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# Can we use starlings' aversion to eyespots as the basis for a novel 'cognitive bias' task?

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### ABSTRACT

Experiments in humans have shown that changes in emotional (affective) state cause adaptive changes in the processing of incoming information, termed "cognitive bias". For instance, the states of anxiety and depression have been shown to be associated with "pessimistic" judgements of ambiguous stimuli intermediate between stimuli associated with positive and negative outcomes. This phenomenon provides a promising method for objectively assessing animal emotional states and has been successfully demonstrated in preliminary studies. However, the experiments yielding these results required extensive training to establish the necessary positive and negative associations. Here we present an experiment using responses to eyespot stimuli that are naturally aversive to many bird species, and require no explicit associative training. We manipulated the state of wild-caught European starlings (*Sturnus vulgaris*) by playing one of four possible sounds: starling "threat call" (control manipulation), a sparrowhawk call (i.e. predator), starling alarm call or white noise, on the assumption that the latter three sounds would cause anxiety. Immediately following the auditory stimulus, we recorded the birds' behaviour in the presence of each of three visual stimuli: eyespots, ambiguous eyespots or no eyespots. We hypothesised that there would be an interaction between the state of the birds and their response to eyespots, with birds showing enhanced aversion to ambiguous eyespots when anxious. We found evidence that white noise and alarm calls generated anxiety, and that eyespots were aversive. However, there was no interaction between state and response to eyespots. In an attempt to understand our failure to obtain the predicted cognitive bias, we discuss evidence that the aversive nature of eyespots is not attributable to predator mimicry, and is therefore not modulated by anxiety.

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## 1. Introduction

Objectively assessing the affective (i.e. emotional) state of animals is one of the primary concerns of welfare science. A promising recent approach focuses on assessing how emotional processes affect cognitive function (Mendl and Paul, 2004; Paul et al., 2005). The underlying theoretical background was initially derived from work

in humans, where differences in "trait" (stable variability between individuals) and "state" (transitory variability within individuals) anxiety are associated with well-defined biases in performance on cognitive tasks that test attention, memory and judgement (reviewed by Paul et al., 2005). For example, this literature suggests that individuals suffering from negative affective states associated with anxiety and depression are more likely to interpret ambiguous stimuli as threatening, or as indicative of negative outcomes (e.g. Eysenck et al., 1991). These "pessimistic" cognitive biases make sense from an evolutionary perspective under the assumption that negative

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affective states are an adaptive response to receiving information that there may be threats present in the environment. Under these circumstances it is adaptive to adopt a more conservative criterion for classifying an ambiguous event as a likely threat (Haselton and Nettle, 2006). To avoid charges of anthropomorphism, we define “pessimism” operationally as an increased probability of classifying an ambiguous stimulus as predicting a negative outcome.

In an attempt to explore whether animals show similar “pessimistic” cognitive biases, Harding et al. (2004) trained rats to press a lever for food reward on hearing a positive 2 Hz tone but to refrain from lever pressing to avoid punishment with white noise on hearing a negative 4 Hz tone. Once trained, rats were then tested with ambiguous intermediate tones (2.5, 3.0 and 3.5 Hz). Rats kept in “unpredictable” housing conditions known to induce symptoms of depression were less likely to lever press in response to the intermediate tones. This result was interpreted as evidence for a “pessimistic” cognitive bias in rats in a negative affective state. Analogous experiments on European starlings (*Sturnus vulgaris*) have provided support for this result, by showing that birds housed in smaller cages lacking environmental enrichment are also more likely to classify an ambiguous stimulus as predicting a more negative outcome (Bateson and Matheson, 2007; Matheson et al., 2008). The design of the tasks used in the above experiments is summarised in Table 1.

In all of the above experiments, the demonstration of cognitive bias relies on the establishment of a continuous or discrete stimulus scale with positive reinforcement associated with one end and negative (or less positive) reinforcement with the other. Extensive training of experimental subjects was required in order for them to learn the necessary positive and negative associations (Harding et al., 2004; Bateson and Matheson, 2007; Matheson et al., 2008; Burman et al., 2008). There are a number of drawbacks associated with this extended training. First, it is very time-consuming and hence may be difficult to transfer to situations where a practical assessment of animal welfare is needed rapidly and cost-effectively. Second, extensive training introduces potential experimental error whereby subjects perceive and learn about additional elements that were not foreseen by the experimenter, e.g. side-biases (Jackson et al., 1998); “superstitious” responses (Doughty et al., 2001); or interactions between the stimuli and reinforcers (Matheson et al., 2008). Finally, a carefully controlled training

regime is only possible where all individuals are currently experiencing the same conditions (as those in experimental conditions are). However, in non-experimental circumstances differences in environmental conditions and prior affective state can occur. These in turn are known to lead to changes in the neuronal processes underlying learning and memory (LeDoux, 1992; McEwen and Sapolsky, 1995) that could impact on the findings of a cognitive bias trial.

