Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

doi:10.1093/beheco/art013
Advance Access publication 20 March 2013

Original Article

Bitter taste enhances predatory biases against aggregations of prey with warning coloration

Hannah M. Rowland,[a] Graeme D. Ruxton,[b] and John Skelhorn[c]
[a]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK, [b]School of Biology, Dyers Brae, University of St Andrews, St Andrews, Fife KY16 9TH, UK, and [c]Centre for Research in Animal Behaviour, College of Life and Environmental Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter EX4 4QG, UK

Aposematic prey that possess chemical defenses advertise these to potential predators using conspicuous warning coloration. Aposematism is often associated with group living, which is hypothesized to enhance the protection of these species. Predators exhibit unlearned biases against foods with warning coloration, and the presentation of a novel sound or bitter-tasting toxin augments these biases. Whether these nonvisual signal components also cause naive predators to more strongly avoid aggregated prey, and whether biases against aggregations are restricted to situations where aggregated prey possess visual signals typically associated with aposematism, is unknown. We conducted an experiment in which naive domestic chicks (*Gallus gallus domesticus*) acted as predators and used artificially colored pastry prey. The experiment had a 2×2 design in which naive birds were offered a drop of either water or bitter-tasting chloroquine solution before being given the choice between solitary and aggregated prey that were either both red, a typical aposematic color, or both green (usually associated with crypsis and palatability). We found that birds were warier of red-aggregated prey and attacked significantly more solitary prey before aggregated prey compared with green. After sampling bitter-tasting chloroquine solution, the birds showed a bias in their attack decisions, attacking significantly fewer aggregated prey in total compared with those who had sampled water, but only when prey were red. Thus, exposure to a bitter-tasting toxin affected predatory preferences. We discuss our findings in relation to the mechanisms of bias, the benefits of group living, and the evolution of warning coloration and aggregation.

Key words: **aggregation, aposematism, bitter taste, chemical defense, domestic chick, evolution, *Gallus gallus domesticus*, innate bias, warning coloration.** *[Behav Ecol]*

INTRODUCTION

Aposematism, the combination of a repellent physical or chemical defense, such as a toxin, with conspicuous coloration (Poulton 1890; Cott 1940), is taxonomically and geographically widespread (including birds, Dumbacher et al. 1993; marine animals, Edmunds 1991; insects, Schmidt 2008; and amphibia, Summers and Clough 2001). Aposematism has been particularly well studied in the insects: the Monarch butterfly (*Danaus plexippus* L.) sequesters toxic cardenolides from its host plant and signals its chemical defense using a highly conspicuous orange-and-black pattern (Brower et al. 1968); the seven-spot ladybird (*Coccinella septempunctata*) synthesizes the toxin coccinelline and advertises using black spots on a red background (Marples et al. 1989); and the yellow-and-black stripes of the common wasp (*Vespula vulgaris*) are an indicator of its venomous sting (Schmidt 2008). These species have in common a tendency to live, migrate, or hibernate in groups, which is hypothesized to enhance their conspicuousness and the power of their advertisements (Sillén-Tullberg and Leimar 1988).

Understanding how prey have evolved aposematic coloration and aggregative behavior that increases the likelihood of detection by predators (Ruxton and Sherratt 2006) is an intriguing question for evolutionary biologists, because attacks are likely to be costly, even if prey possess defenses that increase the likelihood of survival (Higginson et al. 2011). The most widely accepted answer to this question is that naive predators are instinctively averse to the colors and patterns most commonly associated with toxicity (Schuler and Hesse 1985; Sillén-Tullberg 1985; Roper and Cook 1989; Schuler and Roper 1992; Mastrota and Mench 1995), and aggregation of aposematic prey generates unlearned aversions (Gamberale and Tullberg 1996a). Conspicuous coloration also facilitates faster avoidance learning by predators (Gittleman and Harvey 1980; Roper and Wistow 1986; Guilford 1992). Similarly, aggregation enhances the speed at which predators learn to avoid aposematic prey (Sillén-Tullberg and Leimar 1988; Sillén-Tullberg 1990; Gagliardo and Guilford 1993; Tullberg et al. 2000; Riipi et al. 2001).

Address correspondence to H.M. Rowland. E-mail: hr325@cam.ac.uk.
Received 4 July 2012; revised 30 January 2013; accepted 7 February 2013.

