Research paper

Experienced chicks show biased avoidance of stronger signals: an experiment with natural colour variation in live aposematic prey

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Abstract. An important factor for understanding the evolution of warning coloration in unprofitable prey is the synergistic effect produced by predator generalisation behaviour. Warning coloration can arise and become stabilised in a population of solitary prey if more conspicuous prey benefit from a predator's previous interaction with less conspicuous prey. This study investigates whether domestic chicks (*Gallus gallus domesticus*) show a biased generalisation among live aposematic prey by using larvae of three species of seed bugs (Heteroptera: Lygaeidae) that are of similar shape but vary in the amount of red in the coloration. After positive experience of edible brownish prey, chicks in two reciprocal experiments received negative experience of either a slightly red or a more red distasteful larva. Attacking birds were then divided into two treatment groups, – one presented with the same prey again, and one presented with either a less red or a more red larva. Birds with only experience of edible prey showed no difference in attack probability of the two aposematic prey types. Birds with experience of the less red prey biased their avoidance so that prey with a more red coloration was avoided to a higher degree, whereas birds with experience of the more red prey avoided prey with the same, but not less red coloration. Thus, we conclude that bird predators may indeed show a biased generalisation behaviour that could select for and stabilise an aposematic strategy in solitary prey.

Key words: aposematism, defence, *Graptostethus servus*, *Lygaeus equestris*, predation, stimulus generalization, synergism, *Tropidothorax leucopterus*

Introduction

Aposematic prey have defence properties that render them unprofitable as prey, and they benefit from advertising this unprofitability with warning coloration (Poulton, 1898; Cott, 1940; Edmunds, 1974). One problem concerning the evolution of aposematism is that warning coloration often entails conspicuousness and therefore an obvious cost of increased risk of discovery by predators. It is most probable that warning coloration evolved in already defended prey, because for palatable prey to signal its presence would be

devastating. Thus, for warning coloration to be beneficial in a cryptic and defended population, the increased risk of discovery must be balanced by a decreased seizure rate.

Leimar *et al.* (1986) presented a theoretical model for the evolution of warning coloration, incorporating aspects of predator generalisation behaviour known from learning psychology (see also Leimar and Tuomi, 1998; Yachi and Higashi, 1998, 1999). When animals generalise in a stimulus dimension after discrimination learning between two stimuli, a positive and a negative 'peak-shift' often appears. The maximum and the minimum responses do not occur at the experienced positive and negative stimuli, but are shifted in the direction away from the negative and positive stimuli, respectively (Hanson, 1959; Purtle, 1973). Applying this psychological phenomenon to the concept of warning coloration would mean that a predator who gains experience of distasteful prey with a certain coloration, would avoid other prey with stronger warning signals to a higher degree than they would avoid the previously experienced prey (Leimar *et al.*, 1986; Dawkins and Guilford, 1993). Thus, this positively biased synergistic effect could provide a selection pressure that would balance the increased discovery rate that a more conspicuous warning coloration entails (Leimar *et al.*, 1986; Leimar and Tuomi, 1998).

Another often discussed cost in the initial evolution of warning coloration is that a rare novel form would be in disadvantage to the common form due to less efficient associative learning (Guilford, 1988, 1990). However, in the above scenario this supposed cost do not exist due to the bias in predator generalisation, as the novel more conspicuous form is protected to a higher degree than the prior experienced form.

As mentioned above, there already exist numerous of studies on various species showing the existence of biased generalisation behaviour in the form of peak-shifts, both positive and negative, in different types of stimulus dimensions, and with different separation between positive and negative stimuli (see Purtle, 1973 and references therein). Although the existence of biased generalization in animals seems pretty well documented, it still needs to be investigated whether predators facing live prey may generalise from negative experiences in a biased way that benefits more conspicuous prey. There is some empirical evidence that predators show positively biased generalisation behaviour in foraging situations with respect to signal strength, in experiments with artificial signals (Lindström *et al.*, 1999) and among live aposematic prey varying in size (Gamberale and Tullberg, 1996). In the present experiment we use both aposematic and non-aposematic larvae of different seed bug species to investigate how predators may generalise in a stimulus dimension of varying prey coloration.