Our aim in the current paper is to address the above drawbacks of previous cognitive bias tasks by exploiting stimuli that animals find naturally aversive, meaning that no training is required to establish the association between a stimulus and a negative outcome. The eyespot stimuli used by many lepidoperan species to deter bird attacks are a good potential candidate for use in experiments with European starlings. Eyespots are known to be aversive to passerines, and are effective in preventing birds from feeding on both live lepidopterans and paper models (Vallin et al., 2005; Stevens et al., 2007). Though the mechanism for the aversive effect of eyespots is unknown, one theory is that they mimic the eyes of the natural predators of small passerines (mammals and raptors; for a review see Stevens (2005)). In support of this theory, an extensive set of laboratory experiments showed that eyespots enclosed within a head shape (designed to resemble an owl), and displayed adjacent to a feeder, were particularly effective at deterring starlings from feeding (Inglis et al., 1983). These results imply that the negative outcome associated with eyespots could be predation.

On the basis of these findings we chose to use eyespots adjacent to food as our negative stimulus. We used a similarly sized visual stimulus, but with no eyespots, adjacent to food as our positive stimulus. As our intermediate test stimulus we added visual noise to the eyespot stimuli (see Section 2.3 for details) on the grounds that eyespots with reduced contrast have been shown to produce a deterrent effect of reduced magnitude (Stevens et al., 2007).

In order to observe a cognitive bias, it is necessary for the affective state of the experimental subjects to interact with their response to the ambiguous predictors of food reward. In previous experiments experimental manipulations of state have involved changes in housing conditions that are theorised to cause an anxious and/or depressed state (e.g. Harding et al., 2004; Bateson and Matheson, 2007; Burman et al., 2008). The success of these experiments relies on

**Table 1**  
Methodology of previous cognitive bias tasks.

Species	Stimuli		Outcomes		State manipulation	Reference
	Positive	Negative	Positive	Negative		
Rat	2 Hz tone	4 Hz tone	45 mg food pellet	30 s 70 dB white noise	Predictability of housing Housing enrichment	Harding et al. (2004) Burman et al. (2008)
Rat	Location of food bowl in test arena	Alternative location of food bowl	45 mg food pellet	No food reward		
Starling	10 s light	2 s light	Instant food: 45 mg pellet	15-s delayed food: 45 mg pellet	Cage size and enrichment	Matheson et al. (2008)
Starling	White lid	80% grey lid	Palatable mealworm	Unpalatable quinine-injected mealworm	Cage enrichment	Bateson and Matheson (2007)

changes in one form of cognitive input (the negative affective state caused by the housing manipulations) affecting judgements of other forms of cognitive input (ambiguous predictors of food reward). In the current experiment, we aim to clarify this link by using auditory signals to manipulate starlings' perceived predation risk. We will then investigate how this manipulation affects the birds' responses to visual eyespot stimuli assumed to mimic a potential predator.

To manipulate the starlings' perceived risk of predation and induce a fearful and/or anxious affective state we chose to use auditory stimuli. Specifically we used: a predator call (sparrowhawk) assumed to provide the most direct evidence for the presence of a predator, a starling alarm call given when one bird in a flock detects a predator and white noise, which is known to be anxiety-inducing in rats (Windle et al., 1999). We used a starling "threat" call, used in social interactions as a control, on the grounds that this was unlikely to increase perceived predation risk or raise anxiety levels.

We hypothesised that the auditory stimuli causing increases in perceived predation risk and fear/anxiety (i.e. sparrowhawk, alarm call and white noise) would cause starlings to be more likely to respond to the eyespot stimuli "pessimistically", and thus be less likely to approach them to feed. Similarly to other cognitive bias experiments, we predicted that the ambiguous eyespot stimuli would prove to be the most sensitive at detecting changes in affective state. However, the starlings might also show an increased response to the full eyespots, since there is no reason to assume that their normal response to eyespots is maximal (unlike in previous cognitive bias experiments where animals are trained to a high performance criterion with the positive and negative stimuli before manipulations of state are performed). Therefore in summary, we predict an interaction between the state of the birds and their response to the eyespot stimuli (Fig. 1).

## 2. Methods

### 2.1. Experimental subjects

The subjects were 32 (16 male and 16 female counter-balanced across the four treatments) adult European

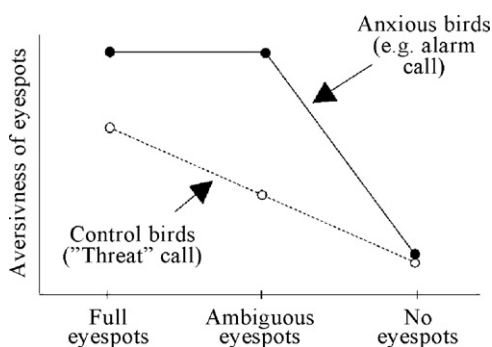


Fig. 1. Diagram showing the approximate predicted pattern of responses to eyespot stimuli in anxious and control birds. The crucial prediction is that there should be an interaction between the birds' state and how aversive they find the eyespots.