Aposematic prey rarely rely on visual signals only and make use of additional nonvisual signals of their unpalatability by using, for example, sounds (Haskell 1966; Brown et al. 2007), odors (Rothschild et al. 1984; Moore et al. 1990), and the secretion of bitter-tasting compounds (De Jong et al. 1991). These are thought to act as "go-slow" signals that cause predators to reduce their attack rates on prey that are more likely to be defended (Guilford 1994). In line with this theory, it has been found consistently that the presentation of a novel sound, odor, or bitter-tasting compound causes naive predators to increase their bias against novel foods or foods with visual traits typically associated with aposematism, such as conspicuousness or a red or yellow color (Marples and Roper 1996; Rowe and Guilford 1996, 1999a, 1999b; Jetz et al. 2001; Lindström et al. 2001; Rowe and Skelhorn 2005; Siddall and Marples 2008, 2011; Skelhorn et al. 2008). It is not currently known whether these additional signal components also cause naive predators to bias their foraging preferences against aggregated prey, and, if so, whether biases against aggregations are restricted to situations where aggregated prey possess visual signals typically associated with aposematism. To answer this question, we examined the foraging behavior of naive domestic chicks (*Gallus gallus domesticus*), which served as visually hunting predators. We produced 2 "species" of prey, which differed in color signal. Prey were either red (a color typically associated with insect warning patterns) or green (a color associated with palatable cryptic prey). We gave separate groups of predators a choice between aggregated and solitary prey that were either all red or all green. Chicks are known to use taste cues in conjunction with visual signals to make foraging decisions (Rowe and Skelhorn 2005; Skelhorn et al. 2008). We therefore used a well-established system of presenting a taste cue prior to a prey preference test to determine whether sampling a bitter-tasting toxin prior to meeting the prey caused birds to bias their attacks away from aggregated prey and toward solitary prey; and whether any bias to avoid aggregations was present both when prey were red and when they were green (see Rowe and Skelhorn 2005; Skelhorn et al. 2008; Skelhorn 2011). This simulates a real ecological scenario that can occur in many predator–prey interactions. Within a given prey species, both chemical defense and coloration commonly vary ontogenetically, with aposematism being more common in later life-history stages and crucially often occurring later in development than the expression of significant chemical defenses (e.g., panic moth caterpillars, *Saucrobotys futilalis*; Grant 2007), giving an ecologically commonplace pathway for predators to experience aversive tastes of cryptic prey before exposure to visual aposematic signals. Another example occurs in phase-changing desert locust (*Schistocera gregaria*), which can occur in 3 different defense conditions: at low densities, they exist in a solitary phase where individuals are palatable and have a cryptic green color (Sword et al. 2000; Despland and Simpson 2005); higher densities trigger changes in behavior in terms of attraction to conspecifics and host plant preferences leading to an aggregation stage where cryptic unpalatable animals also sequester bitter-tasting alkaloids (Despland and Simpson 2005); at the next molt after the density-driven behavioral changes, color change occurs from green to a conspicuous yellow-and-black appearance, giving an aggregation stage with aposematic coloration and chemical defense. Thus during a buildup of these insects over a season, predators could experience unpalatable tastes separately from, and before encountering, an aposematic visual signal.

We predicted that aggregation would increase the efficacy of red, but not green coloration and thus chicks in the red groups would be less willing to attack aggregated prey, and have a lower preference for aggregated prey, than chicks given green prey. Furthermore, we predicted that chloroquine would enhance biases against red, but not green-colored, aggregations. We, therefore, expected chicks in the chloroquine and red prey group to be less willing to attack aggregated prey, and express a lower preference for aggregated prey, than chicks in the water and red prey group, whereas we expected to see no differences between chicks given green prey.

METHODS

Subjects and housing

Fifty-two male chicks (Hyline strain) were obtained from a commercial hatchery on the day of hatching. Chicks were housed at the University of Glasgow in white metal cages measuring 120 cm × 50 cm × 50 cm. Two cages housed the experimental chicks (n = 20 per cage) and a third housed the buddy chicks (n = 12; buddy chicks serve as visual companions to the experimental chicks during the foraging experiment, thereby reducing any potential distress from placing experimental chicks in the arena alone. Buddy chicks never acted as experimental chicks and never had access to artificial prey). Each cage was heated to 27 °C, following guidelines to the operation of the Animal (Scientific Procedures) Act 1986 (UK Government 2009), using either 1 Interbrooda standard (40 cm × 60 cm) or 2 Interbrooda minis (40 cm × 40 cm) (http://www.alphahatch.com/interbrooda-mini-ah630450-104-p.asp). These brooders, also known as "electric hens," consist of an electrically heated square or rectangular plate, which stands on 4 adjustable legs, which enables the adjustment of height and temperature as the chicks grow. The laboratory was held at a constant temperature of 24 °C. Temperatures were monitored and recorded daily. Water was provided ad libitum in two 1 L jam-jar drinkers. Brown chick starter crumbs were also provided ad libitum in 2 ceramic food bowls that contained a clear plastic cylinder, which reduced the tendency of the chicks to sit in the food. The cages were lined with brown paper cage liners, which were replaced daily. During training and experimentation, periods of food deprivation were necessary to promote motivation to forage. During all periods of deprivation, chicks had access to water but not food. All deprivation periods were in accordance with UK Home Office regulations and guidelines and were no longer than 1.5 h.

Chicks were subject to a 14:10 h light:dark cycle and the lighting had no UV component. All subjects were marked with identifying color codes on the top of their heads using nontoxic Sharpie™ marker pens. Markings did not result in aggressive behavior between individuals. Weights were monitored for welfare purposes throughout the experiment, with all experimental chicks gaining as much weight as buddy chicks (who experienced fewer periods of food deprivation) as the experiment progressed. The experiment was conducted following guidelines to the operation of the Animal (Scientific Procedures) Act 1986 (UK Government 2009). The nature of the study meant that we did not require a Home Office license (chicks had free food choice, solutions were offered to the chicks, and deprivation periods less than 1.5 h). At the end of the experiment, all chicks were euthanized following UK Home Office "schedule one" methods (in this case, we employed cervical dislocation).

Artificial prey

Pastry was produced by mixing flour and lard in a 3:1 ratio, into which was mixed 75 mL of water with either 1 mL of green food

dye (Sugarflair™ spruce green) or 2 mL of red food dye (Dr. Oetker™). The pastry was molded into worms measuring 10 mm × 5 mm.

