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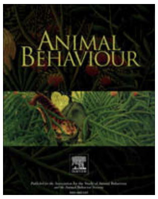

Role of different colours of aposematic insects in learning, memory and generalization of naïve bird predators

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Among the various properties of visual warning signals, colour seems to be especially important for avian predators. We tested the role of particular colours of an aposematic insect (firebug, *Pyrrhocoris apterus*; Heteroptera: Pyrrhocoridae) in unlearned avoidance, learning, memory and generalization of a naïve avian predator (great tit, *Parus major*). The wild type of the firebug is aposematic, red-and-black, and its colour mutants (white, yellow, orange) retain the same black pattern; the bug can be made artificially nonaposematic (painted uniformly brown). Wild-caught great tits avoid the firebug depending on colour, and their reaction to variously coloured prey is a result of avoidance learning and may vary according to their experience. We trained naïve great tits to avoid firebugs of different colours, and then gave some birds a memory test with firebugs of the same colour and other birds a generalization test with firebugs of a different colour. Naïve, hand-reared great tits showed no initial avoidance and attacked firebugs irrespective of colour. They learned to avoid all the colour forms at a similar rate. The generalization was asymmetric: birds that learned to avoid red firebugs did not generalize their experience to yellow or white mutants whereas birds that learned to avoid yellow mutants generalized their experience to red firebugs. The red colour thus represents a more effective signal than the yellow; predation by birds could have played a crucial role in selectively favoured evolutionary transitions from yellow to red coloration in pyrrhocorids.

Aposematic animals advertise their defensive mechanisms to predators by conspicuous signals (Poulton 1890; Ruxton et al. 2004). Some predators possess innate biases against conspicuous signals of aposematic prey such as specific colours and odours (Smith 1975; Schuler & Hesse 1985; Roper 1990; Rowe & Guilford 1996).

Predators with no innate responses to warning signals have to learn the association between the warning signal (conditioned stimulus) and unpalatability (unconditioned stimulus) during encounters with aposematic prey (Järvi et al. 1981; Sillén-Tullberg 1985a; Cardoso 1997; Exnerová et al. 2007). To be effective, aposematic signals should (1) accelerate avoidance learning, (2) prevent or delay forgetting, and (3) facilitate accurate recognition of the prey (Ruxton et al. 2004).

Avoidance learning can be enhanced by a specific quality of the warning signal, such as odour (Rowe & Guilford 1996; Roper & Marples 1997), taste (Hilker & Köpf 1994; Gill et al. 1998), sound (Rowe 2002), size (Marples 1993; Gamberale-Stille 2000; Riipi et al. 2001), pattern symmetry (Forsman & Merilaita 1999), and especially coloration (Sillén-Tullberg 1985a; Roper & Redston 1987; Avery & Nelms 1990; Marples et al. 1994; Lindström et al. 1999b; Gamberale-Stille & Guilford 2003). Retention of the signal in the predator's memory may be influenced by prey coloration (Roper & Redston 1987; Roper 1994; but see Ham et al. 2006) or by the presence of specific odours (Avery & Nelms 1990; Roper & Marples 1997). Surprisingly, studies focused on those features of aposematic signals that enhance the signal memorability are rare (Ruxton et al. 2004).

Once the learning process is completed, particular features of aposematic prey may facilitate its recognition by the predator (Guilford 1986; Gamberale-Stille 2001) or may influence the scope

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and direction of generalization (Ruxton et al. 2004). Generalization is a response to a particular novel stimulus resulting from previous experience with another stimulus (Lieberman 2000). The ability of predators to generalize their experience with aposematic prey is important for the evolution of warning signals and mimetic associations (Leimar et al. 1986; Yachi & Higashi 1998; Balogh & Leimar 2005; ten Cate & Rowe 2007). Generalization may be narrow or broad; the scope is predicted to depend on the strength of the previous negative experience with the aposematic prey (Lindström et al. 1997) and on the number of prey species involved (Beatty et al. 2004). Cases of both narrow (e.g. Sillén-Tullberg et al. 1982) and broad (e.g. Evans et al. 1987) generalization have been demonstrated in experiments with living aposematic prey. The generalization gradient may be asymmetrical, that is, generalization is easier in one direction of the stimulus dimension than in the opposite one. Such asymmetry is usually considered to result from the peak shift: a product of discrimination learning in which the peak of response is shifted away from the training stimulus (Spence 1937). There is growing evidence that some features of aposematic signals may be generalized asymmetrically. Domestic chicks, *Gallus gallus domesticus*, generalized their experience with larvae of aposematic heteropterans towards larger, older instars (Gamberale & Tullberg 1996), larger groups of individuals (Gamberale & Tullberg 1998) and species with more intensive red coloration (Gamberale-Stille & Tullberg 1999) than the prey they learned to avoid. Similarly, great tits, *Parus major*, that were trained to avoid artificial food with various visual markings generalized their experience towards more conspicuous items (Lindström et al. 1999b). On the other hand, when trained to associate visual signals with a positive unconditioned stimulus (food), chickens either remembered the particular colour precisely (Osorio et al. 1999) or generalized towards a colour intermediate between the two different colours they were trained to prefer (Baddeley et al. 2001; Jones et al. 2001). It is possible that rules for generalization of positive and negative signals may be different.