Another reason why we chose to use live prey when investigating predator reactions towards aposematic prey is that predators may deal with different

types of food differently. One would for instance expect a red berry to signal profitability and a red insect to signal unprofitability, and thus the 'same' signal to mean different things in different contexts. Young chicks may show differences in their pecking preferences of different colours depending on if the item is a bead or an insect. For instance, when presented with small beads, chicks prefer those of red or blue colour (Rogers, 1995, and references therein), but when presented with insect prey, red seems to be at least mildly aversive (Roper and Cook, 1989; Roper, 1990).

Methods

Predators

We used domestic chicks (*Gallus gallus domesticus*) as predators. The chicks arrived from the hatchery in batches of about 40 individuals. On arrival, they were less than 20 h old and had not eaten. Each batch was housed in a $100 \times 55 \times 20$ cm cage with wooden sides, steel-net floor and a roof made partly of wood and partly of chicken wire. The floor of the cage was covered with sawdust and a 60 W carbon light bulb provided heat. The chicks were fed chick starter crumbs and water *ad libitum* throughout the housing period and during testing. During the second day after arrival the chicks were occasionally also fed live mealworms (*Tenebrio molitor*), to make them accustomed to live prey.

Prey and coloration

As prey in the experiment we used live larvae of three different species of seed bugs, *Graptostethus servus*, *Lygaeus equestris* and *Tropidothorax leucopterus* (Heteroptera: Lygaeidae) (Fig. 1). The larvae have a similar body-shape, and all have black legs, wing-buds and head pattern, but they vary in the amount of red coloration. *Graptostethus servus* larvae have very thin inconspicuous lines of red on the abdomen, but give an overall brownish-grey impression. We know from a previous experiment that *G. servus* larvae are tasteful to chicks (Gamberale and Tullberg, 1998), and they were used as a positive stimulus in this experiment. The other two species are aposematic and were used as negative stimuli and to test the birds' generalization behaviour. They have previously been used in studies of aposematism (Gamberale and Tullberg, 1996; Sillén-Tullberg, 1985), from which we know that they are distasteful to birds. Both species give an overall red impression but *T. leucopterus* larvae are brighter red and the coloration seems more saturated than for *L. equestris*

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Figure 1. The experimental prey from left to right; a fifth instar *Graptostethus servus* larva, a fourth instar *G. servus* larva, a fourth instar *Lygaeus equestris* larva and a fourth instar *Tropidothorax leucopterus* larva.
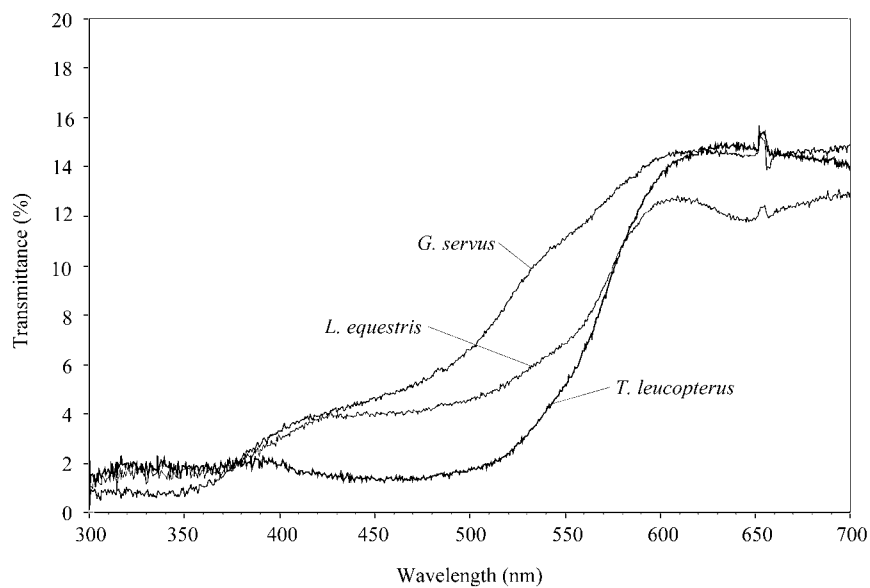