Pretraining

On arrival at the laboratory, chicks were allowed to acclimatize for 3 h, after which food was removed from all of the cages for 1 h. After 1 h of food deprivation, chicks commenced pretraining, which is used to familiarize them with the arena and to foraging alone. Without such training, chicks placed in the arena alone become distressed, they call loudly, and do not eat.

Pretraining was conducted in 3 experimental cages simultaneously. These cages were identical to the home cages, except that there was a mesh divider separating a buddy arena, measuring 20 cm × 50 cm × 50 cm, from an experimental arena of 100 cm × 50 cm × 50 cm (see Skelhorn and Rowe 2006 for a schematic). The floor was covered with the backing paper of sticky-backed plastic (a waxy paper imprinted with a faint red grid whose intersections were at 2.5 cm intervals). All chicks were given six 4-min pretraining sessions, during which they were presented with brown chick starter crumbs scattered on the floor of the experimental arena. In trials 1 and 2, chicks were placed in the experimental arena in groups of 3; in trials 3 and 4, chicks were placed in the arena in pairs; and in trials 5 and 6, lone chicks were placed in the arena. All training was completed in the presence of 2 buddies. By the end of pretraining, all chicks were eating brown starter crumbs from the arena without any signs of distress.

Preference test

Prey presentation

We used a prey presentation method previously employed by Gamberale and Tullberg (1996a) and Skelhorn and Ruxton (2006). We taped the upturned lid of a Petri dish (3 cm diameter) on top of the base of the dish to create a 2-tiered presentation device, so that chicks could only sample prey placed in the lid and not in the base. This permitted the creation of either solitary or visually aggregated prey while controlling olfactory cues and the number of prey that could be attacked. For solitary treatments, 1 pastry worm was presented in the lid of the dish with none in the base; for aggregated treatments, 1 pastry worm was presented in the lid and 7 pastry worms in the base. This allowed us to create 4 categories of prey: aposematic and solitary (1 red worm on top, none below); aposematic visually aggregated prey (1 red worm on top, 7 red below); nonaposematic solitary (1 green worm on top, none below); and nonaposematic visually aggregated (1 green worm on top, 7 green below).

Experimental procedure

On day 2, experimental chicks were food deprived (but had water ad libitum) for approximately 90 min prior to engaging in the task to promote motivation to forage. Buddy chicks had free access to food and water in their home cage, but only access to water during the task. Buddy chicks were used on a rotational basis and changed every 3 trials or after 1 h, whichever came first.

An experimental chick was chosen at random after the deprivation period and allocated to receive a drop of either 0.4% chloroquine phosphate solution (chloroquine group) or distilled water (control group) from a 20 to 100 mL micropipette. Previous work suggests that domestic chicks find this concentration of chloroquine phosphate solution aversive and that this method of tastant delivery has exactly the same effect as allowing predators to sample toxic prey prior to a choice test (Rowe and Skelhorn 2005). The benefit of using a solution over experience of a toxic prey is that the possibility of generalization of color signals of the toxic prey to the test prey is reduced to virtually zero.

Directly before being placed into the experimental cage, we offered each chick the allocated taste solution from the end of micropipette. If chicks did not drink this drop, then the solution was dropped on the tip of the beak, which they could shake and wipe off if they wanted. All chicks consumed some of the solution. The experimental chick was then immediately placed in the experimental arena. Two buddy chicks occupied the buddy arena of the same cage. Inside the experimental arena, the experimental chick encountered 24 Petri dishes (3 cm diameter), 12 of which contained solitary prey and the other 12, visually aggregated prey. For half of the chicks, the prey were all red; for the remaining chicks, the prey were all green.

Chicks were required to attack (peck or eat) 12 of the 24 available prey before being removed from the arena. All chicks attacked 12 prey items. The order of attacks was recorded.

Statistical analyses

We calculated the number of solitary prey attacked before the first aggregated prey as a measure of wariness in the chicks. These data were positively skewed and included zero counts. We, therefore, tested whether a generalized linear model (GLM) with a standard negative binomial regression model provided a better fit than a zero-inflated model using a Vuong test in R (UCLA: Academic Technology Services). The Vuong test showed that a zero-inflated model did not provide a significant improvement ($P = 0.144$), so we tested our predictions that 1) red coloration would increase wariness toward aggregations, so that chicks given red prey would attack significantly more solitary prey before attacking an aggregated prey than chicks given green prey; 2) chloroquine would enhance wariness against red-aggregated prey more than water; and 3) there would be no difference in wariness toward green-aggregated and green-solitary prey after experience of chloroquine or water. We tested this using a standard negative binomial regression model with the 2 predictor variables of color and solution type and the interaction between the two in R (version 2.14; R Development Core Team 2012).

We calculated the total number of aggregated prey attacked by the chicks as a measure of preference. The data satisfied the requirements for parametric statistics. With 3 degrees of freedom among our 4 experimental groups, we used orthogonal contrasts to test our a priori predictions (following Ruxton and Beauchamp 2008) within GLM Anova with the 2 predictor variables of color and solution type and the interaction between the two using R. By only testing the comparisons of interest, we simplify our analyses and reduce the risk of type I errors (Ruxton and Beauchamp 2008). We tested the predictions that 1) aggregation would increase the efficacy of the red signal, so that chicks in the red group would have a lower preference for aggregated prey than chicks in the green group (we compared water and red prey + chloroquine and red prey versus water and green prey + chloroquine and green prey); 2) chloroquine would enhance biases against red-aggregated prey (we compared water and red-aggregated prey versus chloroquine and red-aggregated prey); and 3) chloroquine would not enhance biases against green-aggregated prey (we compared water and green-aggregated prey versus chloroquine and green-aggregated prey).

