as insects (particularly in the order Lepidoptera), birds and reptiles, but they are also common in aquatic taxa such as molluscs, flatworms and fishes [2–7]. Because these markings occur in such a wide range of animal taxa, they have been intriguing biologists for a long time; indeed Poulton [2] was among the first to suggest that eyespots are likely to have an anti-predator function.

Poulton [2], who had introduced a small heath butterfly (Coenonympha pamphilus) into a lizard’s cage, wrote that ‘It was at once obvious that the lizard was greatly interested in the large eye-like mark on the under side of the fore wing: it examined this mark intently, and several times attempted to seize the butterfly at this spot’ [2, p. 207]. This suggested anti-predator utility of eyespots described by Poulton later became known as the ‘deflection hypothesis’ [3,8,9]. The main idea of this hypothesis is that marginal eyespots draw predator attacks towards the less vital parts of the prey’s body or towards the opposite direction of the escape trajectory of the prey, thus increasing the chance of prey survival. The term deflection can, however, be misleading, because it implies that the attack is thwarted or redirected by the physical action of the prey rather than by the predator’s decision about attack direction. Therefore, we prefer to use the word ‘diversion’ instead of deflection.

Finding experimental support for such a divertive effect has, however, turned out to be quite a challenge. There are several studies that have not found any clear support for a diversion hypothesis (e.g. against birds [10,11], lizards [12] and against fish predators [13]). Blest [3] argued that when painted onto mealworms, spots directed the attacks of avian predators towards them.
but his study suffered heavily from pseudo-replication and thus the results were not statistically reliable. However, some empirical evidence for the divertive effect of eyespots has been revealed only recently. Olofsson et al. [14] showed that marginal eyespots of the woodland brown butterfly (Lopinga achine) drew the attacks of blue tits (Cyanistes caeruleus) towards the eyespots, but only under specific light conditions where the UV-intensity of the light was high, and the intensity of longer light wavelengths were low. Vallin et al. [15] found that eyespots on artificial prey items diverted the attacks of blue tits towards these markings. Thus, even though this hypothesis was proposed over 100 years ago, empirical support for the divertive effect is still scarce, and so far no direct, unambiguous behavioural tests have been conducted with aquatic taxa.

A further hypothesis which has been invoked to explain the anti-predator function of eyespots is the predator intimidation hypothesis [2–4,16,17]. According to this hypothesis, some eyespots, for example some relatively large eyespots in Lepidoptera, would intimidate potential predators by resembling the eyes of the predator’s own enemy [2,18]. It has also been suggested that the conspicuousness of eyespots could cause predators to hesitate from attacking the prey [17, but see [19]]. Unlike the diversion hypothesis, there are several studies that have presented convincing support for the intimidating effect of eyespots in terrestrial environments (typically for passerine birds preying on butterflies [17,19–22]). However, the intimidating function of eyespots has also received surprisingly little attention in aquatic systems, although fishes, for example, commonly encounter the eyes of their enemies or eyespots sported by fishes or other taxa.

In order to investigate the anti-predator function of eyespots in aquatic environments and to assess the importance of the diversion hypothesis and the intimidation hypothesis, we tested the behavioural response of fish towards eyespots. We did this by presenting artificial prey items with or without eyespots to laboratory-reared, predator-naive three-spined sticklebacks (Gasterosteus aculeatus) in a series of experiments. In our first set of experiments, we tested whether eyespots smaller than the attacking fish’s own eye could have a diverting effect on attacking fish by observing where the fish directed their attacks. In the second part of our study, we tested if eyespots larger than the fish’s own eye would intimidate attacking fish by comparing attack latency between spotted and spotless prey.

2. Material and methods

(a) Experimental fish and holding conditions

This study was conducted between January and April over 3 consecutive years 2010, 2011 and 2012 in the aquatic laboratory at the Department of Biosciences, Åbo Akademi University in Turku, Finland. To study attack behaviour in fish, we used the three-spined stickleback as a predator in this study. We chose the three-spined stickleback because it is considered to be a primarily visual predator [23–27], it is widely occurring and can be easily maintained in aquaria. In nature, three-spined sticklebacks are often parasitized, and for example, the tapeworm Schistocephalus solidus is known to dramatically affect their behaviour [28–30]. To avoid such confounding effects of parasitism on behavioural responses, all of our fish were bred through artificial fertilization and reared in the laboratory, resulting in a tapeworm-free population. Parental fish were caught at two different occasions, in June 2009 and May 2011 outside Tvärminne Zoological field station (59°50’ N, 23°12’ E). In their early life stages, our F1 laboratory fish were fed three times daily with live brine shrimp, and when they had reached a body size of approximately 2 cm, they were fed one to three times daily with frozen red mosquito larvae (Chironomidae sp.). All test individuals were between 6 and 18 months old during these experiments, and different fishes were used in all three experiments. These experiments were performed with permission from the State Provincial Office of Southern Finland (decision number STH472A).