Figure 2. The reflection spectra of the three species used in the experiment, *Graptostethus servus*, *Lygaeus equestris* and *Tropidothorax leucopterus*, measured with an Ocean Optics S1000-2LOS25U spectrometer and a DH-2000 Deuterium-Halogen Light Source (215–1700 nm). The Reflectance Probe (FCR-7UV200-1.5 × 100-2) was held at an angle of 45° from the measured surface to minimise mirror reflectance.

larvae. We measured the reflectance spectra for the three species (Fig. 2). They all have the major part of reflectance in the orange–red but differ in the proportion of the total reflectance within this area, with *G. servus* having smallest

and *T. leucopterus* having the largest proportion within the orange–red area (Table 1).

The larval instars vary in size between the species, but are about the same size within species. Therefore we chose experimental instars so that the prey stimulus with the brightest red coloration also was the smallest in size. This was done because the size of aposematic prey affects attack behaviour of chicks so that larger aposematic prey are attacked less (Gamberale and Tullberg, 1996). We used fourth (5.3 $\pm$ 0.3 mm; 9.7 $\pm$ 1.5 mg, $n = 10$) and fifth (7.6 $\pm$ 0.7 mm; 22.7 $\pm$ 3.6 mg, $n = 10$) instar *G. servus*, fourth instar *L. equestris* (6.4 $\pm$ 0.4 mm; 16.9 $\pm$ 3.1 mg, $n = 10$) and fourth instar *T. leucopterus* (5.2 $\pm$ 0.2 mm; 8.9 $\pm$ 1.6 mg, $n = 10$), (mean $\pm$ SD). Both fourth and fifth instar *G. servus* where used as positive stimulus to minimise the risk that the birds would use prey size as the stimulus dimension to generalise over.

T. leucopterus was reared on a diet consisting of seeds and green parts of their toxic host plant *Vincetoxicum hirundinaria* (Asclepiadaceae), husked sunflower seeds and water. *G. servus* and *L. equestris* were both reared on a diet of husked sunflower seeds and water, but from the second instar on, *L. equestris* larvae were only fed on seeds from their toxic host plant *Vincetoxicum hirundinaria* (Asclepiadaceae). It has been shown that *L. equestris* larvae might be partly dependent on host plant for defence (Tullberg *et al*., 2000). All larvae were cultured at a 17:7 h light:dark regime, and *G. servus* and *T. leucopterus* at a temperature of 27 °C and *L. equestris* at a temperature of 32 °C.

Experimental arena and procedure

The trials took place in an arena in the same kind of cage that the chicks were housed in, except that the floor of the cage was covered with a transparent plastic sheet with sawdust underneath, as a background. Part of the cage was screened off, leaving a testing floor size of 30 × 55 cm.

On the chicks' third day, they were placed in pairs in the experimental arena and were presented with the positive stimulus, the edible *G. servus* larvae, in a petri-dish. All birds had readily attacked and eaten at least one of

Table 1. The percentage of total reflectance spectra (300–700 nm) that lays within the orange and red, and red area, measured for larvae of the three Lygaeidae species

Species	590–700 nm (%) (orange–red)	650–700 nm (%) (red)
Graptostethus servus	51.25	23.50
Lygaeus equestris	53.65	24.70
Tropidothorax leucopterus	66.96	30.70

both fourth and fifth instar *G. servus* larvae before they were used in the experiment.