RESULTS

There was a significant interaction between the color of the prey and the solution type offered to the chicks on the number of solitary prey attacked before an aggregated prey—from hereon termed "wariness" (likelihood ratio test: $\chi^2_1 = 40.71$, $P = 0.014$; Figure 1). Chicks given red prey attacked significantly more solitary prey before attacking an aggregated prey than chicks given green prey ($z = -2.031$, $P = 0.042$). However, chicks' wariness of aggregated prey did not differ as a function of solution type when prey were red ($z = 1.701$, $P = 0.089$). In line with our predictions, when prey were green, there was no significant difference in wariness of aggregated prey as a function of solution type ($z = -1.659$, $P = 0.097$).

As predicted, chicks given red prey attacked significantly fewer aggregated prey than chicks given green prey ($t = -7.368$, $P < 0.001$). Furthermore, when prey were red, chicks attacked significantly fewer aggregated prey after sampling chloroquine than after sampling water ($t = 2.558$, $P = 0.020$); when prey were green, there was no significant difference in the number of aggregated prey attacked as a function of solution type ($t = -0.624$, $P = 0.540$). This suggests that birds showed a bias against red aggregations, and chloroquine-enhanced biases against red, but not green aggregations. However, the interaction between the color of the prey and the solution type offered on the total number of aggregated prey attacked by the chicks was nonsignificant ($F_{1,40} = 2.831$, $P = 0.09$).

Avian predators have been shown to possess unlearned aversions to particular colors and patterns associated with warning signals. We measured whether chicks in each experimental group had preferences for solitary or aggregated prey by comparing the number of chicks in each group that attacked more aggregated than solitary prey with the number of chicks that attacked more solitary than aggregated prey and analyzed this data with a binomial test. We found that chicks given red prey showed a significant preference for solitary prey over aggregated prey both when given water (binomial test; $P = 0.002$, $n = 10$) and when given chloroquine (binomial test; $P = 0.002$, $n = 10$) prior to the preference test. However, birds given green prey showed no significant preference for either solitary or aggregated prey (binomial test; water group, $P = 0.754$, $n = 10$; chloroquine group, $P = 0.344$, $n = 10$).

DISCUSSION

The main finding from our experiment was that aggregation of prey was more effective at deterring predation when prey were a color typically associated with aposematism (red) than when they were green (usually associated with crypsis and palatability) and that experience of a bitter-tasting toxin caused naive predators to more strongly avoid red-aggregated prey but not green-aggregated prey. This is the first evidence that a nonvisual component of prey's defense (a bitter taste) causes biases against aggregations and that this is restricted to situations where aggregated prey possess visual signals typically associated with aposematism.

Our findings are consistent with those of Gamberale and Tullberg (1998), who demonstrated that the probability of naive domestic chicks attacking live larvae of the aposematic bug *Tropidothorax leucopterus* decreased with increasing prey group size, whereas the probability of chicks attacking larvae of the nonaposematic bug *Graptostethus servus* was unaffected by group size. Because we controlled for factors other than color (e.g., shape and movement) that may have differed between the 2 species used in Gamberale and Tullberg's experiment, our findings represent stronger support for the conclusion that patterns of preference were due to naive predators being disinclined to attack aggregations of prey with an aposematic visual trait but not prey that have a color

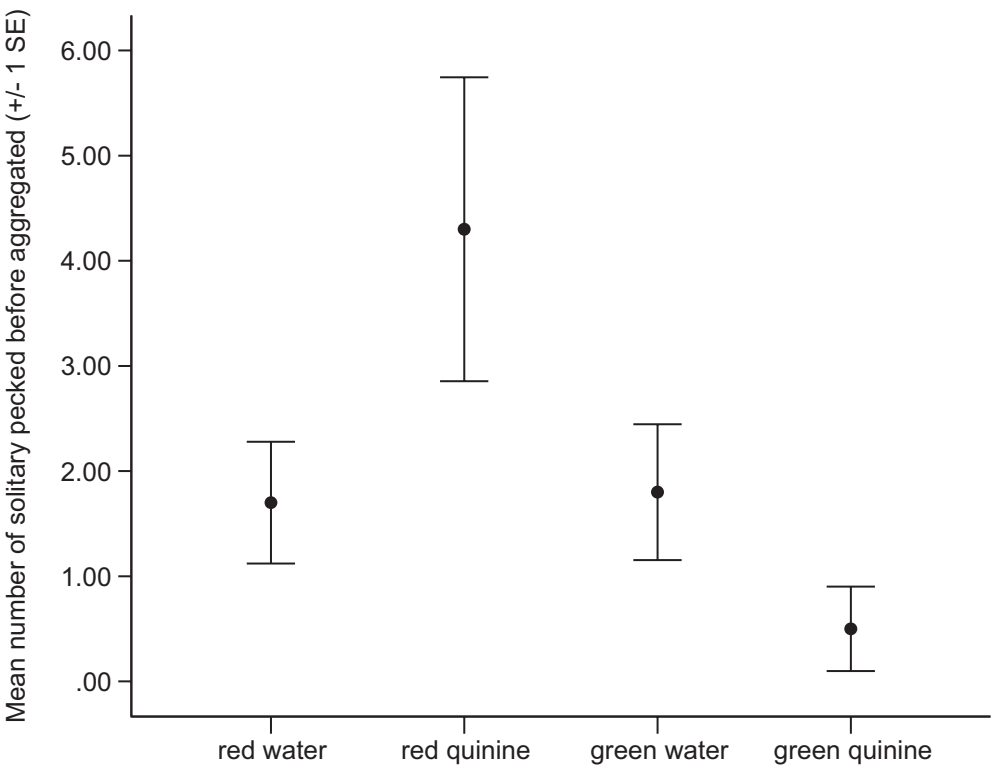

Figure 1
The mean number (±1 SE) of solitary prey attacked before aggregated prey by birds in each of our 4 experimental groups ($n = 10$ chicks for each group).

typically associated with palatability [first hypothesized by Poulton (1890) and subsequently by Beddard (1895), Cott (1940), and Edmunds (1974)].