Warning signals may be multimodal, that is, they may consist of any combination of visual, olfactory, gustatory, acoustic or behavioural components which reinforce themselves or act synergistically (Rowe & Guilford 1999), but for avian predators coloration is considered to be an essential component of multimodal signals (Sillén-Tullberg 1985b; Roper 1990; Marples et al. 1994; Rowe & Guilford 1996). It is not clear which aspects of visual warning signals (colour, pattern, contrast between differently coloured body parts, contrast against the background) are most important for learning and memory; however, the colour itself plays an important role (Sillén-Tullberg 1985a, b; Gamberale-Stille & Guilford 2003; Exnerová et al. 2006). Most studies have focused on comparison of typical warning colours (red, orange, yellow and white) with a nonwarning one (e.g. Sillén-Tullberg 1985a; Roper & Redston 1987; Roper 1990; Lindström et al. 1999a; Rowe & Guilford 1999; Exnerová et al. 2003; Gamberale-Stille & Guilford 2003) whereas studies comparing reactions of birds to different warning colours are scarce. Exnerová et al. (2006) compared reactions of wild-caught birds to yellow, orange and red aposematic insects while Ham et al. (2006) did the same with artificial prey items. Wild-caught great tits readily avoided red-and-black individuals of the firebug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae; Exnerová et al. 2003, 2006) and were able to generalize their experience with red or yellow unpalatable prey to novel orange prey (Exnerová et al. 2006; Ham et al. 2006). However, the behaviour of wild-caught birds in the experiments may have been affected by their previous experience.

In this study we tested (1) the effect of different warning colours on the rate of predator avoidance learning and on the memorability of the learned warning signal, and (2) the ability of predators to generalize different warning colours. More specifically, we investigated the response of naïve (hand-reared) great tits lacking any previous experience to aposematic prey. The effectiveness of different warning colours was compared in live insect prey, the adult red-and-black, wild-type firebugs versus their laboratory-born, yellow and white mutants sharing the black pattern with the wild type (Exnerová et al. 2006), and with brown-painted firebugs whose brown colour is not aposematic (Exnerová et al. 2003). Birds were trained to avoid firebugs of a particular colour to assess whether the rates of avoidance learning are similar for different warning colours and more rapid than for the brown colour. We gave some birds a memory test with firebugs of the same colour they had learned to avoid. This memory test showed whether the birds had remembered their experiences equally well. Other birds had a generalization test with firebugs coloured differently from those they had learned to avoid. Using this test we could detect (1) whether the birds generalized different warning colours, and if they did, (2) whether the generalization was symmetrical.

METHODS

Prey

Brachypterous adults of the red-and-black, wild-type firebug and the white and yellow mutants of this species were used as the warningly coloured, prey. These firebugs are gregarious and phytophagous, warningly coloured and widely distributed in Europe; they are unpalatable for small passerines (Exnerová et al. 2003). The warning coloration of the wild type is formed by red erythropterin-based epidermal pigmentation and a black cuticular melanin pattern. Mutants differ from the wild type only in the composition of the epidermal pteridines. The yellow colour of *yellow* mutants results from a low amount of erythropterin and the presence of xanthopterin; the white colour of *albinotic* mutants is produced by the absence of erythropterin and the presence of xanthopterin and leucopterin (Socha & Němec 1992; Bel et al. 1997). Other characteristics (e.g. size, body shape, locomotion, black melanin pattern) are the same as in the wild type. The defensive secretion produced in the metathoracic glands of wild-type adults contains 35 chemicals, mainly short-chain aldehydes (Farine et al. 1992); the secretions of albinotic and yellow mutants are very similar to those of the wild type (J.-P. Farine, A. Exnerová, P. Štys & R. Socha, unpublished data). For more details about the colour mutants, their occasional occurrence in the wild and the origins of laboratory strains see Exnerová et al. (2006). 'Non-aposematic' firebugs were obtained by painting the wild-type firebugs with dark brown watercolour dye and chalk (as in Exnerová et al. 2003) to cover the original colour and pattern. This treatment did not impair the firebugs' chemical defence; painted individuals were able to release the secretion as well as the non-painted ones. According to our pilot tests with painted house crickets, *Acheta domestica*, the dye used is not aversive to birds in any way. The colour forms are henceforth referred to as the red wild type/form, yellow and white form/mutant and brown (-painted) form.

The wild-type firebugs were collected in the field (populations feeding on small-leaved lime trees, *Tilia cordata*) in Prague, Czech Republic. White and yellow mutants came from strains reared for several years in the laboratory (by R. Socha). All experimental firebugs were reared on linden seeds (*T. cordata*) and water ad libitum, under a long-day photoperiod 18:6 h light:dark, at a temperature of 26 ± 1 °C. Mealworms (larvae of *Tenebrio molitor*) were used as a familiar and palatable control prey to check the foraging motivation of birds during the experiments.

Reflectance spectra of all colour forms of the firebug (Fig. 1) were measured using equipment that allowed for measurements of reflectance in the UV and visible wavelengths from very small areas

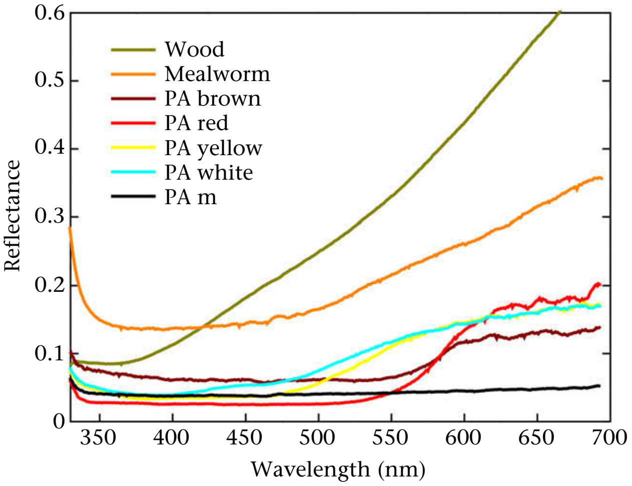

Figure 1. Reflectance spectra of the wooden feeding tray (wood), mealworms, brown-painted firebugs (PA brown), pteridine-coloured body parts of the red (PA red), yellow (PA yellow) and white (PA white) forms of the firebug, and melanized body parts (PA m) of the red, yellow and white forms. Values represent means of eight measurements; data from melanized parts of different colour forms are pooled.

