

# Birds learn to use distastefulness as a signal of toxicity

John Skelhorn<sup>\*,†</sup> and Candy Rowe

Centre for Behaviour and Evolution, Institute of Neuroscience, Newcastle University, Henry Wellcome Building, Framlington Place, Newcastle NE2 4HH, UK

Aposematic prey advertise their toxicity using conspicuous visual signals that predators quickly learn to avoid. However, it is advantageous for predators not to simply avoid toxic prey, but to learn about the amount of toxin that prey contain, and include them in their diets when the nutritional gains are high relative to the costs of ingesting the toxin. Therefore, when foraging on a defended prey population where individuals vary in their toxin concentration, predators should learn to use cues which distinguish prey with different levels of toxicity in order to include less defended individuals in their diets. In this experiment, we found that European starlings (*Sturnus vulgaris*) could learn to use a bitter taste to predict the amount of toxin that individual prey contained, and use that information to preferentially ingest less toxic prey to maximize their nutrient intake relative to the amount of toxin ingested. Our results suggest that bitter tastes could evolve as reliable signals of toxicity, and can help to explain why many toxins taste bitter. They also highlight the need to develop new mathematical simulations of the evolution of prey defences which incorporate the adaptive decision-making processes underlying nutrient and toxin management.

**Keywords:** denatonium; quinine; toxin regulation; avian; aposematism; mimicry

## 1. INTRODUCTION

Many animals possess defensive toxins, which are advertised to predators using conspicuous coloration and other warning signals, such as sounds and odours (Cott 1940; Rothschild & Haskell 1966; Edmunds 1974). This defensive strategy is known as aposematism and is widespread across the animal kingdom (Poulton 1890; Cott 1940; Mappes *et al.* 2005). The predominant view is that aposematism is an effective defence strategy because naive predators can rapidly learn to associate warning signals with the noxious effects of the toxins that they signal, and reduce their attack rates on them accordingly (Gittleman & Harvey 1980; Roper & Redston 1987; Alatalo & Mappes 1996). Indeed, for more than 100 years, the theoretical framework for aposematism and mimicry (where sympatric species share the same warning pattern) has been developed based upon the speed and durability of avoidance learning in naive predators (Müller 1879; Fisher 1930; Guilford 1990; Speed 1993a, 1999).

However, an alternative view is that predators should not simply learn to avoid toxic prey, since this would reduce their opportunities for gaining nutrients. Aposematic prey contain valuable nutrients as well as toxins, and could potentially be profitable sources of food (Sherratt *et al.* 2004; Barnett *et al.* 2007; Skelhorn & Rowe 2007). Therefore, predators should learn about the amount of toxin that aposematic prey contain in order that they can include them in their diets when it is

profitable to do so (Skelhorn & Rowe 2007). Perhaps surprisingly, little is known about what animals learn about toxic prey, or how they trade-off the benefits of eating nutrients with the costs of eating of toxins (e.g. Yearsley *et al.* 2006; Torregrossa & Dearing 2009). However, given that predators are often long-lived and are naive for a relatively short period of their lives, it is essential to know how educated animals learn to regulate their intake of toxic prey in order to fully understand the selection pressures acting on both prey defences and warning signals.

In an aposematic prey population, individuals often vary in the amount of toxin that they contain, with some possessing higher toxin concentrations than others (e.g. Brower *et al.* 1967; de Jong *et al.* 1991). Although it is impossible to say what proportion of insect defence chemicals are unpalatable, many toxins commonly used by defended insects taste bitter (Eisner & Meinwald 1966; Blum 1981; Pasteels *et al.* 1983), which can lead to them being rejected by predators (Järvi *et al.* 1981; Wiklund & Järvi 1982; Brower & Calvert 1985; Sillén-Tullberg 1985; Gamberale-Stille & Guilford 2004; Skelhorn & Rowe 2006a,b; Halpin *et al.* 2008a,b). However, since the distastefulness of bitter-tasting toxins often increases with toxin concentration (Schafer *et al.* 1983), the bitter taste of toxic prey might also allow predators to learn about the toxins contained in prey and preferentially reject individuals with higher quantities of toxin. Because predators appear to have a limit to the amount of toxin that they are willing to ingest (Barnett *et al.* 2007; Skelhorn & Rowe 2007), it would be advantageous for them to be able to discriminate between individuals that vary in their toxin concentration and preferentially ingest the less defended individuals. This would enable them to gain more nutrients from a defended prey population for a given amount of toxin ingested.