It is clear that aggregation itself was not the important stimulus promoting avoidance because aggregated and solitary green prey were attacked at similar rates (see also Gamberale and Tullberg 1998). It is, therefore, reasonable to conclude that aggregation enhances the repellence of prey visual signals if these signals are of a color associated with aposematism. We know that such coloration is more effective at prompting avoidance learning when the prey's body size or color patch is larger (Gamberale and Tullberg 1996b, 1998; Lindström et al. 1999; Mand et al. 2007), and, in a similar fashion, aggregation might increase the salience of a color signal. Alternatively, aggregation may simply increase the conspicuousness of the signal (again by increasing its size): a factor known to generate unconditioned aversions in birds (Gamberale and Tullberg 1996a; Gamberale-Stille 2000; Remmel and Tammaru 2011).

Aversions toward prey that possess colors typically associated with insect warning patterns have previously been reported in a variety of avian species (Caldwell and Rubinoff 1983; Schuler and Hesse 1985; Sillén-Tullberg 1985; Roper and Cook 1989; Mastrota and Mench 1995), but the results of studies assessing color aversions are not always consistent (Roper and Wistow 1986; Roper and Marples 1997). Furthermore, the idea that predators possess unconditioned aversions to aggregations of aposematic prey has received mixed support (Sillén-Tullberg 1990; Gamberale and Tullberg 1996a, 1998). Although this may be due to the fact that some species possess only weak aposematic signals that are insufficient to generate unlearned aversions (Lindstedt et al. 2011), an alternate explanation is that predators may sample potentially valuable novel prey items when their expectation of risk of the prey being defended is perceived to be low, but avoid novel or brightly colored prey items when the risk of their being toxic is perceived to be high (Rowe and Guilford 1999b; Gamberale-Stille and Tullberg 2001). This appears to be the case when predators experience a bitter-tasting toxin prior to encountering brightly colored prey for the first time, as in our experiment.

Our study is the first to show that bitter taste enhances biases against attacking prey that are aggregated in favor of solitary prey when these possess coloration typically associated with aposematism (red), but not with crypsis and palatability (green). Our findings are consistent with other studies that have found additional signal components, such as a novel sound, odor, bitter-tasting toxin, or a conspecific's disgust response, can increase bias against single food items with visual traits typically associated with aposematism, including conspicuousness or a red or yellow color (Marples and Roper 1996; Rowe and Guilford 1996, 1999b; Jetz et al. 2001; Lindström et al. 2001; Rowe and Skelhorn 2005; Skelhorn et al. 2008; Siddall and Marples 2011; Skelhorn 2011). Because the interaction term (color × solution type) in our main Anova for the number of aggregated prey attacked (Figure 2) was nonsignificant, we must treat our conclusions with some caution. However, we note that the contrasts within Anova (a more appropriate form of analysis: see Ruxton and Beauchamp 2008) confirmed our a priori predictions, and therefore we feel justified to discuss the effects of taste on unconditioned color biases.

A likely mechanism that would explain the avoidance of novel and brightly colored aggregated prey items is that bitter taste increases a predators' perceived risk of prey being toxic by inducing an aversive state that results in altered perception of and responses to stimuli in other modalities (e.g., see Nitschke et al. 2006). Peyrot des Gachons et al. (2011) found that a bitter-tasting stimulus induces nausea in human subjects up to 30 min after exposure, showing that the body not only detects potential toxins but anticipates and prevents their ingestion by inducing a prophylactic aversive state. Previous work suggests that domestic chicks find the concentration of chloroquine phosphate solution we used aversive (Rowe and Skelhorn 2005) and that at high doses chloroquine is emetic (Alcock 1970). There is a link, therefore, between the experience of a bitter-tasting compound in the chick's mouth and exhibiting an aversive state.

