The effect of pyrazine odor on avoidance learning and memory in wild robins *Erithacus rubecula*

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**Abstract**  Toxic insects advertise their defended state to potential predators using warning displays. Frequently these displays use cues through more than one sensory modality, and combine color, smell and sound to produce a multimodal warning display. Signalling through more than one sensory pathway may enhance the rate of avoidance learning, and the memorability of the learned avoidance. A common insect warning odor, pyrazine, has previously been shown to increase the rate of learned avoidance of unpalatable yellow prey by domestic chicks (*Gallus gallus domesticus*), and the odor also improved memory of this learned avoidance. However, to date no research has examined this response to pyrazine odor using wild birds under natural conditions. This study used wild robins (*Erithacus rubecula*) to investigate whether wild birds avoided yellow baits that smelled of pyrazine more strongly than those presented with no odor. The results provide some evidence that pyrazine odor does increase the level of protection an aposematic insect gains from a wild avian predator, but that the effect of pyrazine on learned avoidance was much weaker than was found with domestic chicks [Current Zoology 57 (2): 208–214, 2011].

**Keywords**  Aposematism, Learned avoidance, Memory, Multimodal warning display, Pyrazine, Robins

Many prey species have adaptations which make them less profitable to potential predators. These aposematic species frequently advertise their unprofitability using warning displays (Edmunds, 1974). Such displays are often comprised of conspicuous visual signals but may also be accompanied by olfactory or auditory signals producing a multimodal warning display (Rowe, 1999; Partan and Marler, 1999, 2005). The different components of these multimodal signals may be aimed at the same or different predators (Rothschild, 1965; Rowe, 1999; Ratcliffe and Nydam, 2008). Empirical evidence suggests that combined signals can alter predator behaviour in a different manner than the individual components presented alone (Rowe and Guilford, 1996, 1999a, 1999b; Rowe, 1999; Siddall and Marples, 2008).

When a naïve predator encounters a defended prey species for the first time, it does not usually know the meaning of the aposematic signal. The predator needs to learn the meaning of the warning display if it is to avoid the noxious effects of the aposematic prey in the future. Therefore when a predator samples the toxic prey and suffers the effects of the prey’s defence system it learns to associate this with the prey species’ warning display (Pearce, 1997; Ruxton et al., 2004). Once the predator has learned to avoid the toxic insect, it needs to remember this learned avoidance if the insect is to be protected over time. So memory as well as learning plays an important role in the prey’s protection from predation, and the predator’s protection from poisoning. Memorability is defined as how easily a signal can be remembered (Guilford and Dawkins, 1991). Conspicuous warning signals, such as aposematic signals, may increase the rate at which a learned avoidance is acquired (Gittleman and Harvey, 1980), and prolong the memorability of this avoidance (Roper and Redston, 1987).

A common insect warning odor, pyrazine, is used by so many animal and plant taxa across a diversity of geographical ecosystems, that it could almost be considered to be a ubiquitous olfactory warning signal (Moore et al., 1990). The addition of a second signal component, such as an odor or sound, to a visual signal as part of a multimodal display may enhance the effectiveness of the signal in a number of ways. Firstly, it may increase the conspicuousness of the signal. This may serve to increase innate avoidance behaviour, increase the rate of learning and prolong memory. There are several studies that provide empirical evidence of these effects, both with odors not found in defenses against birds, such as almond and vanilla (Marples and Roper, 1997; Roper and Marples, 1997), and with classically warning odors such as pyrazine (Rowe and...
When no such aversion is present in the absence of the predator’s awareness of other signal components (Rothschild and Moore, 1987), thus enhancing both learning and memory of the signal. Finally the combination of a novel odor signal component with colors associated with aposematism (specifically yellow and red) can reveal an unlearned aversion to these colors, even when no such aversion is present in the absence of the odor (Rowe and Guilford, 1996; Jetz et al., 2000). By means of all three of these mechanisms, an unprofitable prey item, signalling through a novel odour and a conspicuous color, would be expected to cause strong avoidance learning and a long lasting memory of that avoidance. However, all these empirical studies were conducted under laboratory conditions using domestic chicks Gallus gallus domesticus as model avian predators. To date no such work has been conducted using free-living wild birds.