\* Author for correspondence (j.skelhorn@bio.gla.ac.uk).

† Present address: Division of Ecology and Evolutionary Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK.

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Our experiment provides a crucial test of whether distastefulness can be used by predators as a signal of prey toxicity, and whether they can learn to use taste cues to strategically manage their intake of toxic prey. Using an established protocol of European starlings (*Sturnus vulgaris*) foraging on insect larvae (Skelhorn & Rowe 2006c, 2007, 2009; Barnett *et al.* 2007), we can independently manipulate the toxin concentration and the level of distastefulness of prey. Defended prey can be injected with different concentrations of quinine solution, a toxin that can cause an emetic response in humans and birds at high concentrations (Alcock 1970; Bateman & Dyson 1986), but at low concentrations is used widely as an aversant in learning experiments across a range of taxa (e.g. Schoenbaum *et al.* 1998; Darmaillacq *et al.* 2004). Although quinine is distasteful, starlings cannot taste it when it is injected into mealworms because they swallow the mealworms whole (Skelhorn & Rowe 2006c, 2007). However, starlings can detect the noxious effects of quinine post-ingestion, and can readily learn to associate the effects of quinine with reliable colour cues, and reduce their intake of quinine-injected mealworms (Skelhorn & Rowe 2006c, 2007; Barnett *et al.* 2007). Distastefulness can be manipulated independently by coating prey with Bitrex, a bitter-tasting substance that starlings find unpleasant, but is not toxic (Skelhorn & Rowe 2009). Therefore, for the first time to our knowledge, we are able to disassociate the toxicity and distastefulness of prey in a realistic foraging setting. Our experiment tests whether predators learn to use distastefulness to discriminate between prey that vary in their toxin concentrations, and whether they use this information to optimize their intake of toxic prey.

## 2. MATERIAL AND METHODS

### (a) *Subjects and housing*

Ten adult European starlings (five males and five females) were caught in Northumberland and held under an English Nature licence (no. 20062404). Birds were housed individually in wire cages measuring 45 × 75 × 45 cm, with a drawer at the bottom that could be pulled in and out. They were subject to a 10 L:14 D cycle using fluorescent lights, and temperatures were maintained at 16–17°C (see Skelhorn & Rowe 2006c for further details). Water was provided ad libitum, as were Zoofood Pheasant breeder pellets and fruit except during training and experimenting when short periods of food deprivation were necessary. At the end of the experiment, birds were returned to a free-flight aviary before being released back into the wild.

### (b) *Preparation of artificial prey*

The prey were mealworms (*Tenebrio molitor*) selected to be of similar length (approx. 20 mm). We manipulated the toxin concentration of prey by injecting different concentrations of quinine sulphate solution through the mouthparts of a mealworm using a hypodermic needle. We produced mildly defended prey by injecting mealworms with 0.02 ml of 1 per cent quinine sulphate solution, and moderately defended prey by injecting mealworms with 0.02 ml of 3 per cent quinine sulphate solution. Our previous studies find no evidence that birds taste bitter chemicals when they are injected into mealworms using this method (Skelhorn & Rowe 2006c, 2009).

In order to manipulate the taste of the defended prey independently of their toxin concentration, we coated them

with 0.02 ml of one of two Bitrex solutions to create mildly distasteful prey (two drops of 2% Bitrex solution diluted to 100 ml with water) or moderately distasteful prey (eight drops of 2% Bitrex solution diluted to 100 ml with water). Starlings do not reduce their consumption of mealworms coated with these solutions, and it has no measurable toxic effects (Skelhorn & Rowe 2009).

We also gave the birds undefended mealworms in our experimental trials, which were injected and coated with 0.02 ml of water, and were entirely palatable.