These taste-related attack biases against red aggregations were only evident when preferences were measured by the total number of each prey attacked in the whole trial and were not evident in chicks' wariness, as measured by the number of solitary prey attacked before an aggregated prey. There are several potential explanations for this, and understanding differences between wariness and longer-term foraging preferences could help us to understand the mechanisms underlying "unlearned" color biases. The difference could simply be due to sampling noise. Alternatively, another explanation is that our measure of wariness is subject to an increased level of variance in the red and chloroquine group: 6 out of 10 chicks attacked an average of 0.83 (±0.98 SD) solitary prey before an aggregated, and 4 of the 10 chicks attacked 9.5 (±1.00 SD) solitary prey before an aggregated prey. This heavily influences the variance of our measurements of wariness, but has less effect on measures of overall preference. This difference could be a result of cognitive or perceptual differences in the predators. For example, birds may not use rules like "avoid scary prey," but instead "sample scary prey less often" or "eat smaller meals when faced with scary prey." Alternatively, this difference in predator behavior could also be explained by individual differences in the birds' perception of the bitter taste, which affects their expectation of risk and expression of wariness (see Davis et al. 2010 who suggest that polymorphisms exist in bitter-taste receptor genes of white-throated sparrows, which could result in differences in perception and behavior). Finally, the number of aggregated/solitary prey attacked across the entire trial may not be a measure of preference per se, but may instead reflect differences in the way that birds learn about different types of prey. We know that predators learn more quickly to avoid aggregations of aposematic prey than solitary aposematic prey (Gagliardo and Guilford 1993; Gamberale and Tullberg 1996a, 1998; Tullberg et al. 2000), but we do not know how aggregation influences the way in which birds learn to associate visual signals with rewards. If aggregation only facilitates aversion learning, then it is possible that it also makes it more difficult for birds to learn to associate aposematic aggregations with positive experiences. Therefore, our measure of preference could simply reflect the fact that birds are learning to associate solitary prey with food rewards more quickly than aggregated aposematic prey.

Irrespective of the exact mechanisms via which color and aggregation influence prey selection, our data suggest an alternative route for the evolution of aggregation behavior. The experience of bitter-tasting toxins of both aggregated green mutants and their solitary conspecifics (and potentially even the toxins of visually distinct aposematic prey) could cause predators to become more wary of other aggregated aposematic mutants, thus allowing them to reproduce and spread through the population. Alternatively, biases against aposematic aggregations may have evolved in response to the presence of aggregated aposematic prey. If this is the case, enhanced biases may not have influenced the initial evolution of aggregation behavior. However, they could certainly increase the benefit of aggregation in existing systems, which could potentially make it more evolutionarily stable among aposematic species.

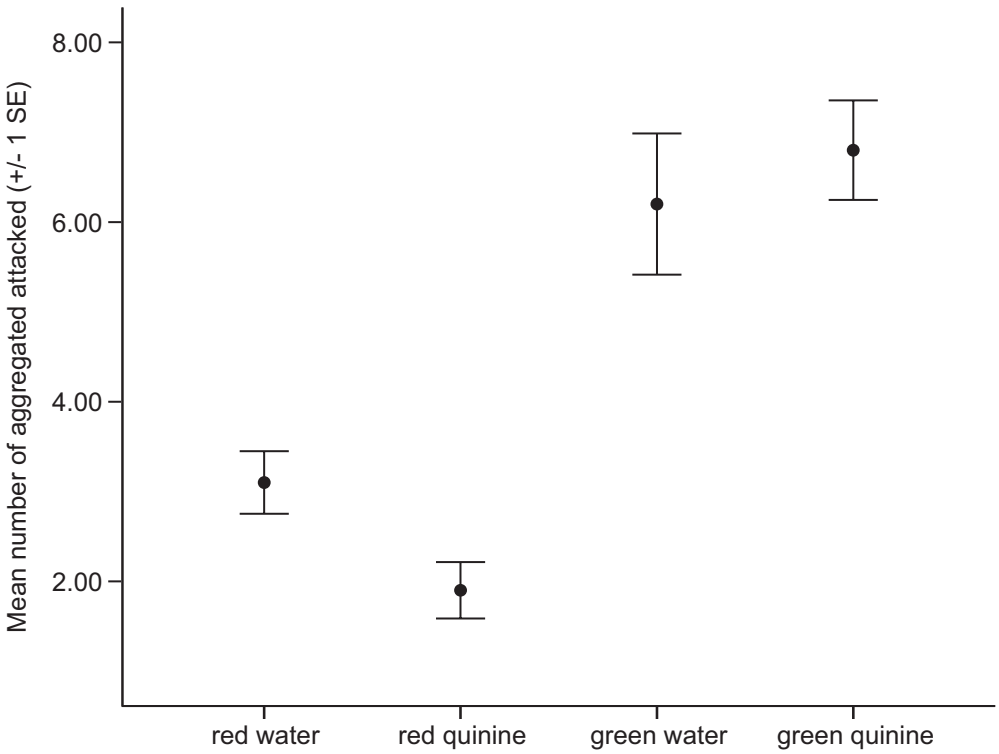

Figure 2
The mean number (±1 SE) of aggregated prey attacked by birds in each of our 4 experimental groups (n = 10 chicks for each group).

In conclusion, we have shown that aggregation enhances chicks' foraging biases against prey with coloration that is typically associated with aposematism (red), but not with crypsis and edibility (green) and that sampling a bitter-tasting toxin enhances this bias further by altering expectation of risk in some individuals. Our findings help to explain why the evolution of aposematic coloration may facilitate the evolution of aggregation behavior and the evolution of complex aposematic signals involving multiple sensory modalities and associated behavioral traits.

FUNDING

This work was funded by Natural Environment Research Council grant NE/E016626/1 and a research fellowship to H.M.R. from the Wingate Foundation. H.M.R. is currently supported by a research fellowship from Churchill College, Cambridge.

We thank 3 anonymous referees for their comments and suggestions.