This current experiment sought to investigate whether wild robins exhibited similar behavioural responses to those shown by domestic chicks in the laboratory, when presented with similarly unpalatable prey displaying the same color and odor signal components. The experiment was designed to complement that of Siddall and Marples (2008), which demonstrated that pyrazine could enhance both the rate of learned avoidance of yellow prey, and the memory of that avoidance, by domestic chicks.

1 Materials and Methods

1.1 Test subjects

The experiment was carried out using 16 wild European robins in Archbishop Ryan Park, Dublin 2, Ireland between October 2006 and January 2007. Robins were chosen as model predators as there was a large population in Archbishop Ryan Park. They were easily trainable, could be ringed using individually identifiable color rings, and held separate territories, thus ensuring that robins could be tested individually. Work of this manner had previously been conducted using robins (Marples et al., 1998; Thomas et al., 2004). The Archbishop Ryan Park is 4.75 hectares of managed public parkland; therefore, birds in the area were habituated to humans, which facilitated direct observation of foraging decisions.

Robins have a broad insectivorous diet and may therefore be good representatives of how birds make foraging decisions about insect prey (Cramp, 1998; Thomas et al., 2003). Prior to testing, the robins were caught using mist nets. The trapping was carried out under license (BTO license, #F/CF/4601 to NMM and Irish NPWS license, R (B) 17/2006 to NMM) and each bird was given an individually recognisable combination of color rings on its left leg. During this time the winter territories of each individual were also mapped, to ensure that the experimental arena was placed in an area where only one robin would come to the tray. As they were winter territories, only one individual was present at each site, rather than a pair, as occurs in the summer territories (Cramp, 1998).

1.2 Artificial prey

Uncooked pastry baits were used as artificial prey. The pastry was made using 70 g flour: 30 g lard: 10 ml distilled water and dye solution (Marples et al., 1998; Thomas et al., 2004). This was rolled out to a thickness of 1.5 mm and then cut into small rectangles, to make baits which were 5 mm × 2.5 mm × 1.5 mm in size.

1.3 Color and taste cues

Commercial food dyes were used to color the pastry, 1 ml of Sugarflair Colors© Ltd. (Benfleet, Essex, UK) “Egg Yellow” or “Spruce Green” dye was diluted to make 90 ml of solution using distilled water, and then 10 ml of this solution was added to the flour and lard mixture to make the pastry. The yellow baits were made unpalatable by dipping them in a solution of one part 2.5% W/V Denatonium benzoate (Macfarlan Smith Ltd.), commercially available as “bitrex”, to eight parts distilled water, and allowing them to dry overnight prior to testing.

1.4 Odor cues

The concentration of pyrazine solution was the same as that used in the laboratory experiments by Siddall and Marples (2008), 100 μl of 2-isobutyl-3-methoxypyrazine diluted to 1000 ml using distilled water. In the odor treatment the pyrazine solution was placed on filter paper beneath the yellow baits, and distilled water was placed beneath the green baits. In the odorless treatment distilled water was placed beneath all baits.

1.5 Experimental arena

The experimental arena was a black plastic tray measuring 35 cm long × 21 cm wide × 5.5 cm deep (Fig. 1) with white paper on the floor, so that both bait colors appeared equally conspicuous against the background (tested by reflectance spectroscopy; Thomas et al., 2004). One standard size (90 mm diameter) plastic Petri dish was placed in its lid in each corner of the tray. Each Petri dish was perforated with a radial pattern of holes.
in its base. The lid of the dish on which the Petri dish sat contained a piece of filter paper soaked in either the pyrazine odor solution or distilled water. The Petri dish and the base were separated using spacer pads. This prevented the transfer of the odor or water onto the baits, which might otherwise have affected the palatability of the baits. The holes in the base of the Petri dish allowed the odor to permeate up from the filter paper, exposing the birds to volatile odor cues as they ate the pastry baits. The Petri dish was sanded on the underside and painted white, so that the tray blended in with the base of the experimental arena and provided a contrasting background against which the baits were presented.