The experiment started on the birds' fourth day, when they were less than 84 h old. Each chick was tested together with a companion chick that, prior to testing, had been fed with as many mealworms as it wanted, which made it inactive and not interested in the experimental prey. The use of a companion chick was necessary, because the experimental chicks became distressed and were not interested in food when alone.

Ninety-five birds received a *L. equestris* larva, the less red prey, on the first experimental day. Birds that attacked the prey were divided into two treatment groups, one of which received a *L. equestris* larva again ($n = 41$), and the other of which received a *T. leucopterus* larva ($n = 41$) on the second experimental day.

A reciprocal experiment was carried out with 80 new chicks that received a *T. leucopterus* larva, the more red prey, on the first experimental day. Birds that attacked the prey were divided into two treatment groups, one of which received a *T. leucopterus* larva again ($n = 35$), and the other of which received a *L. equestris* larva ($n = 35$) on the second experimental day.

The larvae were presented singly in petri dishes with a diameter of 5 cm. A thin layer of Fluon was applied to the rim of the dishes to prevent the insect's escape. To find out if the birds were active and interested in attacking live prey, we also presented a mealworm to each bird at the same time as the experimental prey, and in the same type of petri-dish. An experimental trial lasted 2 min. If the bird attacked the experimental prey, the trial was terminated when the bird had finished handling the prey. If a bird failed to attack the prey during the 2 min, the trial was repeated approximately 15 min later. We noted if the chick attacked the prey or not and the time to attack, measured as the total time the chick had access to the prey before an attack. An attacked insect was investigated after the trial, and was counted as effectively dead, even if it was only severely injured.

The proportion attacking birds and the proportion prey individuals killed when attacked were compared between treatment groups and between experimental days using contingency tables, or Fisher Exact Test when expected values were less than 5. The time to attack was compared using the Mann–Whitney U-test. To investigate whether there was an interaction between novelty and prey coloration on attack probability of experienced birds, we used log-linear analysis (STATISTICA 5.1). It is important to note that we could only control for that the aposematic prey were distasteful, but not to what degree. Thus, we do not know if the two species differed in the degree of distastefulness. As it would be expected that avoidance learning is dependent on prey taste as well as the warning signal, it would be improper here to discuss and compare attack probabilities of birds with different prey experiences.

Results

Inexperienced birds

Birds with no prior experience of aposematic prey showed no difference in attack probability when presented the two aposematic forms. During the first experimental day 86% of chicks presented with the less red *L. equestris* prey attacked, as compared to 89% of the birds presented with the more red *T. leucopterus* prey ($\chi^2 = 0.053$, d.f. = 1, $p = 0.82$; Fig. 3a). Neither was there any difference in attack latency between the groups (Mann–Whitney U, $Z = -0.96$, $p = 0.92$). However, attacking birds killed significantly more *L. equestris* prey than the more red *T. leucopterus* prey ($\chi^2 = 5.58$, d.f. = 1, $p = 0.02$), but there was no difference between prey species in how many that were eaten by birds ($\chi^2 = 0.741$, d.f. = 1, $p = 0.39$).