Handling editor: Wolfgang Forstmeier

REFERENCES

Alcock J. 1970. Punishment levels and the response of blackcapped chickadees (*Parus atricapillus*) to three kinds of artificial seeds. Anim Behav. 18:592–599.
Beddard FE. 1895. Animal coloration; an account of the principle facts and theories relating to the colors and markings of animals. 2nd ed. London: Swan Sonnenschein.
Brower LP, Ryerson WN, Coppinger LL, Glazier SC. 1968. Ecological chemistry and the palatability spectrum. Science. 161:1349–1350.
Brown SG, Boettner GH, Yack JE. 2007. Clicking caterpillars: acoustic aposematism in *Antheraea polyphemus* and other Bombycoidea. J Exp Biol. 210:993–1005.
Caldwell GS, Rubinoff RW. 1983. Avoidance of venomous sea snakes by naïve herons and egrets. Auk. 100:195–198.
Cott HB. 1940. Adaptive coloration in animals. London: Methuen.
Davis JK, Lowman JJ, Thomas PJ, ten Hallers BFH, Koriabine M, Huynh LY, Manye DL, de Jong P, Martin CL, Program NCS, et al. 2010. Evolution of a bitter taste receptor gene cluster in a new world sparrow. Genome Biol Evol. 2:358–370.
Despland E, Simpson SJ. 2005. Food choices of solitarious and gregarious locusts reflect cryptic and aposematic antipredator strategies. Anim Behav 69(2), 471–479.
Dumbacher JP, Beehler BM, Spande TF, Garraffo HM, Daly JW. 1993. Pitohui—how toxic and to whom. Science. 259:582–583.
Edmunds M. 1974. Defence in animals: a survey of anti-predator defences. Harlowe (Essex): Longman.
Edmunds M. 1991. Does warning coloration occur in nudibranchs. Malacologia. 32:241–255.
Gagliardo A, Guilford T. 1993. Why do warning-colored prey live gregariously. Proc R Soc Lond B Biol Sci. 251:69–74.
Gamberale G, Tullberg BS. 1996a. Evidence for a more effective signal in aggregated aposematic prey. Anim Behav. 52:597–601.
Gamberale G, Tullberg BS. 1996b. Evidence for a peak-shift in predator generalization among aposematic prey. Proc R Soc Lond B Biol Sci. 263:1329–1334.
Gamberale G, Tullberg BS. 1998. Aposematism and gregariousness: the combined effect of group size and coloration on signal repellence. Proc R Soc Lond B Biol Sci. 265:889–894.
Gamberale-Stille G. 2000. Decision time and prey gregariousness influence attack probability in naïve and experienced predators. Anim Behav. 60:95–99.
Gamberale-Stille G, Tullberg BS. 2001. Fruit or aposematic insect? Context-dependent color preferences in domestic chicks. Proc R Soc Lond B Biol Sci. 268:2525–2529.
Gittleman JL, Harvey PH. 1980. Why are distasteful prey not cryptic. Nature. 28:897–899.
Grant JB. 2007. Ontogenetic colour change and the evolution of aposematism: a case study in panic moth caterpillars. J Anim Ecol. 76:439–447.
Guilford T. 1992. Predator psychology and the evolution of prey coloration. In: Crawley MJ, editor. Natural enemies: the population biology