### (c) *General procedure*

The experiment had four distinct and consecutive phases: training (2 days); learning (5 days); toxin manipulation (2 days); and, simultaneous choices (2 days; see the electronic supplementary material). In all phases, every subject was given a single trial on each day. Birds were food deprived for 2 h before being moved in their cages behind a white curtain erected in the same room. The bottom of the curtain was level with the drawer in the base of the cage, this visually isolated subjects from both the experimenter and conspecifics, and allowed the experimenter to present prey to the birds without being seen. Birds' behaviour was monitored using video cameras connected to television screens. Each bird had 5 min to acclimatize behind the curtain before the start of a trial.

### (d) *Training trials*

In this phase, birds were trained to eat sequentially presented mealworms (which had not been experimentally manipulated). Birds received two trials of 18 prey presentations, in which the drawer of the cage was pulled out, a Petri dish containing a mealworm was placed in the drawer, and the drawer replaced. Subjects were given 1 min to attack the mealworm before the drawer was pulled out and the Petri dish and the mealworm were removed. If the mealworm was attacked and eaten, the Petri dish was removed immediately. Mealworms were presented every 3 min. In the second trial, all subjects readily attacked the prey and ate all 18 mealworms presented.

### (e) *Learning trials*

All birds were given five learning trials in which they received a sequence of six undefended prey and 12 defended prey (six mildly defended and six moderately defended mealworms). Undefended and defended prey were made visually discriminable by placing purple and green paper discs under the Petri dishes, but mildly and moderately defended prey were visually identical (the colour which signalled defended mealworms was balanced across subjects). Unique prey sequences were produced for each trial for each bird, where prey type was randomized across trial in order that birds could not use temporal cues to determine the palatability of prey.

Before the start of these trials, birds were assigned to one of two experimental groups: the reliable group (three males and two females) and the unreliable group (two males and three females). These two groups differed in whether or not Bitrex reliably predicted the concentration of quinine in the defended prey. Therefore, birds in the reliable group were given six mealworms that were moderately toxic and moderately distasteful, and six mealworms that were mildly toxic and mildly distasteful. However, birds in the unreliable group received six moderately toxic mealworms where half were moderately distasteful and half were mildly distasteful,

and six mildly toxic prey where half the mealworms were moderately distasteful and the other half were mildly distasteful. Therefore, the level of distastefulness in the unreliable group could not be used to predict the concentration of toxin in the mealworms. The unreliable group acted as a control to ensure that any discrimination between mildly and moderately defended prey by birds in the reliable group was owing to differences in toxin concentration rather than the level of distastefulness. We recorded the numbers of each prey type attacked and eaten during each training trial.

#### (f) Toxin manipulation trials

At the end of the training trials, subjects received two toxin manipulation trials on consecutive days. These trials followed the same protocol as the training trials, however, prior to the start of each trial, birds received three additional prey presentations. The additional presentations took the same form as experimental presentations (birds had 1 min to eat each prey item, and prey items were presented every 3 min), and the third additional presentation was made 3 min before the start of the experimental trial. In these presentations, we manipulated the birds' toxin burden, by giving them three mealworms that either contained 0.02 ml of water or 0.02 ml of 3 per cent quinine sulphate solution. When quinine is presented in this way, birds are known to detect its noxious effects (but not its taste) during the experimental trial (Skelhorn & Rowe 2007). These trials tested how birds used what they had learned about the prey to manage their intake of quinine when their toxin burden was raised. Mealworms used in these three pre-feeding presentations did not have any colour or taste associated with them. Three birds in each group received the water-injected mealworms on the first day and quinine-injected mealworms on the second day, while for the other birds the order of the trials was reversed. We recorded the numbers of each prey type attacked and eaten during each trial. We then performed a series of simultaneous choice trials to determine what cues birds were using to make their foraging decisions (see the electronic supplementary material).

### 3. RESULTS

#### (a) Learning trials

Birds in both groups readily learned to discriminate between quinine-injected and water-injected mealworms. All the birds invariably attacked and ate all the water-injected mealworms in every daily trial, but as expected, learned to reduce the total number of quinine-injected mealworms ingested in each trial over the five days, and ate fewer defended prey in trial 5 compared with trial 1 (Wilcoxon test: reliable group  $Z = 2.04$ ,  $p = 0.041$ ,  $n = 5$ ; unreliable group  $Z = 2.04$ ,  $p = 0.041$ ,  $n = 5$ ; figure 1).