(about 0.1 × 0.1 mm) of the insects (Xe lamp, imaging-spectrometer Triax 320, microscopic imaging system, ICCD camera PI-Max Princeton Instruments, Princeton, NJ, U.S.A.). Measurements were taken from eight pteridine-coloured and eight melanized areas on the dorsum (head, pronotum, scutellum, forewings, abdomen). Reflectance spectra of brown-painted firebugs, mealworms and the feeding tray were measured as well. Contrasts of mealworms and individual colour forms of firebugs (R) against the background of the wooden feeding tray (R_{wood}) were computed as a reflectance ratio $(R-R_{\mathrm{wood}})/R_{\mathrm{wood}}$ (according to Andersson et al. 1998, Fig. 2).

Predators

We used 120 naïve, hand-reared great tits as predators (Table 1); naïve predators are those that do not have any previous experience with aposematic prey (Ruxton et al. 2004). Great tits are suitable for avoidance-learning experiments, because they have no innate bias against firebugs (Exnerová et al. 2007). Nestlings, 12–15 days old, were taken from nestboxes placed in a mixed forest near Hradec Králové, Eastern Bohemia. They were fed a mixed diet, consisting of mealworms, crickets (*Acheta domestica*), boiled eggs, commercial food for insectivorous birds (Vitakraft, Bremen, Germany), and insects swept in the field (beetles, grasshoppers and caterpillars, excluding any warningly coloured species) with the addition of vitamins for insectivorous birds (Sera, Vitakraft). We kept the young birds in family groups of about three birds in artificial nestboxes until the 17th day of life and then in cages (50 × 30 cm and 30 cm high) with food (same diet used for nestlings and for older juveniles housed in the cages) and water ad libitum. Photoperiod was the same as outdoors (about 16:8 h light: dark) and the temperature was 18–22 °C. Experiments took place between the 35th and 45th day of the bird's life.

Ethical Note

We obtained permission to take nestling great tits from the Environmental Department of Municipality in Hradec Králové, Czech Republic and permission for laboratory experimentation with birds from the Czech Animal Welfare Commission. Each bird was ringed (under licence from the Czech Ringing Centre Praha) and released back to the locality of its origin within a few days of the termination of the experiment. Several feeders were kept at this locality where the released birds were provided with food (mealworms and a commercial mixture for insectivorous birds). All the birds were released (in groups of six to eight) at a healthy weight and well before they started their postjuvenile moult.

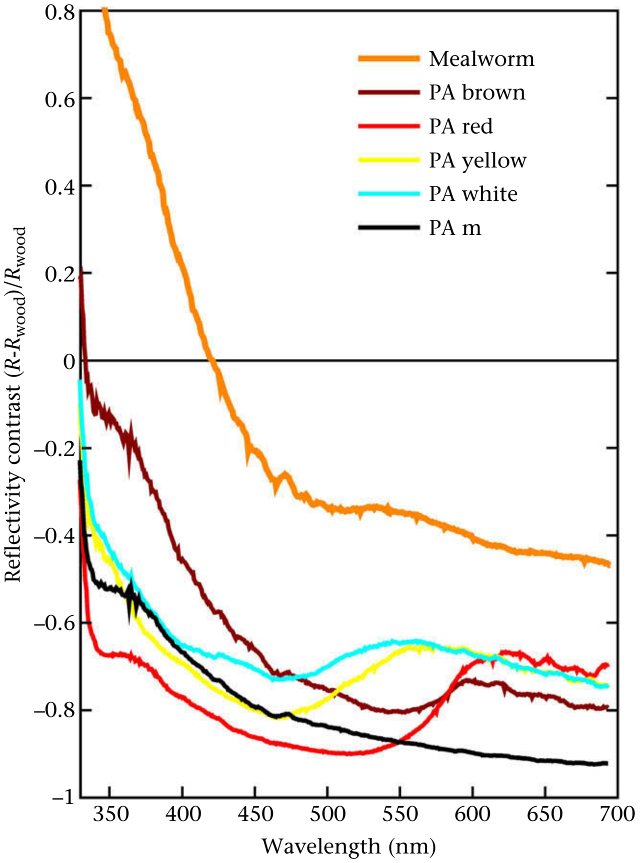

Figure 2. Spectral contrast of mealworms, brown-painted firebugs (PA brown), pteridine-coloured body parts of the red (PA red), yellow (PA yellow) and white (PA white) forms of the firebug, and melanized body parts (PA m) of the red, yellow and white forms against the background of the wooden feeding tray used in the experiments. Data from melanized parts of different colour forms are pooled.

Experiments

Experiments were conducted in wooden experimental cages (70 × 70 × 70 cm) with wire-mesh walls, perch, circular feeding tray with beige (pine wood) cups, and front wall made of one-way glass (for details see Exnerová et al. 2003). Cage illumination (Biolux Combi 18 W, Osram) simulated the full daylight spectrum. Each bird was trained to search for food in one of the cups of the feeding tray and then deprived of food for 2 h before the experiment.

All the experiments consisted of a sequence of 5 min trials. If the bird ate the whole prey the trial was terminated immediately; otherwise it lasted 5 min. We offered each bird one mealworm to check its foraging motivation in the first trial and then in every other trial after that. In even-numbered trials each bird was offered a single firebug (Table 1). The number of trials in a sequence depended on the phase of the experiment (see below). Each bird was tested alone and only once.

The experiment consisted of two phases carried out on 2 consecutive days: (1) avoidance learning the first day, and (2) a memory or generalization test the second day (always overnight and starting 18–22 h after the avoidance-learning session).

Table 1
Experimental design

	Day 1		Day 2	
	Avoidance learning	Number of birds	Test	Number of birds
Memory test				
Group 1	Red form	20	Red form	20
Group 2	Yellow form	20	Yellow form	18*
Group 3	Brown form	20	Brown form	19*
Generalization test				
Group 4	Red form	20	Yellow form	20
Group 5	Yellow form	20	Red form	17*
Group 6	Red form	20	White form	20

Great tits were divided into six groups and participated in two successive phases of the experiment: (1) avoidance learning and (2) a memory test (groups 1–3) or generalization test (groups 4–6). Each group received a unique combination of firebug colour forms during learning and the test phase.