(b) Experimental set-up

Before the actual experiments, we trained the laboratory-reared three-spined sticklebacks to search for artificial prey items on artificial backgrounds (see below). Each prey item consisted of two components: (i) a rectangular piece of printed paper that covered (ii) a frozen red mosquito larva (Chironomidae sp.) which served as a reward and ensured that the fish would be motivated to attack the artificial prey items. We created the patterning of our artificial prey items and backgrounds with a purpose-written programs using the software MATLAB R2008b (The MathWorks, Inc., Natick, MA, USA) and then printed them with a laser printer (HP LaserJet P4015x with 1200 dpi resolution) on water resistant paper (‘Rite in the rain’, J. L. Darling Corporation, Tacoma, WA, USA). To ensure that the artificial prey items would remain still, we attached a thread of non-corrosive metal as a weight to the underside of each prey item. This was achieved by attaching the metal thread with double-sided tape to the prey item and then covering the underside with self-adhesive tape.

We used eight identical 30-l (I x w x h: 50 × 20 × 30 cm) glass aquaria for these experiments. The experimental aquaria were lit from above by four (one per two aquaria) natural light simulating fluorescent lamps (36 W, BIOvital, NARVA). Each aquarium was divided into three main sections: the start zone (SZ), mid zone (MZ) and the foraging zone (FZ). The SZ was divided from the MZ by an opaque divider. Each aquarium had a prey search area (9.5 × 7 cm in size) which was located in the FZ (figure 1), and the side in which the FZ and the SZ were located was altered between aquaria. We made the bottoms of the experimental aquaria dark by covering them with dark brown self-adhesive foil to create a less stressful environment for the fish, but also to make the prey search area stand out from the rest of the zone. To prevent any disturbance from outside, the area in front of and between the aquaria was covered with black plastic that had small viewing holes (one for each aquarium, 10 × 5 cm in size) to allow observations.

(c) Training of the fish

In order to motivate the fish to attack the artificial prey items, they first received four training sessions to facilitate the association between the artificial prey items and the food reward.
The fish were first given a white, non-patterned artificial prey item with one red mosquito larva placed on top for fishes entering the diversion experiment, and two for fish entering the intimidation experiment (see ‘diversion experiment’ for explanation). The reward was gradually hidden over the four training sessions, until it was completely covered by the artificial prey item in session four. At the beginning of a training session, a randomly chosen fish was placed in the SZ of an experimental aquarium (figure 1). A white prey item was then placed at the centre of the prey search area (on top of respective experimental background, see figure 1), and the food reward was added. Shortly thereafter, the fish was released from the start compartment and was allowed to feed on the reward and acclimate to all the zones of the experimental aquarium overnight. The following morning, the fish was gently moved back to the start compartment (if it was not already positioned there) and allowed to recuperate for a minimum of 5 min before the first actual training trial started. Fish that did not eat the prey item during acclimation were immediately removed from the experiment and replaced with a new individual; however, this happened extremely rarely (only three out of 100 individuals). Each training and experimental session lasted until the fish had found the prey or 15 min had passed. The fish was then gently moved back to the SZ, until the next trial. Each fish received a total of four training trials before an experiment started. Training and experimental trials were distributed two per day, one in the morning and one in the afternoon, allowing the fish 5 h rest between the trials to ensure regained hunger level. If a fish did not attack the prey item by the third training session, it was removed from the experiment. When a fish had completed all four training sessions, it entered one of the experiments described below.

**Figure 2.** The prey items and backgrounds used in the diversion and detectability experiment (upper row), and intimidation experiment (lower row). (a) Prey type, (b) shape and (c) samples of the backgrounds are shown. Placement of the eyespot in the intimidation experiment is indicated by a circle on the folded white prey.

In the first experiment, we wanted to investigate whether eyespots on prey could be used to manipulate where the fish direct their attacks. We did this using black and white speckled prey items that had one eyespot (size: 2.5 mm in diameter of which the central spot was 1 mm in diameter, see ‘intimidation experiment’ for information about the fish’s own eye size), located near one end of the prey (figure 2). Because we wanted to test the effect of the eyespot pattern, we needed to control for possible biases owing to differences in black-to-white ratio. Therefore, we set the density of black in the speckled pattern equal to that in the eyespot, namely 23%. To create a prey item that was not exceptionally conspicuous, or too difficult to detect, we used the same speckled pattern in the background plate but with twice as much (46%) black (figure 2).