of predators, parasites and diseases. Oxford: Blackwell Scientific. p. 377–394.
Guilford T. 1994. "Go-slow" signalling and the problem of automimicry. J Theor Biol. 170(3):311–316.
Haskell PJ. 1966. Insect sounds. London: H.F. & G. Witherby.
Higginson AD, Delf J, Ruxton GD, Speed MP. 2011. Growth and reproductive costs of larval defence in the aposematic lepidopteran *Pieris brassicae*. J Anim Ecol. 80:384–392.
Jetz W, Rowe C, Guilford T. 2001. Non-warning odors trigger innate color aversions—as long as they are novel. Behav Ecol. 12:134–139.
de Jong P, Holloway G, Brakefield PM, de Vos H. 1991. Chemical defence in ladybird beetles (Coccinellidae) II. Amount of reflex fluid, the alkalois adaline and individual variation in defence in 2-spot ladybirds (*Adalia bipunctata*). Chemoecology. 2:15–19.
Lindstedt C, Huttunen H, Kakko M, Mappes J. 2011. Disengtangling the evolution of weak warning signals: high detection risk and low production costs of chemical defences in gregarious pine sawfly larvae. Evol Ecol. 25:1029–1046.
Lindström L, Alatalo RV, Mappes J, Riipi M, Vertainen L. 1999. Can aposematic signals evolve by gradual change? Nature. 397:249–251.
Lindström L, Rowe C, Guilford T. 2001. Pyrazine odour makes visually conspicuous prey aversive. Proc R Soc Lond B Biol Sci. 268:159–162.
Mand T, Tammaru T, Mappes J. 2007. Size dependent predation risk in cryptic and conspicuous insects. Evol Ecol. 21:485–498.
Marples NM, Brakefield PM, Cowie RJ. 1989. Differences between the 7-spot and 2-spot ladybird beetles (Coccinellidae) in their toxic effects on a bird predator. Ecol Entomol. 14:79–84.
Marples NM, Roper TJ. 1996. Effects of novel color and smell on the response of naive chicks towards food and water. Anim Behav. 51:1417–1424.
Mastrota FN, Mench JA. 1995. Color avoidance in northern bobwhites—effects of age, sex and previous experience. Anim Behav. 50:519–526.
Moore BP, Vance Brown W, Rothschild M. 1990. Methylalkylpyrazines in aposematic insects, their hostplants and mimics. Chemoecology. 1:43–51.
Nitschke JB, Dixon GE, Sarinopoulos I, Short SJ, Cohen JD, Smith EE, Kosslyn SM, Rose RM, Davidson RJ. 2006. Altering expectancy dampens neural response to aversive taste in primary taste cortex. Nat Neurosci. 9:435–442.
Peyrot des Gachons C, Beauchamp GK, Stern RM, Koch KL, Breslin PAS. 2011. Bitter taste induces nausea. Curr Biol. 21:R247–R248.
Poulton EB. 1890. The colors of animals: their meaning and use. Especially considered in the case of insects. London: Kegan Paul, Trench Trubner & Co.
R Development Core Team. 2012. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.
Remmel T, Tammaru T. 2011. Evidence for the higher importance of signal size over body size in aposematic signaling in insects. J Insect Sci. 11:1–11.
Riipi M, Alatalo RV, Lindström L, Mappes J. 2001. Multiple benefits of gregariousness cover detectability costs in aposematic aggregations. Nature. 413:512–514.
Roper TJ, Cook SE. 1989. Responses of chicks to brightly colored insect prey. Behav Process. 110:276–293.
Roper TJ, Marples NM. 1997. Color preferences of domestic chicks in relation to food and water presentation. Appl Anim Behav Sci. 54:207–213.
Roper TJ, Wistow R. 1986. Aposematic coloration and avoidance-learning in chicks. Q J Exp Psychol B. 38:141–149.
Rothschild M, Moore BP, Vance Brown W. 1984. Pyrazines as warning odour components in the Monarch butterfly, *Danaus plexippus*, and in moths of the genera Zygaena and Amata (Lepidoptera). Biol J Linn Soc. 23:375–380.
Rowe C, Guilford T. 1996. Hidden color aversions in domestic clicks triggered by pyrazine odours of insect warning displays. Nature. 383:520–522.
Rowe C, Guilford T. 1999a. The evolution of multimodal warning displays. Evol Ecol. 13:655–671.
Rowe C, Guilford T. 1999b. Novelty effects in a multimodal warning signal. Anim Behav. 57:341–346.
Rowe C, Skelhorn J. 2005. Color biases are a question of taste. Anim Behav. 69:587–594.
Ruxton GD, Beauchamp G. 2008. Time for some a priori thinking about post hoc testing. Behav Ecol. 19:690–693.
Ruxton GD, Sherratt TN. 2006. Aggregation, defence and warning signals: the evolutionary relationship. Proc R Soc Lond B Biol Sci. 273:2417–2424.
Schmidt JO. 2008. Venoms and toxins in insects. In: Capinera JL, editor. Encyclopedia of entomology. The Netherlands: Springer. p. 4076–4089.
Schuler W, Hesse E. 1985. On the function of warning coloration—a black and yellow pattern inhibits prey-attack by naive domestic chicks. Behav Ecol Sociobiol. 16:249–255.
Schuler W, Roper TJ. 1992. Responses to warning coloration in avian predators. Adv Stud Behav. 21:111–146.
Siddall EC, Marples NM. 2008. Better to be bimodal: the interaction of color and odor on learning and memory. Behav Ecol. 19:425–432.
Siddall EC, Marples NM. 2011. Hear no evil: the effect of auditory warning signals on avian innate avoidance, learned avoidance and memory. Curr Zool. 57:197–207.
Sillén-Tullberg B. 1985. Higher survival of an aposematic than of a cryptic form of a distasteful bug. Oecologia. 67:411–415.
Sillén-Tullberg B. 1990. Do predators avoid groups of aposematic prey? An experimental test. Anim Behav. 40:856–860.
Sillén-Tullberg B, Leimar O. 1988. The evolution of gregariousness in distasteful insects as a defense against predators. Am Nat. 132:723–734.
Skelhorn J. 2011. Color biases are a question of conspecifics' taste. Anim Behav. 81:825–829.
Skelhorn J, Griksaitis D, Rowe C. 2008. Color biases are more than a question of taste. Anim Behav. 75:827–835.
Skelhorn J, Rowe C. 2006. Prey palatability influences predator learning and memory. Anim Behav. 71:1111–1118.
Skelhorn J, Ruxton GD. 2006. Avian predators attack aposematic prey more forcefully when they are part of an aggregation. Biol Lett. 2:488–490.
Summers K, Clough ME. 2001. The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). Proc Natl Acad Sci USA. 98:6227–6232.
Sword GA, Simpson SJ, El Hadi OTM, Wilps H. 2000 Density–dependent aposematism in the desert locust. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 267(1438), 63–68.
Tullberg BS, Gamberale-Stille G, Solbreck C. 2000. Effects of food plant and group size on predator defence: differences between two co-occurring aposematic Lygaeinae bugs. Ecol Entomol. 25:220–225.
Tullberg BS, Hunter AH. 1996. Evolution of larval gregariousness in relation to repellent defences and warning coloration in tree-feeding Macrolepidoptera: a phylogenetic analysis based on independent contrasts. Biol J Linn Soc. 57:253–276.
Tullberg BS, Leimar O, Gamberale-Stille G. 2000. Did aggregation favour the initial evolution of warning coloration? A novel world revisited. Anim Behav. 59:281–287.
UCLA: Academic Technology Services, S. C. G. Zero-inflated negative binomial regression. UCLA: Statistical Consulting Group. Available from: http://statistics.ats.ucla.edu/stat/r/dae/zinbreg.htm.
UK Government. 2009. Guidance to the operation of the Animals (Scientific Procedures) 1986. In: ScotPIL manual—avian species. London: Stationery Office.