starlings caught from the wild under licence from Natural England. Prior to the experiment all birds were housed in indoor aviaries (2.4 m × 2.15 m × 2.3 m) (2.25 m high × 3.60 m wide × 2.40 m deep) in groups of a maximum of 20 individuals. Aviaries were furnished with water baths, wood chippings covering the floor and a number of dead trees to provide perches and cover. Throughout the study birds were fed a diet of Purina kitten food *ad libitum*, supplemented with assorted fruit and mealworms (*Tenebrio larvae*). Aviaries and the experimental room (see below) were kept under a 14:10 light:dark cycle, with lights coming on at 0700. Light was provided by Phillips Master bulbs (50 kHz). The temperature was maintained between 17 and 21 °C.

### 2.2. Apparatus

Three days prior to experimental testing birds were transferred to individual cages (Fig. 2) located in a separate experimental room. The procedures of capture and transfer are known to be acute stressors in starlings (Rich and Romero, 2005). However, the experimental results show that the elevated anxiety levels putatively caused by the transfer protocol did not cause a ceiling effect. The experimental cages (150 mm × 44 mm × 45 mm) contained a number of environmental enrichments including four natural branch perches, a plastic tray filled with bark and a plastic tray half-filled with water. The cage was visually divided into four equal length sections by means of black tape attached to the outside of the cage.

At one end of the cage, a bowl (diameter 85 mm, height 35 mm) was placed 5 cm from the wall. The subjects' food was placed in this bowl and birds were allowed to feed *ad libitum*. A card (85 mm × 155 mm) featuring the positive stimulus (see below) was hung on the cage side above this bowl. The subjects had therefore habituated to the presence of the card used as the positive stimulus and the location of food rewards in the cage prior to the experimental manipulations.

### 2.3. Eyespot stimuli

The eyespot stimuli (Fig. 3) were based on a photograph of owl eyes with a contrasting light iris and dark pupil.

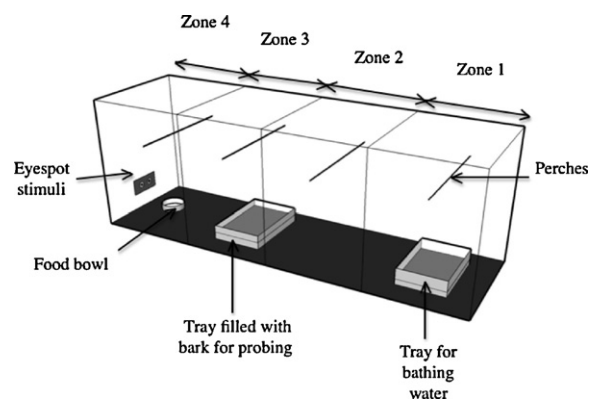


Fig. 2. Diagram of the experimental cage.

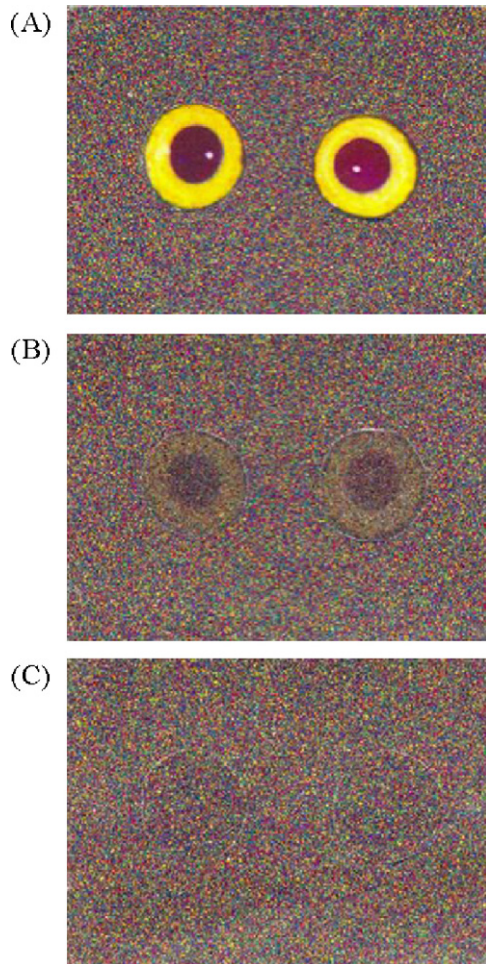


Fig. 3. The three eyespot stimuli used during the experiment: (A) Full eyespots. (B) Ambiguous eyespots. (C) No eyespots.