* Smaller sample sizes in some memory (groups 2, 3) and generalization (group 5) tests are due to omission of those birds that failed to learn the avoidance during the first day.

(1) Avoidance learning consisted of a sequence of trials that were repeated until the bird refused to handle the firebug during three trials in a row (learning criterion). Such birds were considered to have learned to avoid the firebugs successfully. Consequently, the number of trials included in the learning session was variable, depending on how fast the bird had learned the avoidance. If a bird did not achieve the learning criterion within the sequence of 30 firebug trials, the experiment was terminated, and the bird was excluded from the following experimental treatment (Table 1).

(2) Both the memory and generalization tests consisted of 10 consecutive 5 min trials in which the birds were alternately offered either a mealworm or a firebug; each bird was thus sequentially presented with five firebugs in either of these tests (Table 1).

Of all the possible colour combinations we chose to test those that appeared most important in the study of reactions of wild-caught birds towards different mutants of *P. apterus* (Exnerová et al. 2006). The combinations of learning and test treatments are shown in Table 1. In the avoidance learning the birds learned to avoid either red firebugs (60 birds; groups 1, 4 and 6), yellow mutants (40 birds; groups 2 and 5) or brown firebugs (20 birds; group 3). The birds given the memory test were offered firebugs of the same colour as they had learned to avoid (groups 1, 2 and 3). The birds given the generalization test were offered a different colour form from the one they had learned to avoid: birds that had learned to avoid the red firebugs were offered the yellow mutants (group 4) or white mutants (group 6), and birds that had learned to avoid yellow mutants were offered the red firebugs (group 5).

During every trial, we recorded (1) attack latencies, (2) whether the bird handled (pecked or seized) the firebug, and (3) whether the firebug was killed by the bird.

(1) Attack latencies. Naïve great tits have no innate avoidance of red wild-type firebugs (Exnerová et al. 2007); nevertheless, they may still hesitate longer to attack a certain colour form. To test this possibility we measured attack latencies as the time from the beginning of the first trial to the first handling (pecking or seizing) of the offered prey (separately for the firebug and the mealworm). To assess the effects of a bird's experience on the previous day we also measured the attack latency of the first firebug handled during the memory test.

(2) Counts of firebugs handled. Counts of trials in which the bird handled firebugs during avoidance learning until it reached the learning criterion were considered to be a measure of avoidance-learning rate since it reflects the amount of olfactory and gustatory experience required in the learning process. As another measure of the rate of avoidance learning, we used the total number of firebug trials until the bird reached the learning criterion. Although the sequence usually included one to several trials in which the birds did not come into contact with a firebug, the birds could get the visual information needed for the process of learning from those trials during which they did not handle the prey. We used counts of trials in which the bird handled the firebugs during the memory test as a measure of remembering the avoidance of a particular colour form of the firebug. Similarly, counts of firebugs handled during the generalization test indicated whether the bird generalized between the colour form it had learned to avoid and a new colour form.

(3) Counts of firebugs killed. Counts of trials in which the bird killed the firebug offered were considered as a measure of mortality of various colour forms of firebugs during the encounter with great tits, either naïve or experienced with firebugs of the same or a different colour.

Statistics

Since the data did not have a normal distribution we used nonparametric statistics in all the analyses. To analyse attack latencies we used (1) a Kruskal–Wallis test to assess the influence of the colour form of the firebug on the attack latency in the avoidance learning, (2) a Wilcoxon signed-ranks test to compare attack latencies between the first firebug and the first mealworm, and (3) a Wilcoxon signed-ranks test to compare attack latencies for the first firebug handled in the avoidance learning and in the memory test (groups 1–3 pooled).

Total counts of firebug trials until the birds that were trained to avoid different colour forms of firebugs reached the learning criterion, and counts of firebugs of different colour forms that were handled and killed by individual birds during the avoidance-learning process, were compared by a Kruskal–Wallis test. The variances were compared by *F* ratio tests.

We assessed the birds' memory for a particular colour of firebugs by measuring the effect of avoidance learning on the bird's response to firebugs of the same colour form during the memory test on day 2. We compared counts of trials in which the bird (1) handled and (2) killed the firebug from the five firebug trials of the memory test with the corresponding first five firebug trials of the avoidance-learning session. Comparisons were carried out separately for the different colour forms (groups 1, 2 and 3) using the Wilcoxon signed-ranks test.

Similar tests were performed to find out whether birds generalized their experience towards different colour forms of the firebugs. Comparisons were carried out separately for different combinations of colour forms (groups 4, 5 and 6) using the Wilcoxon signed-ranks test.

We used the Kruskal–Wallis test to compare the counts of red firebugs (1) handled and (2) killed by birds that had previously learned to avoid yellow mutants with the counts of red firebugs (1) handled and (2) killed by naïve birds by comparing data from the generalization test of group 5 with data from the first five firebug trials of the avoidance learning of group 1. The same comparison was carried out for the counts of yellow mutants (1) handled and (2) killed by birds experienced with red firebugs (data from the generalization test of group 4) and those handled and killed by naïve birds (data from the first five firebug trials of the avoidance learning of group 2).

All *P* values result from two-tailed tests. All calculations were made using S-PLUS 4.0 (MathSoft 1997).

RESULTS

Attack Latencies

Attack latencies to the first firebug and to the first mealworm were significantly different (Wilcoxon signed-ranks test: $Z = 3.87$, $N = 120$, $P = 0.0001$; groups 1–6 pooled). Birds attacked the firebugs with a 2.5 s delay (median) in comparison with the mealworms. There was no effect of the firebug colour form on the attack latencies (Kruskal–Wallis test: $H_2 = 1.85$, $P = 0.396$; groups 1, 2 and 3). The attack latencies to the first firebug handled in the memory test were significantly longer than the corresponding latencies in the avoidance learning (Wilcoxon signed-ranks test: $Z = 3.52$, $N = 57$, $P = 0.0004$; groups 1–3 pooled); the median of the differences was 13.2 s (Fig. 3).