During pre-training, mealworms *Tenebrio molitor* were put into each of these four Petri dishes. The pre-training method is discussed below. During the learning and memory trials the robins were given a choice test between palatable green and unpalatable yellow baits. Four baits of the same color were placed in a cruciform pattern in each Petri dish, and Petri dishes containing the same color of baits were placed in opposite corners of the feeding tray (Fig. 1). The robins were therefore offered a total of eight green and eight yellow baits during each trial. A small Petri dish (35 mm diameter) with one mealworm was placed in the centre of the experimental arena, to ensure that each robin came into the arena at the start of the learning trial. See below for details of the experimental arena.

### 1.6 Pre-training

Each bird was pre-trained using mealworms to come to a fixed feeding site within its own territory. The feeding tray was placed at the feeding site with one mealworm in each of the four Petri dishes. Once the bird came down to feed, a signature whistle was given and repeated while the bird remained at the tray. This pre-training process was continued over a six week period until the robins readily came to the feeding tray upon hearing the signature whistle and were tame enough to continue to forage while allowing close observation of their food choice (from 2–3 m away).

### 1.7 Learning trials

Once the robins had completed their pre-training they were split into two treatment groups, one in which all the baits were odorless and another where the yellow baits smelt of pyrazine. There were nine replicate individuals in the odorless treatment and ten in the pyrazine treatment. More robins were pre-trained than took part in the experiment, as several individuals disappeared during the course of the experiment. The Archbishop Ryan Park was divided into quarters with robins from diagonally opposite quarters of the park sharing the same treatments, so as to minimize any effects of territory location on the results obtained.

The robins were offered a choice test between eight palatable green baits and eight unpalatable yellow baits. The experimental arena was left in place for ten minutes, or until all the baits of one color had been eaten. The number of yellow and green baits eaten during each trial was noted. The robins were given seven learning trials over the course of a week, with one trial performed in each territory between 8 am and 11 am each morning. The position of the Petri dishes was rotated one place anti-clockwise each day, so that the robins could not learn to associate the position of the baits on the tray with their palatability, which may have affected their food choice. This also removed any effects that a favoured approach direction may have had on food choice.

A trial was considered to be a learning trial if the robin came to the experimental arena and ate the mealworm (so necessarily saw the other baits). Only robins that attacked at least one yellow bait during the first learning trial were included as experimental subjects. This excluded two individuals from the odorless treatment and one individual from the pyrazine treatment, resulting in seven replicates in the odorless treatment.
and nine in the pyrazine treatment. These exclusions were deemed necessary as those individuals had no opportunity to learn avoidance of the yellow baits.

The learning trials were not conducted in wet or windy weather, as rain may have washed off the bitrex and therefore affected palatability or diluted the pyrazine odor. The wind may also have blown the pyrazine odor around the experimental arena, thus reducing the odor gradient between the odor and non-odor dishes. Finally, wind tended to cause the feeding trays to overturn, making it difficult to conduct the experiment during adverse weather conditions.

1.8 Memory trials

Once the seven learning trials were complete, the memory trials were conducted. The first was the memory trials conducted after 96 hours, the next one week after the first memory trial, and the last one month after the second. During the memory trials the green baits were palatable, but the yellow baits were unpalatable. These memory trials were therefore by definition extinction trials, as an extinction trial is one that tests for the presence of the conditioned response (in this case avoidance of the yellow bait) in the absence of the conditioned stimulus (in this case the bitrex) (Pearce, 1997).

This type of memory test was done because no studies have yet shown how long wild birds remember learned avoidance and it was necessary to be able to test the same individual multiple times after different retention intervals. If a traditional extinction trial had been conducted the birds may have formed an association between the yellow baits and palatability, which would have eroded their learned avoidance, and affected subsequent memory tests.