Out of the 40 fish that entered training sessions for this experiment, 22 individuals passed the training and entered the experiment. Each individual was used in four identical trials, two per day, with the first starting on the following morning after their last training session. Each fish was tested four times in order to investigate whether repeated encounters influence the response towards the eyespot. Before the start of each trial, we placed two pieces of red mosquito larvae under an experimental prey item, close to each end of the prey, and waited 2 min before starting the trial. These fish had received only one mosquito larva (placed under the centre) of each prey item during the training, because we did not want them to learn that the experimental prey item was covering more than one food reward, as it might have affected the choice of which side of the prey to attack. Also, we wanted to ensure that any olfactory cues were evenly distributed between the two sides of the prey. Each trial started when the opaque divider was lifted, allowing the fish to swim out from the SZ, and search for the prey item located in the FZ (figure 1). The side the eyespot was on during the first experimental trial was randomized for each fish and altered between each trial. For each individual and trial, we recorded two variables with the event-recording software J-WATCHER (v. 1.0, available at; http://www.jwatcher.ucla.edu): (i) attack latency (defined as the time the fish took the fish to attack the prey item from its first entry to the FZ, that is, when the fish’s mouth made contact with the rectangular piece of paper, see the electronic supplementary material, video clip), and (ii) side of the prey attacked (i.e. on the eyespot side or on the spotless side of the midline, figure 2).

**Experiment 1b: control for potential influence of detectability**

Because the eyespot part of the prey in the diversion experiment might have been more conspicuous than the non-eyespot side of the prey, we wanted to establish whether any possible attacks towards the eyespot side could be due to a divertive effect (i.e. the eyespot drawing attacks towards it rather than simply being easier to detect than the rest of the prey). Therefore, to be able to test the effect of the eyespot on the detectability of the prey, we made a spotless prey with an identical size and black-to-white ratio as the spotted prey (figure 2). The experimental procedure was the same as in the diversion experiment, and we recorded the attack latency for each trial. In total, 33 fish entered training for this experiment, and 17 individuals completed the experiment.

**Experiment 2: intimidation experiment**

In the final experiment, we investigated whether eyespots larger than the eye of the attacking fish could have an intimidating effect on the attacker. The training and experimental protocol were similar as in the two previous experiments with the exception that size of the prey (2.1 × 0.7 cm) was different and it was folded in a 90° angle along its midline. This made the eyespot part of the prey always face toward the fish in the SZ, whereas the other half laid flat against the substrate (figure 2). The food reward (mosquito larva) was placed under the horizontal half of the prey item. We folded the prey because we wanted the experimental prey to display its eyespot towards the fish to enhance any possible intimidating effect. This was because it has been suggested that some fish (butterflyfishes, Chaetodon spp.) displaying potentially intimidating eyespots pause and present their spots before they try to flee [4], thus displaying them in an upright position.

The experimental prey was mottled and had an eyespot larger than the sticklebacks’ own eye, the average diameter...
significant after this correction. All analyses were conducted instead of 0.05. However, all our significant results remained significant when corrected for multiple testing by using an adjusted \( p \)-value of 0.0125 instead of 0.05 (31). As these data did not meet the assumptions for parametric tests, we used non-parametric analyses. To analyse treatment effects on the dependent variable attack latency, we conducted survival analysis (Cox regression) separately for each of the four trials in experiments 1b and 2. The Cox regression analysis method allowed us to include cases where fish did not attack the prey within the observation time by simply defining a prey attack as censored. As each fish received four prey presentations and hence the trials were not statistically independent, we corrected for multiple testing by using an adjusted \( \alpha \)-value of 0.0125 instead of 0.05 (31). However, all our significant results remained significant after this correction. All analyses were conducted using the statistical software R for Windows (v. 2.9.2, (32)).

3. Results

(a) Experiment 1a: diversion experiment

The eyespot caused a strong behavioural response in the fish. Significantly more of the sticklebacks directed their attacks towards the eyespot side compared with the spotless side of the prey \( (\chi^2 = 11.64, \text{d.f.} = 1, p < 0.001); \) figure 3. This effect remained significant in trial 2 \( (\chi^2 = 6.35, \text{d.f.} = 1, p = 0.01) \) and in trial 3 \( (\chi^2 = 8.05, \text{d.f.} = 1, p = 0.004). \) In the fourth trial, however, the diverting effect was no longer significant \( (\chi^2 = 2.58, \text{d.f.} = 1, p\text{-value} = 0.11). \)