Avoidance Learning

All birds except one handled at least one firebug during the avoidance learning. Six of 60 birds that had been offered the red form (groups 1, 4 and 6) killed none during the avoidance learning. The counts for the yellow (groups 2 and 5) and brown (group 3) forms were two of 40 and three of 20 birds, respectively (Table 2).

There was no effect of colour form of the firebug on the number of trials required by birds to reach the learning criterion (Kruskal–Wallis test: $H_2 = 3.37$, $P = 0.186$; Table 1, Fig. 4). The number of firebugs handled by individual birds during the avoidance learning was not influenced by their colour ($H_2 = 2.91$, $P = 0.234$; Table 1, Fig. 4), and the same is true for the number of firebugs killed by individual birds ($H_2 = 2.50$, $P = 0.287$; Table 1, Fig. 4).

There was greater variance in the number of trials required to reach the learning criterion among birds trained to avoid the yellow (groups 2 and 5 pooled) form than among birds trained to avoid the red (groups 1 and 4 pooled) form (*F* ratio test: $F_{39,39} = 3.15$, $P = 0.0005$); the corresponding variances did not differ between the birds trained to avoid the brown (group 3) and yellow (group 2) forms ($F_{19,19} = 1.47$, $P = 0.405$) and between the birds trained to avoid the red (group 1) and brown (group 3) forms ($F_{19,19} = 1.30$, $P = 0.406$). The variances in number of firebugs handled and killed by individual birds during avoidance learning differed between the red (groups 1 and 4 pooled) and yellow (groups 2 and 5 pooled) forms (handling: $F_{39,39} = 2.88$, $P = 0.001$; killing: $F_{39,39} = 2.03$, $P = 0.030$), and differed marginally between the brown (group 3) and yellow (group 2) forms (handling: $F_{19,19} = 2.40$, $P = 0.064$; killing: $F_{19,19} = 2.61$, $P = 0.043$). The variances in numbers of handled and killed firebugs of the yellow form were greater than corresponding variances for the red and brown forms (Fig. 4). No difference in variances was found between the red (group 1) and brown (group 3) forms (handling: $F_{19,19} = 1.07$, $P = 0.891$; killing: $F_{19,19} = 2.26$, $P = 0.083$).

All 60 birds offered the red firebugs (groups 1, 4 and 6) learned to avoid them during the 30 firebug trials of the avoidance-learning session. In contrast, five of 40 birds offered the yellow mutants (groups 2 and 5) and one of 20 birds offered the brown firebugs (group 3) failed to learn to avoid them.

Memory Test

Birds that learned to avoid the red firebugs (group 1) handled (Wilcoxon signed-ranks test: $Z = 3.18$, $N = 20$, $P = 0.002$; Fig. 5) and killed ($Z = 3.12$, $N = 20$, $P = 0.002$; Fig. 6) significantly fewer during the memory test than during the avoidance-learning session.

Table 2
Total numbers (*N*) and percentages of hand-reared and wild-caught great tits that handled (H%), and killed (K%) firebugs of different colour forms

Colour form	Hand-reared			Wild-caught		
	N	H%	K%	*N*	H%	K%
Red	60	100	92	50	22	2
Yellow	40	98	95	50	58	42
Brown	20	100	85	50	78	58

Data on hand-reared birds are taken from the avoidance-learning phase: groups 1, 4 and 6 for the red colour form; groups 2 and 5 for the yellow colour form; group 3 for the brown colour form. Data on wild-caught birds are taken from Exnerová et al. (2006).

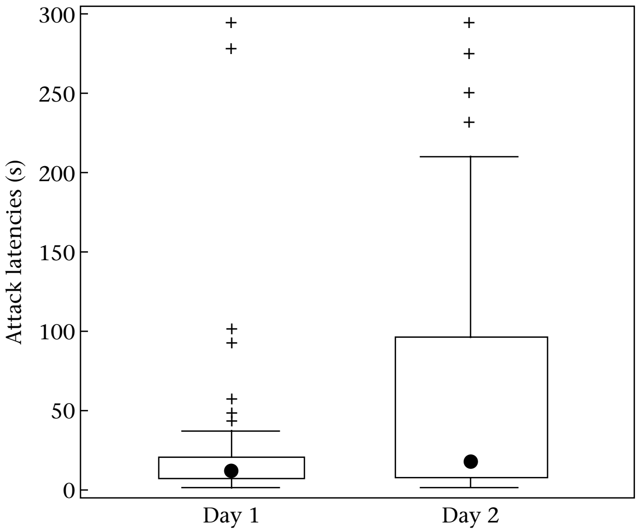

Figure 3. Attack latencies of naïve great tits from the beginning of the first trial to the first handling of firebugs in the avoidance learning (day 1) compared with those in the memory test (day 2). Total number of tested birds: 57 (groups 1–3 pooled). Dots indicate the median, the box the lower and upper quartiles (interquartile range), the whiskers the nonoutlier range (values within 1 times the interquartile range outside the closest quartile) and crosses the outliers.

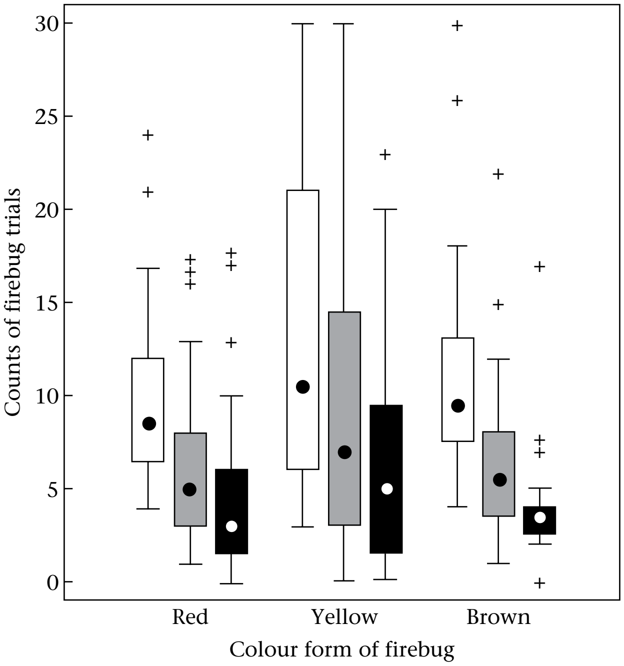

Figure 4. Counts of firebug trials until the naïve great tits reached the avoidance-learning criterion (white bars), and of trials in which the birds handled (grey bars) and killed (black bars) firebugs of different colour forms during the avoidance learning. Total number of birds tested: 60 for the red form (groups 1, 4, 6), 40 for the yellow form (groups 2 and 5) and 20 for the brown form (group 3). Box plots as in Fig. 3.