1.9 Data analysis

Most of the data were non-normal and not transformable by any standard method, therefore non-parametric statistics were used for the analysis (Zar, 2005). Where tests were carried out across trials, so the test followed a repeated measures design, the Friedman test was used for multiple comparisons, and the Wilcoxon signed ranks test for paired comparisons. Where tests compared the responses of different birds between treatments, a Mann Whitney U-test was performed. The only exception to this was when the mean number of yellow baits eaten by robins in each treatment group was compared, when a t-test was used since these data were normally distributed.

2 Results

There were no significant differences between the number of yellow baits attacked across the seven learning trials in either the odorless (Friedman test, \( \chi^2 = 9.713, df = 6, P = 0.137 \); Fig. 2) or pyrazine treatments (Friedman test, \( \chi^2 = 9.405, df = 6, P = 0.152 \); Fig. 2). In a comparison of the first and last learning trials, the robins in the odorless treatment did not learn to avoid the yellow baits (Wilcoxon signed ranks test, \( Z = \text{-}1.377, n = 7, P = 0.047 \)).

When corrected for multiple testing this difference is also not statistically significant. Despite this lack of significance, the apparent trend in Fig 3, together with the consistently lower consumption of yellow in the presence of pyrazine odor seen in Fig. 2, suggests some effect of the pyrazine odor in increasing the robins’ avoidance of the yellow baits.

In the first of the memory tests at 96 hours after their previous encounter with the unpalatable yellow baits (Fig. 4), the birds in the odorless treatment ate significantly fewer yellow baits than they had in the first learning trial (Wilcoxon signed ranks test, \( Z = \text{-}2.251, n = 7, P = 0.024 \)). The birds in the pyrazine treatment did not avoid the yellow baits at all in their 96 hour memory trial (Wilcoxon signed ranks test, \( Z = 0.957, n = 9, P = 0.339 \)). This difference in the results between the treatments might suggest that avoidance learning had occurred in both treatments but that it took longer to take effect in the odorless treatment. Alternatively, it could suggest that the presence of pyrazine made forgetting of the avoidance proceed more quickly.
The consumption of green baits varied between trials, with a decrease in the number of green baits attacked across the seven learning trials by robins in the odorless treatment (Friedman test, \( \chi^2 = 12.939, df = 6, P = 0.044; \) Fig. 2) but no such difference was found in the pyrazine treatment (Friedman test, \( \chi^2 = 3.132, df = 6, P = 0.792; \) Fig. 2). There was, however, no significant difference between the number of green baits attacked during the first and last learning trial by birds in either the odorless (Wilcoxon signed ranks test, \( Z = -1.342, n = 7, P = 0.180 \)) or pyrazine (Wilcoxon signed ranks test, \( Z = -1.00, n = 9, P = 0.317 \)) treatments.

In the first trial, the robins attacked the green baits to the same degree, whether they were accompanied by pyrazine odor or not (Mann-Whitney U test, \( U = 26.00, n = 7, 9, P = 0.503 \)). This suggests that the robins’ innate response towards green food was not affected by the presence or absence of the pyrazine odor associated with yellow baits. From the second learning trial onwards there was an apparently greater consumption of green baits in the pyrazine treatment than in the odorless treatment (Fig. 2), but this fails to reach significance in any one trial. The total number of green baits eaten across all learning trials also did not differ between the odorless and pyrazine treatments (Mann-Whitney U test, \( U = 27, n = 7, 9, P = 0.611 \)).

There were no significant differences in the number of yellow and green baits attacked by robins in the odorless treatment until trial 4 at which point the birds attacked significantly more yellow than green baits (Wilcoxon signed ranks test, \( Z = -2.264, n = 7, P = 0.024; \) Fig. 2). This difference was also present in trial 6 (Wilcoxon signed ranks test, \( Z = -2.214, n = 7, P = 0.027 \) and after a week of retention interval (Wilcoxon signed ranks test, \( Z = -2.226, n = 7, P = 0.026 \)). This suggests that the birds in the odorless treatment were more willing to attack the yellow than the green baits even though the yellow baits were unpalatable.
Fig. 4 The mean number (± SE) of yellow baits attacked in the first and last learning trials, and the 96-hour, 1 week and 1 month memory trials.