Highlights on the pupil and iris suggest a three dimensional object (as per Inglis et al., 1983). The eyes were cut out and pasted on to a background formed from the colours of the eyes subjected to a Gaussian noise manipulation such that a random background pattern was formed (600% level in Adobe Photoshop CS3 (Adobe Systems Inc.)). Three levels of eyespot stimulus were created: full eyespots, in which the eyes were unmanipulated (in combination with the food bowl, this can be thought of as analogous to the negative stimulus in previous cognitive bias experiments); ambiguous eyespots, in which the eyes had 200% Gaussian noise added and no eyespots, in which the eyespots were completely obscured by the addition of 600% Gaussian noise (in combination with the food bowl, this latter stimulus can be thought of as analogous to the positive stimulus in previous cognitive bias experiments).

#### 2.4. Manipulation of state

Four auditory stimuli were used: a starling “threat” call used in mild antagonistic encounters between conspecifics (Hartby, 1969) which are generally quickly resolved (Feare, 1984); the call of a male sparrowhawk (*Accipiter nisus*) on

presenting food to its mate; the alarm call of a starling in the proximity of its nest when a domestic cat (*Felis catus*) was present; and broad-spectrum white noise (a randomly generated audio signal with constant power spectral density across its frequency range—86 Hz to 22 kHz in this case) generated in Audacity 1.2.4 (a freeware audio analysis programme).

Each recording was digitised in a lossless format and edited to a 10-s sample that was repeated four times across a period of 2 min. This minimised the variance in sound intensity that could be expected from natural calls recorded over 2 min. The resulting 2-min recording was broadcast through an Apple Nano ipod and a pair of Yamaha YST-M20DSP active speakers. Playback was standardised such that the sound pressure level was 75 dB at approximately the location of a bird perched on the perch furthest from the eyespots (the location where the majority of the subjects began the observational period).

#### 2.5. Procedure and experimental design

The four birds housed in the experimental room were tested simultaneously. All food was removed from the birds' cages at 0730 on the day of testing. The first trial began at 0900. The lights in the room were switched off and the experimenter inserted one of the three eyespot stimuli into the holder in each of the four cages, filled each food bowl with ten fresh mealworms and vacated the room. The birds were left in the dark to settle for 5 min before one of the four auditory stimuli was played in the room. The lights in the room were then switched on and the birds' behaviour recorded using two Sony DCR-SR32 Camcorders (two cages were captured on each camera) for the next 30 min. At the end of the recording, the stimulus cards were removed from the holders and replaced with the no eyespot cards. There was then a break of approximately 30 min before the next trial began.

Subsequent trials followed the same procedure outlined above. There were three trials in total, one for each of the eyespot stimuli, with the order of presentation of the three stimuli randomised across birds. The auditory stimulus was kept constant within each replicate of four birds. Following the final trial of the day the birds were given *ad libitum* food prior to being caught and returned to the aviary in the course of the afternoon.

The entire experiment comprised eight replicates of the above procedure, two for each of the four different auditory stimuli we used, with the order of replicates randomised. Thus, the experiment had a mixed design, with one within-subjects factor (three levels of eyespot stimuli) and one between-subjects factor (four levels of auditory stimuli). Eight different birds were tested with each auditory stimulus.

#### 2.6. Ethical considerations

No long-term adverse effects of the experiment were observed in behavioural patterns and subjects fed freely on completion of the trials. The experimental protocol was subject to internal review and followed the guidelines laid

out by The Association for the Study of Animal Behaviour on the use of aversive stimuli. All birds were released to the wild at the site of original capture on completion of our studies.

## 2.7. Dependent variables

Using the behavioural analysis software JWatcher (Blumstein et al., 2000) the following events and states were recorded for the first 20 min of each trial: the bird's position in the cage, divided into zones 1–4 (see Fig. 2) and zone 5, which was defined as being in physical contact with the food bowl; whether the bird was facing towards or away from the eyespot stimuli; and any eating or drinking motions made by the bird, specifically eating mealworms from the food bowl. From these data we were able to compute the following measures: the proportion of time the bird spent in each zone; the latency for the bird to make its first movement; the latency for the bird to first approach the food bowl; and the proportion of time the bird spent facing the eyespot stimulus.

## 2.8. Analysis

There was a large effect of one of the auditory stimuli on the initial latency to move (see results below) resulting in the proportion of time spent in each zone being influenced by the birds' initial decision of whether to move or not. Hence, we subtracted the time until each bird's first movement in calculating the proportion of time in each zone, the proportion of time facing the eyespots and the latency to approach the food bowl.

All measures were transformed as necessary to allow for the use of parametric statistics. We used a mixed ANOVA with eyespot stimulus level as a within-subjects factor and auditory stimulus as a between-subjects factor. Where post-hoc tests were necessary to identify the source of significant effects, we used a Bonferroni correction for multiple comparisons. All data were analysed using SPSS 16.0 for Mac.

The data from one trial in the group subjected to white noise had to be discarded due to equipment failure. Though this failure resulted in the loss of only one data point for three individuals (for each of the respective eyespot levels they were undergoing in that trial), the repeated measures element of the analysis would have required the removal of all three birds from the ANOVA. This would have resulted in a significant increase in the possibility of a type II error. Hence, we chose to replace the three missing data points with the mean for the group (those hearing white noise and either full, 200% noise added or no eyespots as appropriate). Since this reduces the variance in the data, we take into consideration the increased probability of committing a type I error, particularly in pairwise comparisons involving this group.