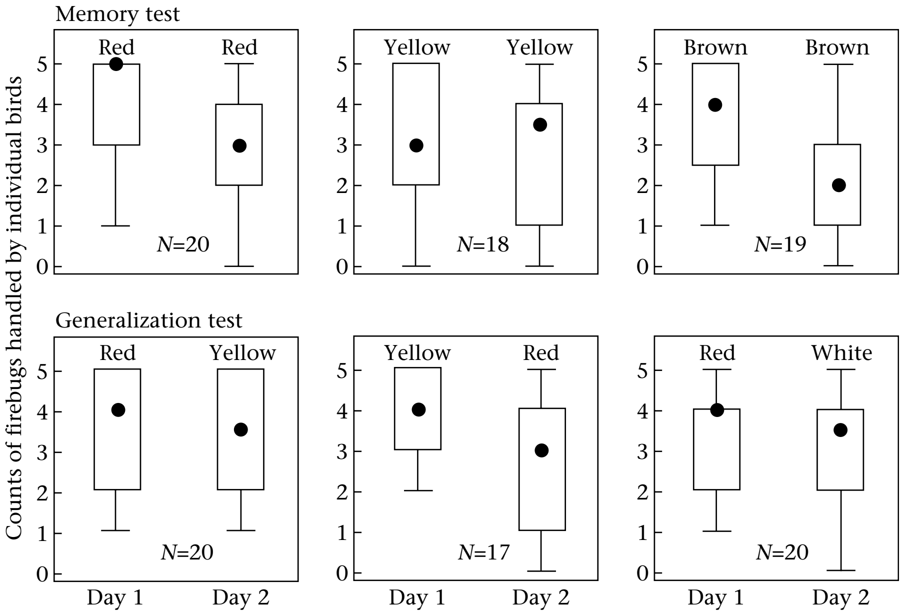

Figure 5. Counts of individuals of different colour forms of the firebug handled by naïve great tits in the first five trials of the avoidance learning (day 1, groups 1–6) and the memory test (day 2, groups 1–3) or generalization test (day 2, groups 4–6). Box plots as in Fig. 3.

Similarly, birds that learned to avoid the brown firebugs (group 3) handled ($Z = 2.77$, $N = 19$, $P = 0.006$; Fig. 5) and killed ($Z = 2.51$, $N = 19$, $P = 0.012$; Fig. 6) significantly fewer in the memory test than during the avoidance-learning session.

For yellow mutants (group 2) we found no difference between the avoidance learning and the memory test in the numbers of mutants handled ($Z = 1.11$, $N = 18$, $P = 0.266$; Fig. 5) and killed ($Z = 1.07$, $N = 18$, $P = 0.287$; Fig. 6) by individual birds.

Generalization Test

Birds that learned to avoid the red firebugs did not generalize their experience to the yellow (group 4) and to the white (group 6) form. They handled and killed similar numbers of the yellow and white mutants during the generalization test as they did the day before with the red form during the avoidance learning (group 4, yellow mutants: Wilcoxon signed-ranks test: handling: $Z = 0.82$, $N = 20$, $P = 0.415$; killing: $Z = 0.02$, $N = 20$, $P = 0.983$; group 6, white mutants: handling: $Z = 1.10$, $N = 20$, $P = 0.272$; killing: $Z = 1.85$, $N = 20$, $P = 0.064$; Figs 5, 6).

In contrast, birds that learned to avoid the yellow mutants (group 5) handled and killed significantly fewer red firebugs during the generalization test (handling: $Z = 2.49$, $N = 17$, $P = 0.013$; killing: $Z = 2.86$, $N = 17$, $P = 0.004$) than yellow mutants during the avoidance learning the day before (Figs 5, 6).

Birds experienced with the yellow mutants (group 5, generalization test) handled and killed fewer red firebugs than the naïve birds (group 1, first five firebug trials of the avoidance learning; Kruskal–Wallis test: handling $H_1 = 4.72$, $P = 0.030$; killing: $H_1 = 6.82$, $P = 0.009$; Figs. 5, 6). The birds experienced with the red firebugs (group 4, generalization test) did not differ from naïve birds (group 2, first five firebug trials of the avoidance learning) in the number of handled and killed yellow mutants (handling: $H_1 = 0.01$, $P = 0.916$; killing: $H_1 = 0.01$, $P = 0.929$; Figs 5, 6).

DISCUSSION

Unlearned Biases

The first factor that could play a role in predators' prey choice is the existence of initial innate biases: preferences for or avoidance of certain colours. Schuler & Hesse (1985) attributed domestic chicks' avoidance of black-and-yellow prey to a genetically fixed predisposition. Similarly, wild birds show strong unlearned avoidance particularly of venomous aposematic coral snakes (Smith 1975, 1977). Lindström et al. (1999a) referred to an inherited component in behaviour towards colour signals in great tits. However, simultaneous presentation of the different prey types used in their experiment did not allow for a distinction between innate avoidance of warning coloration (yellow-and-black) and preference for alternatively coloured (brown) prey. Nevertheless, there may still be something special in the yellow-and-black coloration that could elicit an unlearned component of avoidance. In our previous experiments we found that naïve great tits do not have any innate avoidance of red-and-black, wild-type firebugs (Exnerová et al. 2007) whereas blue tits, *Cyanistes caeruleus*, and coal tits, *Periparus ater*, do. Our present results show an absence of innate avoidance in great tits for the yellow mutants as well. Ham et al. (2006) also found no initial avoidance of new, warningly coloured, artificial prey (red, orange and yellow).