(b) Experiment 1b: control for potential influence of detectability

There was no difference in attack latency between the fish that searched for the spotted prey and the fish that searched for the spotless prey in any of the four trials (trial 1: Cox regression, Wald = 0.71, \( n = 39, p = 0.40); \) trial 2: Wald = 0.05, \( n = 38, p = 0.82); \) trial 3: Wald = 0.20, \( n = 38, p = 0.66); \) trial 4: Wald = 0, \( n = 36, p = 0.99). \) Attack latency decreased successively in the spotless group (Friedman test, \( \chi^2 = 13.5, n = 16, p = 0.004; \) figure 4) but not in the spotted group \( (\chi^2 = 3.19, n = 19, p = 0.36). \)

(c) Experiment 2: intimidation experiment

There was no significant difference in attack latency between the spotless prey and the prey that had the large eyespot in any of the four trials (trial 1: Cox regression, Wald = 0, \( n = 41, p = 0.98); \) trial 2: Wald = 2.91, \( n = 41, p = 0.09); \) trial 3: Wald = 1.98, \( n = 41, p = 0.16); \) trial 4: Wald = 1.03, \( n = 41, p = 0.31). \) The attack latency decreased as the fish gained more learning experience in the spotless group (Friedman test, \( \chi^2 = 9.29, n = 21, p = 0.003) \) but not in the eyespot group \( (\chi^2 = 4.48, n = 20, p = 0.21); \) figure 5. The latter result...
was probably caused by the high variation in the eyespot group (figure 5).

4. Discussion

With these experiments, we have demonstrated a possible anti-predator function of eyespots in aquatic prey against predation by fish. As all sticklebacks used in these experiments were naive (i.e. had no previous experience of predators, or prey with eyespots), any behavioural responses they showed at least during their first encounter with a spotted prey can be considered innate. The strong response in the first experiment that tested for the diverting effect of eyespots indicates that eyespots very effectively manipulate the behaviour of three-spined sticklebacks and direct their attacks towards them. This is an important finding, because it indicates that eyespots can be used to manipulate the behaviour of aquatic predators and direct their attacks towards the eyespots for protective purposes.

Importantly, the results from experiment 1b revealed that there was no difference in attack latency between the spotted and the spotless prey, indicating that the spotless area of the prey was not markedly more difficult to detect than the eyespot. Consequently, the diverting effect was not simply a result of the spotless part of the prey being more concealed than the eyespot. This may not be that surprising, considering that although both the prey and the background were speckled, the density of the dots in the background was twice as high as on the prey. Importantly, these two experiments demonstrated that the fish were attracted to attack towards the eyespot rather than being directed away from the spotless half of the prey. Our results are also in accordance with a recent study by Vallin et al. [15], who presented artificial prey items to passerine birds. Vallin et al. [15] used prey items that had a small marginal eyespot and presented these items to birds on backgrounds that were either conceal- ing or exposing. Although the effect was not very strong, their result showed that small eyespots directed the attacks of birds towards them, regardless of how well the rest of the prey body blended into the background [33].

Why would the attack-directing effect of eyespots be advantageous for a prey? Particularly in Lepidoptera, it has been argued that if attacks are directed to the small eyespots located near the wing margins of many species instead of the more vital parts, such as the head or the body, the prey is more likely to escape and survive the attack [3,8,9,14,34]. Torn wing edges in Lepidoptera are not very costly damage, but many other species do not have body parts that would be likely to tolerate an attack and enable a successful escape [6,34]. However, it has been observed in butterflyflies (Chaetodontidae) that some individuals that have lost up to 10% of their posterior body region (probably in an attack by a predator) have still been able to survive, recover, and be reproductively active after this type of damage [4].

In addition to redirecting attacks towards less vital body parts, eyespots could also be used to direct attacks in a direction that, in combination with the escape trajectory of the prey, would increase the chance of a successful escape [35]. For example, many fish (e.g. members of Chaetodontidae, Pomacentridae, Gobiidae, Labridae, Apogonidae and Pomacanthidae [36]) have spots located in the areas on, or close to, their caudal fins, and because fish primarily swim head first, an attack directed towards the posterior region could facilitate the escape of a fish [4]. Interestingly, in many species of fish that have an eyespot in the posterior region, the real eye is obscured by an eye stripe (a stripe running through the eye [4,8,35]) or the eyespot is larger than the real eye, which might also indicate that competition for the predator’s visual attention between the eyespot and the real eye may influence the evolution of colour patterns. This also raises the question of whether such eyespots rely on eye mimicry (i.e. that the posterior eyespot gives the appearance that the head is located at that end of the body; cf. the false head hypothesis [2,23,37]) or whether the pattern attracts attention and directs attacks. Dale & Pappantoniou [38] suggest that eye mimicry might be important for the anti-predator function of eyespots against eye-picking predators (such as the cutlip minnow, Exoglossum maxillella), but unfortunately their study lacks proper statistical analysis [6]. Importantly, our findings from the two first experiments suggest that even though eyespots effectively divert the attacks of fish towards them, eyespots do not necessarily make an individual more susceptible to predation risk by increased conspicuousness. It is clear, however, that the function of divertive eyespots against fish predators warrants further experimental studies. For example, although we have shown that the eyespot pattern has a divertive effect against fish, we cannot exclude that also some other patterns might have similar effect. Future studies could investigate which properties of markings (e.g. eyelikeness) are important for predator response.