## 3. Results

### 3.1. Latency to first movement (Fig. 4A)

The data for this measure were  $\log_{10}$  transformed prior to analysis. There was no significant effect of the eyespot stimulus on the latency to the first movement ( $F(2,56) = 1.066$ ,  $p = 0.351$ ). However, there was a significant effect of the auditory stimulus ( $F(3,28) = 11.696$ ,  $p < 0.001$ ). Post-hoc tests show that latency to first movement was significantly greater following the alarm call than any of the other three auditory stimuli (all pair-wise comparisons  $p < 0.002$ ). There was no significant interaction between the eyespot stimulus and auditory stimulus on latency to first movement ( $F(6,56) = 0.443$ ,  $p = 0.847$ ).

### 3.2. Latency to approach the food bowl (Fig. 4B)

The data for this measure were  $\log_{10}$  transformed prior to analysis. There was a significant effect of the eyespot stimulus on the latency to approach the food bowl ( $F(2,56) = 31.891$ ,  $p < 0.001$ ). Post-hoc tests revealed that latency to approach the food bowl was significantly higher

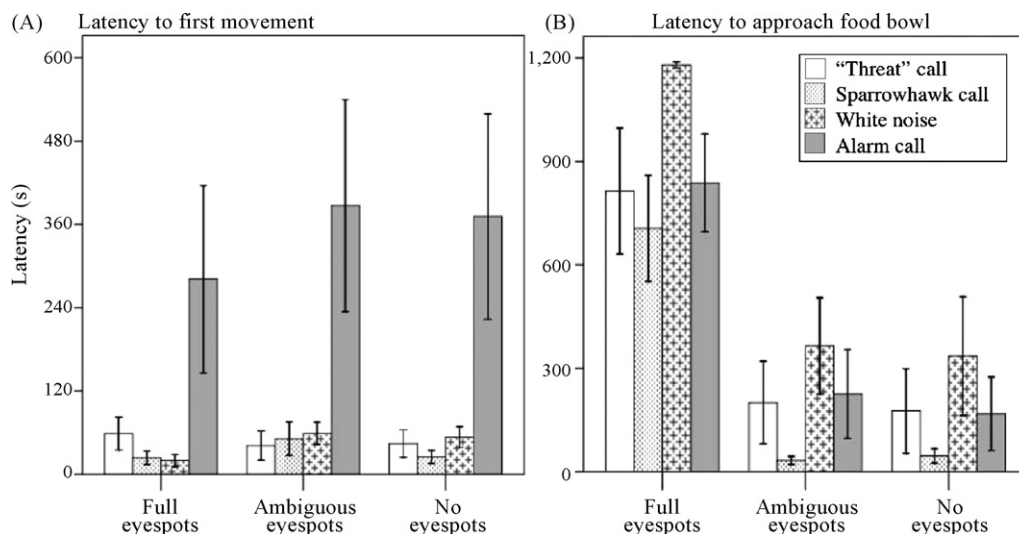


Fig. 4. Mean ( $\pm 1$  S.E.M.) latencies to (A) first movement and (B) approach the food bowl adjacent to location of eyespot stimuli.

with the full eyespots than for the other two eyespot stimuli ( $p < 0.001$ ). There was a significant effect of the auditory stimulus ( $F(3,28) = 3.696$ ,  $p = 0.023$ ). Post-hoc tests show that latency to approach the food bowl was higher following white noise than the sparrowhawk call ( $p = 0.019$ ). There was no significant interaction between the eyespot stimulus and auditory stimulus ( $F(6,56) = 1.066$ ,  $p = 0.394$ ).

### 3.3. Proportion of time spent in different zones of the cage

The data for zones 2 and 3 (the central areas of the cages) were not analysed since the significance of these zones relative to the eyespot or auditory stimuli has no obvious interpretation. We therefore restrict our analysis to the proportion of time spent furthest from the eyespot stimuli (zone 1); the proportion of time spent nearest to

the eyespot stimuli (zone 4); and the proportion of time spent on the food bowl (zone 5).