There was no difference in the total amount of quinine ingested by each group during the learning trials (reliable group, mean =  $20.5 \pm 0.33$  mg; unreliable group, mean =  $19.1 \pm 0.67$  mg; Mann-Whitney test:  $U = 5.5$ ,  $p = 0.142$ ,  $n = 10$ ), indicating that birds were defending a specific toxin burden (see also Skelhorn & Rowe 2007). However, birds in the reliable group ate significantly more defended prey than birds in the unreliable group (Mann-Whitney test:  $U = 5$ ,  $p = 0.012$ ,  $n = 10$ ; figure 1). This can be explained by examining the birds' decision-making behaviour in more detail, and comparing the numbers of moderately and mildly toxic prey that

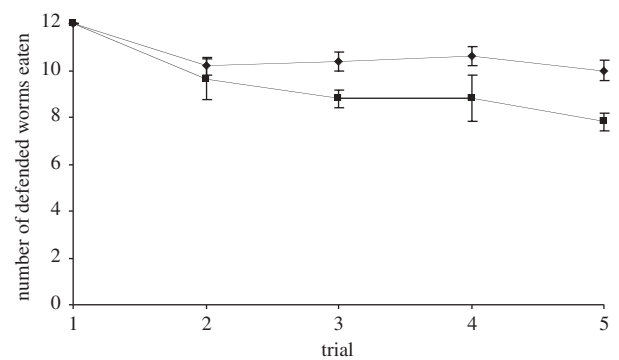


Figure 1. The mean number ( $\pm$ s.e.m.) of defended mealworms eaten in each of the five training trials (filled squares, unreliable; filled diamonds, reliable).

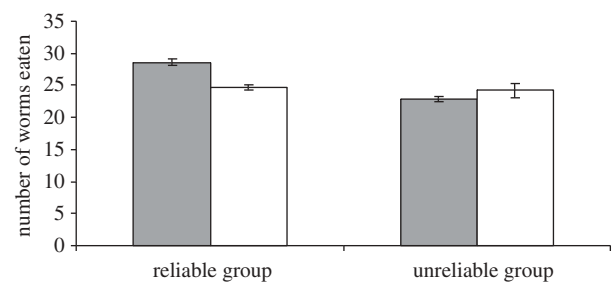


Figure 2. The mean number ( $\pm$ s.e.m.) of mildly defended mealworms (filled bars) and moderately defended mealworms (open bars) eaten across the five training trials for birds in each experimental group.

were eaten. Birds in the reliable group attacked all 12 of the quinine-injected mealworms that were presented, and used the taste of Bitrex to preferentially ingest more of the mildly toxic than the moderately toxic prey (Wilcoxon test:  $Z = 2.06$ ,  $p = 0.039$ ,  $n = 5$ ; figure 2). By contrast, birds in the unreliable group were unable to use taste to discriminate among the defended prey (they did not taste-reject prey, and ate equal numbers of mildly and moderately distasteful prey (Wilcoxon test:  $Z = 0.272$ ,  $p = 0.785$ ,  $n = 5$ )), and consequently ate equal numbers of moderately toxic and mildly toxic mealworms (Wilcoxon test:  $Z = 1.22$ ,  $p = 0.223$ ,  $n = 5$ ; figure 2). Therefore, reliable taste cues enabled birds to eat more of the defended prey and maximize their nutritional intake for the same amount of toxin ingested.

#### (b) Toxin manipulation trials

Birds in both groups ate significantly fewer defended mealworms in the trial when they had been pre-fed with quinine compared with when they had been pre-fed with water (Wilcoxon test: reliable group  $Z = 2.041$ ,  $p = 0.041$ ,  $n = 5$ ; unreliable group  $Z = 2.06$ ,  $p = 0.039$ ,  $n = 5$ ; figure 3), although birds in the unreliable group reduced their consumption more than those in the reliable group (Mann-Whitney test:  $Z = 2.47$ ,  $p = 0.013$ ,  $n = 10$ ; figure 3). This difference can again be explained by examining their consumption of mildly and moderately toxic prey. Birds in the reliable group reduced their consumption of moderately toxic mealworms more than their consumption of mildly toxic mealworms when pre-fed