Birds with L. equestris *experience, the less red prey*

Birds with experience of *L. equestris* prey the first experimental day were less prone to attack aposematic prey the second day (Fig. 3b), whether this was again *L. equestris* prey ($\chi^2 = 11.0$, d.f. = 1, $p < 0.001$) or the more red *T. leucopterus* prey ($\chi^2 = 31.8$, d.f. = 1, $p < 0.0001$). This shows that the
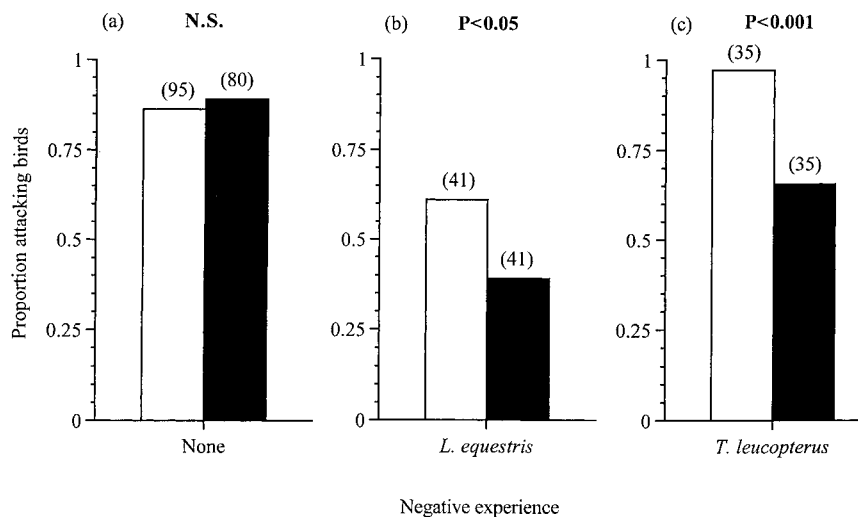

Figure 3. Proportion of birds (sample sizes above bars) attacking two forms of aposematic prey, the slightly red larva of *Lygaeus equestris* (open bars) and the more red larva of *Tropidothorax leucopterus* (closed bars) when birds had (a) no prior experience of aposematic prey, but of palatable *Graptostethus servus* prey, (b) one prior experience of a less red distasteful *L. equestris* larva and (c) one prior experience of a more red distasteful *T. leucopterus* larva.

L. equestris prey were distasteful to the chicks and acted as a negative stimulus.

However, most important, the reaction towards these two aposematic prey types the second test day differed. Thus, attack probability was significantly lower for the more red *T. leucopterus* prey than for the previously experienced *L. equestris* prey ($\chi^2 = 3.95$, *d.f.* = 1, $p = 0.047$; Fig. 3b). Also, there was a tendency for the birds to wait longer to attack *T. leucopterus* than *L. equestris* (Mann–Whitney *U*, $Z = -1.76$, $p = 0.08$). The two experimental groups showed no difference in the proportion of attacked prey that were killed the second experimental day (Fisher Exact Test, $p = 0.72$).

Birds with T. leucopterus *experience, the more red prey*

Also the more red *T. leucopterus* prey seemed to be distasteful and function as a negative stimulus, as fewer birds with experience of the *T. leucopterus* from the first experimental day attacked a *T. leucopterus* larvae again the second experimental day ($\chi^2 = 7.47$, *d.f.* = 1, $p < 0.01$; Fig. 3c). However, the birds did not seem to generalise the negative experience of the more red *T. leucopterus* from the first day to the less red *L. equestris* the second experimental day as 97% of birds with *T. leucopterus* experience attacked the novel *L. equestris* prey. Thus, for these birds, there was no decrease in attack probability between test days (Fisher Exact Test, $p = 0.169$; Fig. 3c). Accordingly, there was a difference in birds' reactions towards the two aposematic prey types the second experimental day in that more birds avoided the more red prey than the less red prey (Fisher Exact Test, $p = 0.0013$; Fig. 3c). Also, birds presented with the more red *T. leucopterus* again waited longer to attack than did birds presented with the less red *L. equestris* (Mann–Whitney *U*, $Z = -3.007$, $p < 0.01$), but there was no difference between species in mortality when attacked (Fisher Exact Test, $p = 0.229$).