The effectiveness of warning signals is increased by their multimodality. Certain olfactants (typically pyrazines) may evoke innate aversion to warningly coloured prey or reinforce innate neophobic reactions: an effect described as 'hidden colour aversion' (Marples et al. 1994; Rowe & Guilford 1996, 1999; Kelly & Marples 2004). We did not find any such effect in our experiments. There are two most likely explanations: (1) aldehydes that dominate the secretion of metathoracic glands of adult firebugs (Farine et al. 1992) do not, in contrast to pyrazines, evoke unlearned avoidance of warning colours; (2) scent glands of pyrrhocorids are reduced relative to those of other true bug families (Schuh & Slater 1995) and the semiochemicals are mainly low volatiles (Farine et al. 1992).

Naïve Versus Wild-caught Birds

In contrast to the naïve birds, the majority of our wild-caught great tits (Exnerová et al. 2006) avoided red-and-black wild-type firebugs ($\chi^2_1 = 72.51$, $P < 0.001$; Table 2) and about half of the wild-caught birds also avoided the yellow mutants ($\chi^2_1 = 18.77$, $P < 0.001$; Table 2). Brown-painted firebugs were attacked not only

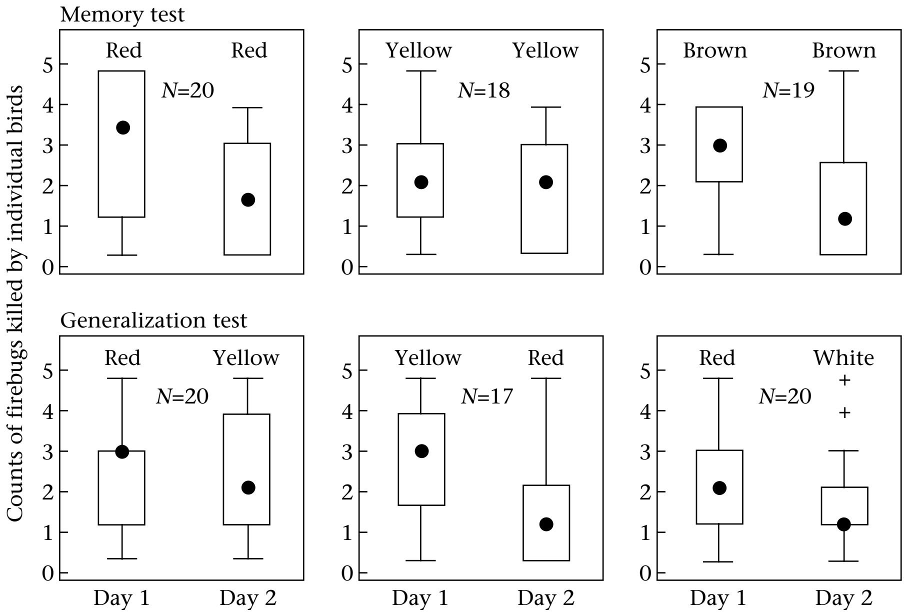

Figure 6. Counts of individuals of different colour forms of the firebug killed by naïve great tits in the first five trials of the avoidance learning (day 1, groups 1–6) and the memory test (day 2, groups 1–3) or generalization test (day 2, groups 4–6). Box plots as in Fig. 3.

by all hand-reared birds, but also frequently by wild-caught birds (Yates corrected $\chi^2_1 = 3.69$, $P = 0.055$; Table 2). The avoidance of red firebugs by adult great tits is most likely to be the result of avoidance learning, and the same may be true for the avoidance of yellow mutants. Even though young birds can learn to avoid brown firebugs, adult wild-caught birds do not avoid them, possibly because of their previous experience with palatable brown insects. Therefore, the reactions of great tits seem to be more influenced by their different experience with variously coloured prey than by their initial unlearned biases.

Attack Latencies

Even if the risk of being attacked is not influenced by prey coloration, the speed of an attack may be. Warning coloration may function as a 'go-slow' signal (Guilford 1994) and may cause longer latency between the prey detection and attack. Such a delay could increase prey survival, because the prey could hide or escape. Attack latency may be influenced by various properties of the warning signals. Gamberale-Stille (2000) found longer attack latencies in naïve domestic chicks when offered groups of aposematic larvae of *Tropidothorax leucopterus* (Heteroptera: Lygaeidae) than when offered solitary larvae. Attack latencies may be influenced by the conspicuousness of the prey against the background. Domestic chicks took longer to peck coloured beads presented on a contrasting rather than on a matching background (Roper & Redston 1987). In contrast, latencies of naïve birds do not seem to vary with different prey coloration when all types of prey are conspicuous (presented on a nonmatching background). Gamberale-Stille & Tullberg (1999) observed no difference in the attack latencies of chicks offered larvae of two species of Lygaeidae (Heteroptera) that differed in the intensity of red coloration.

Similarly, attack latencies in our experiments did not differ between firebugs of different warning colours (red and yellow), or between aposematic and nonaposematic (brown) forms. A difference in the attack latencies was observed only between firebugs and mealworms used as control prey. This difference is probably caused by the novelty effect, because the mealworms were familiar to the birds whereas the firebugs were novel to them. However, there may be a synergistic effect of novel prey appearance and its chemical signal eliciting unlearned wariness and increasing the latency of first contact with the novel prey (Marples & Roper 1996, 1997; Kelly & Marples 2004). In our experiments such an effect was small in comparison with those reported in the above studies.