The proportion of time spent in the area furthest from the eyespots (zone 1) is shown in Fig. 5A. These data were arcsine square root transformed prior to analysis. There was a significant effect of the eyespot stimulus on the amount of time the subjects spent in zone 1 ( $F(2,56) = 6.056$ ,  $p = 0.004$ ). Post-hoc tests show that this effect is due to the difference in the birds' response to full eyespots and no eyespots, with birds spending a greater proportion of their time in zone 1 when the stimulus was full eyespots than no eyespots ( $p = 0.002$ ). There was also a significant effect of auditory stimulus ( $F(3,28) = 3.621$ ,  $p = 0.025$ ). Post-hoc tests show that the effect was due to a significant difference between the alarm call and the sparrowhawk ( $p = 0.046$ ) stimuli, with birds spending a greater proportion of their time in zone 1 following an

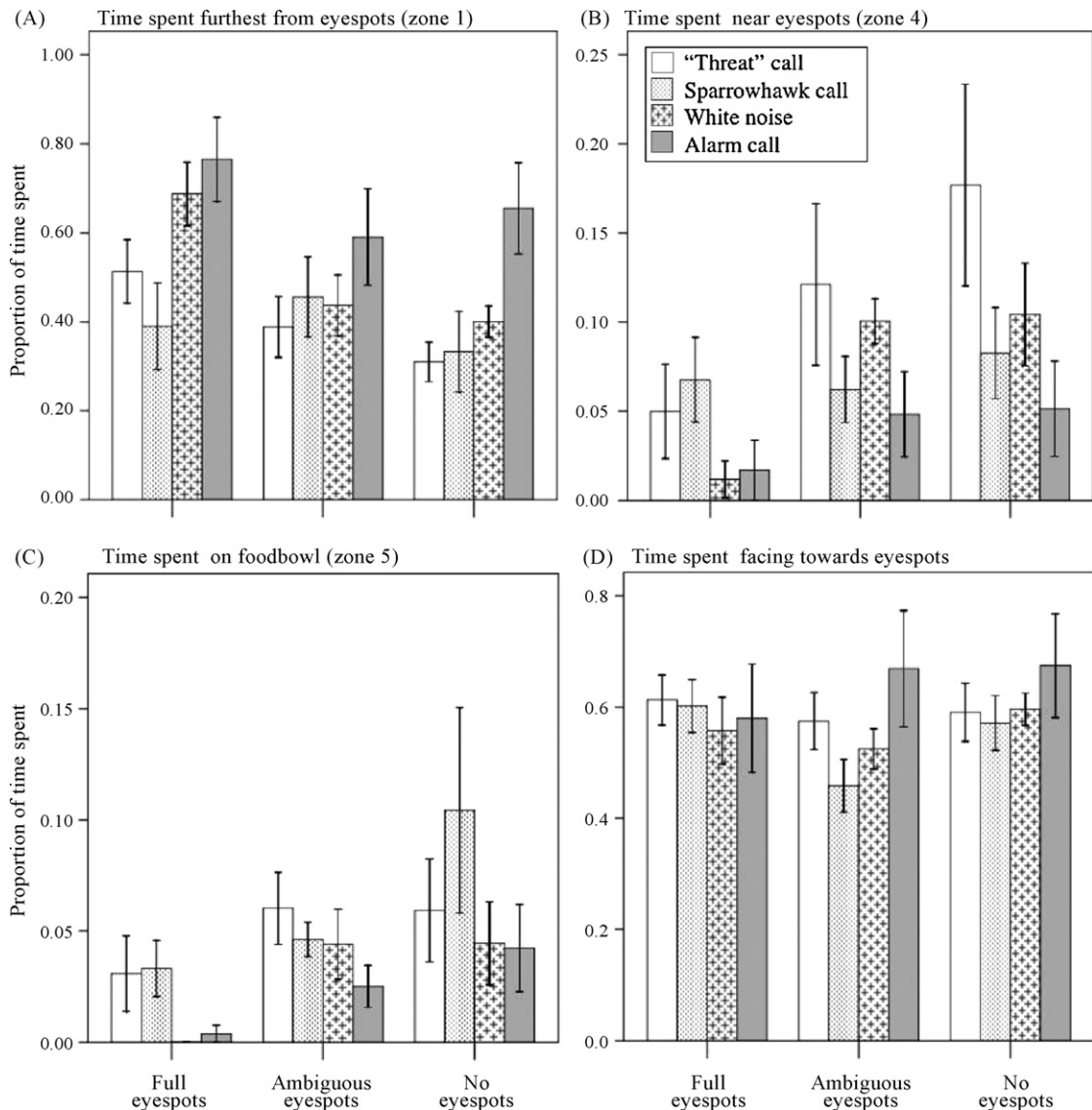


Fig. 5. Mean proportion of time ( $\pm 1$  S.E.M.) spent by birds in (A) the area of the cage furthest from the food bowl/eyespots (zone 1), (B) the area of the cage nearest the food bowl/eyespots (zone 4), (C) on the food bowl adjacent to the location of the eyespot stimuli (zone 5), and (D) facing towards the eyespot stimuli.

alarm call than a sparrowhawk call. There was also a marginally non-significant difference between the alarm call and “threat” call stimuli ( $p = 0.060$ ). There was no significant effect of the interaction between the auditory and eyespot stimuli on the proportion of time spent in zone 1 ( $F(6,56) = 0.962$ ,  $p = 0.459$ ).