Effect of novelty

Figure 3b shows that birds with prior negative experience of the less red *L. equestris* prey showed a biased generalisation of this experience in favour of the more red prey. It could be argued that this result is an effect of birds' negative experience of a red prey the first day may have elicited neophobic reactions in the birds that where not present when they were naive. However, if the results could be explained by an evoked neophobia, one would expect that birds with negative *T. leucopterus* experience also would show a biased generalisation towards the more novel prey, here the less red *L. equestris*. This does not seem to be the case as these birds do not avoid the more novel *L. equestris* prey (Fig. 3c). Also, a log-linear analysis show significant interaction between

novelty (new or similar prey second experimental day) and coloration (less red *L. equestris* or more red *T. leucopterus*) on attack probability ($\chi^2 = 20.45$, $d.f. = 1$, $p < 0.00001$). Thus, experienced birds show different reactions to the novel prey depending on its coloration.

Discussion

The main result from the experiment was that after a negative experience of a prey stimulus, the chicks showed a bias against redness in the form of a stronger aversive reaction to prey with more saturated coloration than the one they had prior experience of. One possible explanation for this bias could be that a negative peak-shift produces this biased generalization towards stronger signals.

Such a result could also be explained by a differential innate avoidance of the two prey types. However, this explanation is not likely since there was no difference in how birds without any previous experience of aposematic prey reacted to the two species.

Another possible explanation could be that the negative prey experience elicited a neophobic response to novel prey per se (Schlernoff, 1984). If that were the case, one would also expect birds with a prior experience of the more red *T. leucopterus* to show greater avoidance of the less red but novel *L. equestris* than to the familiar prey. However, data suggests the opposite, namely that the familiar more red prey were more aversive.

It is important to note that the prey species also differed in the size of the instars. In a previous experiment, it was found that the size of the aposematic prey affects the attack probability of both naive and experienced chicks in a negative manner (Gamberale and Tullberg, 1996). Therefore, in the present experiment we have chosen experimental prey so that the aposematic prey with the most amount of red also were the smallest in size. To avoid birds using size as a stimulus, they received both fourth and fifth instars of the positive *G. servus* prey, where the fifth instar is larger than any of the aposematic prey and the fourth instar is about the size of the smallest, the *T. leucopterus* prey. On the whole, it seems rather likely that the amount of red coloration was responsible for the lower seizure rate on *T. leucopterus*.

There was no effect of prey colour on attack probability of naive birds. However, during the first experimental day, more of the less red *L. equestris* were killed than the more red *T. leucopterus* prey. This could mean that the birds were more careful when handling the more red prey, suggesting some difference between the prey types in bird avoidance also when they were naive. Differences in mortality between the two aposematic prey may also be due to

differences in distastefulness between species, but this effect was only present for naive birds, and the birds did not differ in how many prey were eaten. In any case, both aposematic species were distasteful, as shown by a lower attack probability the second experimental day, and the generalisation behaviour was compared between birds with experience of the same prey species from the first day.

Several authors have discussed and modelled the importance of synergistic effects, and signal form in general, for the evolution of warning coloration (Guilford, 1985; Queller, 1985; Leimar et al., 1986; Arak and Enquist, 1995; Leimar and Tuomi, 1998; Yachi and Higashi, 1998; Lindström et al., 1999). This experiment provides further evidence that bird predators can show a biased generalization behaviour when encountering real aposematic prey in a dimension of natural colour variation (see also Gamberale and Tullberg, 1996). If such a positively biased synergistic effect entails a decreased seizure rate of more conspicuous prey it could balance the increased risk of discovery. In this way an aposematic strategy could arise and be stabilised in a solitary species.

The traditional explanation for the similarity in aspect found among Müllerian mimics, is that of convergence of colour patterns due to more effective avoidance learning (Edmunds, 1974; Turner, 1984). Our present experiment suggests, however, that this may not be the sole explanation for a certain colour pattern. Thus, one could envisage a situation where, although deviating from a common and shared aspect, a more exaggerated pattern would be beneficial. Such selection of a deviating aspect, however, would only be temporary, since it is expected that the other, less exaggerated form would eventually converge upon the more exaggerated one. The point is that selection for convergence could at times be truncated by selection for a deviating pattern.

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