Gagliano [13] interpreted the lack of bite marks towards the eyespot on the posterior dorsal fin of recaptured reef fish (Pomacentrus amboinensis) as evidence against the diversion hypothesis, but as we state, another possibility is that the lack of bite marks instead indicated that the eyespots increased the chance of successfully avoiding an attack [39]. Similarly, McPhail [40] suggested that diversion marks could also increase the probability of a prey successfully avoiding predator attacks, and not only direct attacks towards less vital or defended parts of the prey body. McPhail [40] concluded that caudal, simple spots found in many fish may function as such diversion marks. Unfortunately, as already pointed out [6], sections on McPhail’s [40] experimental methods and data analysis are incomplete, and his data suffered heavily from pseudo-replication. Thus, even though the idea that eyespots in aquatic prey may serve to misdirect predator attacks towards them is widely spread [4,6,40], we contend that our study is the first to present firm experimental support for this.

As our second question, we investigated whether large eyespots could have an intimidating effect against attacking fish. Our result from experiment 2 did not lend support for the intimidation hypothesis. This is interesting, considering the strong support this hypothesis has received in numerous terrestrial studies using passerine predators and butterflies as prey [17,19,20,22,41]. It has also been suggested multiple times that spots could have an intimidating effect in fish [4,16,42]. Yet, at least so far, experimental evidence to support this hypothesis in aquatic environments remains scarce (but see [16]).

It is noteworthy, however, that the fish that received spotless prey items in both the detectability and the intimidation experiment decreased their attack latency with successive trials, whereas the fish that received a spotted prey did not. This may suggest that even though we did not find any increased attack latency owing to intimidation caused by the
eyespots, eyespots might interfere with learnt improvement of attack performance.

One possible explanation for why the large eyespots did not induce any immediate flight response is that, when viewed from the front, in (predatory) fish the projection of eyes is quite different owing to their lateral placement compared with many terrestrial predators, such as birds of prey. Therefore, an eyespot may not mimic a threat owing to the presence of a predator as closely in aquatic environments as it does in many terrestrial systems. Clearly, this is based on the assumption that eye mimicry is important for the intimidating effect of eyespots (3,7,16,43,44), but see also (45,46). Moreover, in experiment 2, the artificial prey items had only one eyespot, and one possibility is that two spots could be more likely to cause an intimidating effect in fish, because it might better reflect a more honest signal about potential threat by mimicking the frontal view of an approaching predator [16,43].

Another possibility is that our experiment lacks some condition such as movement. Many butterflies enhance their intimidating effect of an eyespot by a startle display, that is, by suddenly revealing it and also by moving so that the projection of the spot will be maximized towards the predator [3,17,47]. There are also anecdotal notes that fish appear to turn sideways towards a predator to present their lateral eyespot [4,48]. However, it is important to note that even fixed displays without such additional behaviours have been shown to elicit a response in birds encountering a butterfly [19,22].

Collectively, our results clearly showed that eyespots effectively draw the attacks of fish towards them, although our experiment does not exclude the possibility that also other markings could have a similar effect. Nevertheless, our results suggest that the existence of eyespots in many aquatic prey species may be explained by the diversion hypothesis. This finding also suggests that the diverting effect in fish may not only select for the occurrence of eyespots in aquatic prey, but it may also select for the location, possibly because directing a predator towards the opposite direction in which a prey is moving might enhance the probability of a successful escape.

Acknowledgements. We are grateful to Jennifer Blyth for assistance in the field. We thank Jennifer Kelley, Jörgen I. Johnsson, Ron Rutowski, Ullassa Kodandaramaiah, Innes Cuthill and two anonymous referees for discussions and helpful comments that significantly improved the quality of this paper. We are also grateful to Miranda Grönholm and Annika Wiksten for assistance with the experiments and to Levente Bacso for technical assistance.

Funding statement. This work has been supported by the Academy of Finland (S.M.).

References