The proportion of time spent in the area nearest the eyespot stimulus (zone 4) is shown in Fig. 5B. These data were arcsine square root transformed prior to analysis. There was a significant effect of the eyespot stimulus on the amount of time spent in the section nearest the eyespots ( $F(2,56) = 13.142$ ,  $p < 0.001$ ). Post-hoc tests showed that this effect was due to the birds spending a smaller proportion of their time in zone 4 in the full eyespot condition than the other two eyespot conditions ( $p < 0.002$  for both). There was also a significant effect of auditory stimulus on the amount of time spent nearest the eyespot stimulus ( $F(3,28) = 3.049$ ,  $p = 0.045$ ). Post-hoc tests revealed that the effect was due to the difference in the birds’ response to the alarm call and “threat” call ( $p = 0.033$ ), with birds spending a greater proportion of their time in zone 4 following the “threat” than the alarm call. There was no significant interaction between the eyespot and auditory stimuli on the proportion of time spent in zone 4 ( $F(6,56) = 1.299$ ,  $p = 0.273$ ).

The proportion of time spent on the food bowl (zone 5) is shown in Fig. 5C. These data were arcsine square root transformed prior to analysis. There was a significant effect of eyespot stimulus ( $F(2,56) = 18.629$ ,  $p < 0.001$ ). Post-hoc tests revealed that the effect was due to the difference in the birds’ response to full eyespots and the other eyespot levels ( $p < 0.001$  for both), with birds spending a smaller proportion of their time on the food bowl in the presence of the full eyespots than with the other two stimuli. There was no significant effect of the auditory stimuli on the proportion of time spent on the food bowl ( $F(3,28) = 1.731$ ,  $p = 0.183$ ) and no significant interaction between the eyespot and auditory stimuli ( $F(6,56) = 0.671$ ,  $p = 0.673$ ).

### 3.4. Proportion of time spent facing the eyespots (Fig. 5D)

There was no effect of eyespot stimulus ( $F(2,56) = 0.909$ ,  $p = 0.409$ ), auditory stimulus ( $F(3,28) = 2.475$ ,  $p = 0.082$ ) or their interaction ( $F(6,56) = 0.906$ ,  $p = 0.498$ ) on the proportion of time that the birds spent facing towards the eyespots.

## 4. Discussion

The experiment produced some useful positive findings. Both a starling alarm call and white noise produced evidence of fear/anxiety in the birds. The alarm call caused an increase in the initial freezing response, an increase in the proportion of time spent furthest from the eyespots and a reduction in the proportion of time spent in the vicinity of the food bowl. White noise caused an increase in the latency to feed. These data support the use of auditory stimuli to elicit short-term fear/anxiety in caged birds. Starlings also found static full eyespots aversive, as measured by an increased latency to approach an adjacent food bowl and a reduced proportion of time spent near to

the eyespot stimulus. Ambiguous eyespots were treated no differently from the visual stimulus without eyespots. Most importantly however, there were no interactions between the starlings’ responses to the auditory stimuli and either the full or ambiguous eyespot stimuli. Therefore, contrary to our main hypothesis, there was no evidence that the auditory stimuli eliciting fear/anxiety caused increased aversion to ambiguous eyespots. Below we further discuss the two positive findings from the experiment, and follow this with a discussion of the possible reasons why anxiety did not negatively bias the birds’ judgements of eyespot stimuli as we originally predicted.

### 4.1. Responses to auditory stimuli

Birds that heard a starling alarm call took significantly longer to move once observation began than the subjects that heard the other auditory stimuli. We posit that this measure represents a freezing response, replicating the findings from recent research on starlings freezing when presented with videos of starling flocks being preyed (Carere, personal communication). There is evidence to suggest that freezing is adaptive in wild birds under certain circumstances (Ficken and Witkin, 1977; Lind, 2002). In particular, a study of great tits (*Parus major*) showed that freezing occurred only once some uncertainty about predator presence occurred (a predator had been presented but was now absent) (Kullberg and Lind, 2002). Freezing as an anti-predator adaptation may be particularly adaptive in the experimental conditions where no predator was actually presented and where escape routes are limited/blocked.

Interestingly, the birds failed to show any freezing response after hearing white noise, despite this being a potentially anxiety-inducing unpleasant auditory stimulus (e.g. Windle et al., 1999; Harding et al., 2004). This would suggest that freezing is a specific anti-predator mechanism, which is elicited only by the imminent threat of predation. A non-specific auditory cue such as white noise would therefore elicit general anxiety and increased vigilance without causing freezing.

The lack of freezing behaviour in response to the sparrowhawk call might be explained due to the sparrowhawk being an ambush predator. In hindsight it is of little surprise that hearing clear auditory cues offered by an ambush predator is probably a sign that it is not hunting.

We found evidence that both alarm calls and white noise reduced the birds’ motivation to feed. The white noise resulted in an increased latency to approach the food bowl, and the alarm call reduced the proportion of time spent in the vicinity of the food bowl. Since reduced feeding motivation is a common response to stress, this is suggestive that both the alarm call and white noise caused anxiety in the birds.