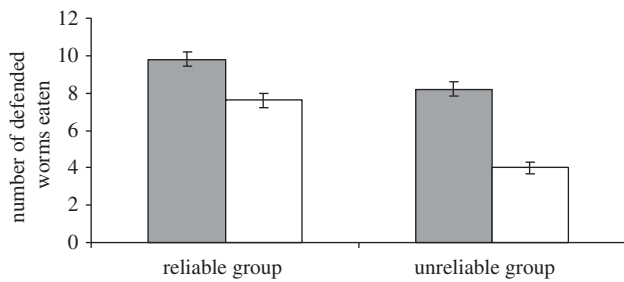


Figure 3. The mean number ( $\pm$ s.e.m.) of defended mealworms eaten after being pre-fed with water-injected worms (filled bars) and after being pre-fed with quinine-injected worms (open bars) by birds in each experimental group.

with quinine (Wilcoxon test:  $Z = 2.070$ ,  $p = 0.038$ ,  $n = 5$ ; figure 4). Therefore, they had not only learned to use taste cues to predict preys' toxin concentration, but they could use taste to reduce their ingestion of the moderately toxic prey relative to the mildly toxic prey when their toxin burden increased. However, birds in the unreliable group reduced their ingestion of both moderately and mildly toxic worms equally (Wilcoxon test:  $Z = 1.134$ ,  $p = 0.257$ ,  $n = 5$ ). As in the learning phase, no bird in this group showed any taste-rejection behaviour, and there was no preference for mildly distasteful prey (Wilcoxon test:  $Z = 1.142$ ,  $p = 0.180$ ,  $n = 5$ ). The results of our simultaneous choice trials show that all birds had learned to associate preys' coloration with their defences, and that in the absence of colour cues, birds could not detect chemical cues prior to attack (see the electronic supplementary material).

#### 4. DISCUSSION

This study, to our knowledge, provides the first evidence that predators learn to associate variation in a bitter taste with different levels of toxin in order to both manage their toxin burdens, and optimize their nutrient intake. We found that when taste was a reliable predictor of toxicity, birds learned about the toxin concentrations of their prey, and used taste to selectively-reject moderately toxic prey. This allowed birds given prey with reliable taste cues to consume more toxic prey items than birds given unreliable taste cues, while maintaining the same toxin burden. Consequently, the presence of reliable bitter taste cues allowed birds to obtain more nutrients from the defended prey population.

Previous studies of taste-rejection behaviour in birds have implicitly assumed that the rejection behaviour is instinctive or that the bitter taste itself is a feeding deterrent (e.g. Skelhorn & Rowe 2006a; Halpin *et al.* 2008a,b). However, by manipulating the association between bitter taste and toxicity, we are able to show instead that the taste-rejection of distasteful prey depends upon a learned association between taste and toxicity. Indeed, we found no evidence that the starlings rejected prey on the basis of taste unless it was a reliable indicator of toxicity. Furthermore, our data clearly show that birds in the reliable group learned the relative toxicity of mildly and moderately distasteful prey, since when their toxin burdens were artificially increased they reduced their

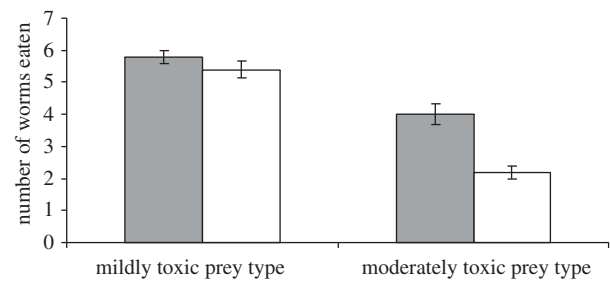


Figure 4. The mean number ( $\pm$ s.e.m.) of mildly defended mealworms and moderately defended mealworms eaten by birds in the reliable group after being pre-fed with water-injected worms (filled bars) and after being pre-fed with quinine-injected worms (open bars).

consumption of moderately defended prey more than mildly defended prey.