Robins in the pyrazine treatment attacked the same number of green and yellow baits throughout the learning and memory trials (Fig. 2). This suggests that in the presence of the pyrazine odor the birds did not differentiate between the two colors despite the yellow being unpalatable and the green palatable.

3 Discussion

The results from this experiment are unexpected, given the avoidance learning shown by chicks in the laboratory (Siddall and Marples, 2008) when offered food dyed with the same yellow dyes, and coated with the same aversant in concentrations previously found to be aversive to robins (Marples unpublished data). The wild robins in this study showed learned avoidance of the yellow baits far less clearly than chicks did, highlighting the importance of repeating laboratory studies with wild birds.

There was, however, some indication that pyrazine odor increased avoidance of the yellow baits. There was some evidence of both a reduction in the total number of yellow baits attacked by the robins and a reduction in consumption of yellow baits during the course of the seven learning trials in the pyrazine treatment which was not evident in the odorless treatment. The only evidence that the birds in the odorless treatment had any learned avoidance occurred after the learning trials, in the first of the memory trials. This suggests that the robins learned their avoidance more slowly in the absence of pyrazine, although perhaps forget that learning more readily.

A second explanation for the results is also possible. It may be that the robins did not acquire a learned avoidance across the seven learning trials, but that the presence of pyrazine caused a revealed innate aversion to yellow, as found in previous studies using chicks as predators (Rowe and Guilford, 1996; Jetz et al., 2000). To argue this we must assume that the robins on the odorless treatment reduced their consumption of yellow in the first memory trial for other reasons.

If birds’ responses to insects reflect those shown towards the pastry baits, then a toxic insect population that advertised its defended state using both a yellow visual display and pyrazine odor may be better protected than insects that used the yellow visual signal alone. When the yellow baits were odorless the robins attacked more yellow than green baits overall, and significantly reduced their consumption of green baits across the learning trials despite the fact that the yellow baits were unpalatable and the green baits fully palatable. There was no suggestion that the birds in the pyrazine treatment avoided green in favour of yellow baits. This result is somewhat surprising, suggesting that the pyrazine odor may have enhanced the birds’ perception that the yellow baits were unpalatable. Rothschild and Moore (1987) suggested that pyrazine may act as an ‘alerting stimulus’, causing the predator to pay more attention to their predatory decisions. The results of the present study do, to some extent, support this theory. There was no evidence of an effect of pyrazine on the innate re-
spontaneous of the birds to green baits in the first learning trial, as one might expect there to be if pyrazine alerts the receiver to the prey, but as the birds were likely to consider green items as potentially palatable, there may have been little opportunity for the pyrazine to increase their acceptance of these baits.

The differences between these robin data and the chick data (Siddall and Marples, 2008) may be due to several factors. The aversive substance, bitrex, may not have been strong enough to cause a robust learned avoidance in wild birds, which will have experienced food with a range of palatabilities. Chicks would have encountered only palatable foods and unpalatable non-foods, so would be expected to be more extreme in their rejection of unpalatable items. The low levels of learning in the present experiment would preclude the detection of any effect pyrazine might have on the memorability of the signal.

Given the many reasons one might expect differences between a bird foraging in the wild and a chick foraging in the laboratory, the results from the wild birds do support the findings of Siddall and Marples (2008) using laboratory chicks that pyrazine odor enhances avoidance of unpalatable yellow prey. The present study is the first demonstration of such behaviour by wild birds, and adds weight to the argument that a toxic insect that advertises its defended state using both a yellow signal and pyrazine odor might well be better protected than an insect using a yellow visual signal alone.

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