Though we did not measure any physiological correlates of anxiety, previous experiments have shown that an auditory stimulus can have significant effects in starlings. Nephew et al. (2003) showed that playing a radio in close proximity to captive starlings produces: an increase in

heart rate, an increased latency for heart rate to return to basal levels; a decrease in the number of preening events; and an increase in blood corticosterone levels (see also Rich and Romero, 2005). We therefore consider it likely that our alarm call and white noise manipulations produced an anxiety response in the birds.

#### 4.2. Responses to eyespot stimuli

The presence of full eyespot stimuli caused subjects to be slower to approach the adjacent food bowl and to spend significantly less time in the vicinity of the eyespot stimuli and food bowl. The full eyespots also caused the subjects to spend significantly more time in the area furthest away (but only in comparison to no eyespots and not to the ambiguous eyespots). Thus our results replicate previous studies showing that starlings find eyespots aversive, especially when presented adjacent to food (Inglis et al., 1983; Avery and Matteson, 1993). A previous study that failed to find a deterrent effect of static eyespot stimuli in starlings paired the stimuli with nest boxes as opposed to food (Belant et al., 1998).

For all measures there was no significant difference between behaviour in the presence of ambiguous eyespots and no eyespots. This suggests that our manipulation of adding noise (and therefore ambiguity) to the eyespots was too extreme, and that the birds made no distinction between the ambiguous and no eyespot stimuli. This interpretation is supported by evidence that contrast levels and conspicuousness are a prime factor in the effectiveness of eyespots (Stevens et al., 2007, 2008).

There was no effect of the eyespot stimuli on the amount of time which the subjects spent facing the eyespots. Although this is a coarse measure of the aversiveness of a stimulus, there is evidence to suggest that animals direct more attention towards anxiety-inducing or threatening stimuli (Eysenck et al., 2007). We therefore consider this as suggestive evidence that the birds did not find the eyespots aversive, at least not in the sense of being an active threat.

#### 4.3. Why did anxiety not modulate responses to eyespots?

In considering why our fear/anxiety inducing auditory stimuli failed to modulate the birds' aversive responses to the eyespot stimuli, it is important to consider how eyespots are hypothesised to function. There is as yet no clear consensus on how eyespots exert an aversive effect. Stevens (2005) reviews two hypotheses for the aversive effect of eyespots in Lepidoptera: first, eyespots intimidate passerine predators through the mimicry of genuine eyes (further hypothesised to represent a specific threat, e.g. mammalian predators); or second, eyespots rely on the intrinsic properties of the avian sensory system and are effective by being highly conspicuous. Unfortunately, our experimental design makes it difficult to distinguish between these hypotheses because a reduction in the amount of time near the food bowl can equally well be explained either by the aversive effect of the eyespots (hypothesis one) or a reduction in motivation to feed (hypothesis two). However, below we argue that the lack

of an interaction between the auditory stimuli and eyespot stimuli provides evidence against the first hypothesis.

There is plentiful physiological and behavioural evidence that the potential presence of a predator causes an anxiety and/or fear response (e.g. mammals: Eilam et al., 1999; passerines: Cockrem and Silverin, 2002; fish: Bell et al., 2007). Thus, if the first hypothesis regarding the function of eyespots is correct, eyespots should cause such a response. The second hypothesis (conspicuousness) does not posit a resemblance of eyespots to any biologically relevant stimulus; hence there is no *a priori* reason to assume an anxiety/fear response. Above we hypothesised that alarm calls and white noise produced evidence of a fear and/or anxiety response in the birds. Should our eyespots have also had some form of anxiety effect, then from a mechanistic perspective we would have expected there to be an interaction between the auditory and visual stimuli in our experiment. Since we found none, we tentatively suggest that the eyespots used in this experiment were aversive for reasons other than eliciting a fear/anxiety response via resemblance to a biologically relevant stimulus (predator eyes). This conclusion is supported by recent experiments that provide strong evidence in favour of the second, conspicuousness-based hypothesis for eyespot function (Stevens et al., 2008).

As already noted above, there is additional corroborative evidence for the above conclusion in the lack of any differences in the amount of time that the birds spent facing towards the eyespots. Also, the eyespots had no effect on the initial latency to move, a measure that was very sensitive to the alarm call, and would therefore have also been expected to be sensitive to the eyespots if these mimicked the presence of a predator. This suggests that eyespots were not perceived to be part of the same predation-threat cue as the anxiety-inducing auditory stimuli.

## 5. Conclusions

Our data show that both an alarm call and white noise produced some evidence of fear or anxiety in captive wild-caught starlings. The data also show that the birds found static eyespot stimuli aversive. However, there was no evidence that the auditory stimuli eliciting fear or anxiety caused increased aversion to either full or ambiguous eyespots. This may be because starlings do not perceive eyespots as threatening. On this basis, responses to static eyespot stimuli are not supported as a novel measure of affective state in birds.

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