It is well established that animals use taste to detect toxins in the foods that they eat, and that they can learn to associate pre-ingestive cues (e.g. the colour, odour or taste) with the ill effects of toxins in order to reduce their intake of toxic foods (e.g. Darmaillacq *et al.* 2004; Glendinning 2007). Indeed, conditioned taste aversion (CTA), where animals learn to associate a taste with the post-ingestive effects of a toxin, is perhaps one of the most well-studied learning paradigms (e.g. Bernstein 1999 for review). CTA studies primarily focus on the acquisition of the aversion by investigating the specific cues which are associated with toxicity or the speed or strength of the learned avoidance. By contrast, our approach demonstrates that birds use learned information about toxins in food in a naturalistic foraging task, and shows that what birds learn about toxic food affects the foraging strategies they employ to regulate their nutritional intake. What is clear from our data is that animals do not just learn to reduce their consumption of toxic food, but rather learn to select foods to maximize their nutrient intake relative to the amount of toxin that they are willing to ingest. Although long-term data suggest that free-ranging herbivores are able to select plants that are relatively high in nutrients and low in toxins (Moore & Foley 2005), this is, to our knowledge, the first demonstration of the way that a reliable gustatory signal could mediate this foraging strategy.

Knowing how predators learn to use distasteful cues to forage on toxic prey potentially changes the way in which we think about and study the evolution of prey defences and warning signals. First, our study may help to explain why many defence chemicals are often bitter tasting (Eisner & Meinwald 1966; Blum 1981; Pasteels *et al.* 1983). Reliable taste signals allow predators to selectively reject individuals that have invested more heavily in toxins, which would increase their chance of surviving predatory attacks, and benefit more from their investment in costly toxins. Conversely, individuals that invested less in toxins would be less likely to survive attack. Clearly it would benefit less defended individuals to dishonestly signal their level of toxicity. However, if predators have evolved bitter taste receptors in order to detect toxins, their bitter taste will provide inherently reliable information about the toxin concentration of the individuals that possess them. This will promote investment in

chemical defences, which has been a major challenge to evolutionary theories of aposematism (Brower *et al.* 1967; Guilford 1994), and make distasteful toxins more evolutionarily stable than those that cannot be detected upon attack.

We are therefore left with the question: if distasteful toxins can evolve as reliable signals of toxicity, why have warning signals in other sensory modalities evolved (Rowe 1999; Rowe & Guilford 1999)? In contrast to distastefulness, warning signals in other sensory modalities, such as colours, odours, sounds and behavioural displays can all be perceived by predators prior to attack. Therefore these signals can provide information to predators that could reduce either the probability of an attack, or the probability of being damaged through more cautious handling by the predator (Sillén-Tullberg 1985; Rowe & Guilford 1999). Intriguingly, our experiment suggests that birds use colour signals if they provide the best information about toxin ingestion (i.e. if there are no reliable taste signals), or if it allows them to preferentially select more palatable prey. In addition, we might also expect that colour signals reduce the attack probability once a predator's maximum toxin burden is reached, since the predator no longer needs to discriminate between prey with different toxin concentrations. Selection on aposematic coloration is therefore likely to depend upon the variability in prey toxin concentrations and the amount of toxin the predator is willing to ingest, and not just the predator's ability to associate the coloration with the toxin. While there have been a few recent studies of the role of toxin regulation in the evolution of prey defences (Sherratt 2003; Sherratt *et al.* 2004; Barnett *et al.* 2007; Skelhorn & Rowe 2007), little is known about the cognitive and physiological processes underlying toxin regulation and decision-making when foraging on toxic prey. Therefore, this finding opens a novel avenue for understanding the evolutionary dynamics of warning coloration and prey defences more generally.

Finally, our findings demonstrate an important role for 'educated predators' in the evolution of prey defences. The theoretical foundations of aposematism and mimicry rest predominantly upon learning algorithms (Müller 1879; Fisher 1930; Guilford 1990; Speed 1993a, 1999), which are tested empirically by measuring the acquisition behaviour of naive predators (e.g. Gittleman & Harvey 1980; Alatalo & Mappes 1996; Rowe *et al.* 2004; Rowland *et al.* 2007). However, predators will not always be naive, and are likely to spend more of their lives as educated rather than naive predators. Educated predators will have learned to use different signals to predict prey toxicity, and use this knowledge in their foraging decisions, which will generate different selection pressures on warning signals. However, if we can understand this and integrate it with what we know about avoidance behaviour in naive predators, we might start to fully understand the variety and complexity of prey defences.

All deprivation periods and experimental procedures were in accordance with Home Office regulations and ASAB ethical guidelines.

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