Avoidance Learning

Predators that do not possess any kind of innate avoidance learn the characteristic features of aposematic prey during their individual experience. Generally, two main questions concerning learning may be addressed: (1) what do predators actually learn (contents of learning), and (2) what are the conditions that bring learning about and affect it (Shettleworth 1998)?

Visual signals of aposematic prey usually have several features, which predators may associate with unpalatability, either independently, or as elements of a compound stimulus: (1) colour (Sillén-Tullberg 1985a; Gamberale-Stille & Guilford 2003); (2) pattern (Evans et al. 1987; Forsman & Merilaita 1999); (3) intrinsic contrast between differently coloured body parts; and (4) contrast against the background (Gittleman et al. 1980; Roper 1990; but see Gamberale-Stille & Guilford 2003).

The birds in our experiments learned an association between a specific colour (conditioned stimulus) and unpalatability of the prey (unconditioned stimulus). With the exception of those birds that generalized from yellow to red firebugs, the great tits did not avoid the firebug when it was a different colour, even though its other characteristics (shape, size, pattern, defensive secretion, way of locomotion) were the same. It seems that, as in the experiments carried out by Aronsson & Gamberale-Stille (2008), a particular colour overshadowed pattern and other features of aposematic prey in the avoidance-learning process. On the other hand, the birds learned to avoid firebugs of all colours and the rate of learning of different colour forms did not differ in our experiments. This result agrees with those of Ham et al. (2006) who observed no effect of a specific colour on the rate of avoidance learning in experiments with wild-caught great tits and artificial red, yellow and grey food items. This is surprising, because typical warning colours (red, orange, yellow) are thought to facilitate avoidance learning (Cott 1940). This facilitation was suggested also in other experimental studies, particularly in an investigation where naïve great tits and larvae of *Lygaeus equestris* (Heteroptera: Lygaeidae) were used (Sillén-Tullberg 1985a). All colour forms of the prey were conspicuous against the nonmatching background in Ham et al.'s (2006) study as in our investigation, whereas in Sillén-Tullberg's (1985a) experiments one of the forms was cryptic on a grey background. This methodological difference may have contributed to the different results obtained. Together, the experimental results indicate that birds associate the specific colour of prey with its unpalatability and that contrast of the prey against the background may speed up the learning process (Sillén-Tullberg 1985a; Gamberale-Stille 2001; Riipi et al. 2001), although the contrast itself is not used as a cue (Gamberale-Stille & Guilford 2003). This could also explain the greater variance in learning rate among the birds that learned to avoid the yellow form than among those learning to avoid the red or brown forms (Fig. 4) since the yellow form was less contrasting with the beige background than the other two colour forms (Fig. 2).

Signal Memorability

Even though the rate at which predators learn to avoid variously coloured prey may be the same, some colour forms may gain an advantage because of their better memorability (Speed 2000). Despite the obvious importance of signal memorability and its frequent use in theoretical models (e.g. Turner & Speed 1996; Speed 1999, 2001; Servedio 2000) only a few experimental studies have focused on the comparison of memorability of different colours and patterns of prey. Results of existing studies are often difficult to compare owing to the use of different experimental designs; the differences concern single- versus multiple-trial learning, simultaneous versus consecutive presentation of palatable and unpalatable prey, various lengths of intervals between learning sessions, and memory tests ranging from 1 h to 1 week. It is practically impossible to distinguish between the effect the signal has on learning from its effect on memory in studies using a single-trial learning design (e.g. Roper & Redston 1987; Roper 1994).

It is generally assumed that predators remember the typically aposematic colours as unpalatability signals better than other colours (reviewed in Speed 2000). However, experimental studies do not support this assumption. In experiments with larvae of two lygaeid species (*T. leucopterus* and *L. equestris*) Gamberale-Stille & Tullberg (1999) found no effect of the intensity of red coloration on memory in domestic chicks. Ham et al. (2006) compared the memorability of variously coloured artificial prey items (yellow, red and grey) for wild-caught great tits and found that all colours (including grey) were equally well remembered as unpalatability signals. Our results suggest the same: birds can remember their experience with unpalatable prey of typical aposematic coloration (red-and-black) equally well as their experience with unpalatable prey of nonaposematic (uniformly brown) coloration. Relatively short periods between learning sessions and memory tests (up to 1 week) were used in all the experiments mentioned above. Consequently, it is possible that the difference in memorability between aposematic and nonaposematic colours may concern longer periods of maintaining the memory. A conspicuous, aposematic prey may, under natural conditions, also benefit from jogging the predator's memory (Speed 2000), that is, refreshing the memory by mere observation of the aposematic prey without the necessity of closer contact. Since the aposematic prey is easy to detect, predators may frequently observe it, which in turn could slow down or even eliminate forgetting (Speed 2000).

It seems surprising that birds in our experiments with firebugs remembered the yellow form worse than both the red and brown forms. The explanation may not be that the yellow mutants are less distasteful then the red firebugs: all colour forms have a similar composition of defensive secretion (J. P. Farine, A. Exnerová, P. Štys & R. Socha, unpublished data), were reared on the same seeds (*T. cordata*), and caused the same apparent discomfort when eaten by the birds. However, the yellow form was less contrasting with the beige background than the other two colour forms of the firebug (Fig. 2). This result is consistent with the influence of the contrast of prey against the background found in previous studies: prey contrasting with the background is remembered better than prey matching it (Roper & Redston 1987; Roper 1994; Alatalo & Mappes 1996). However, contrast alone does not seem to be a sufficient discrimination cue; those birds that had to rely only on contrast failed to learn a discrimination task (Gamberale-Stille & Guilford 2003).

Generalization

Bird predators readily generalize their experience with aposematic prey onto the prey with similar signals (Ruxton et al. 2004). The factors influencing this generalization are still debated. Generalization is thought to be broad when it follows an experience with strongly unpalatable prey (Lindström et al. 1997) and when the predator encounters only a small number of various prey species (Beatty et al. 2004). Experimental evidence demonstrates broad as well as narrow generalization under simplified laboratory conditions. Evans et al. (1987) showed that quails, *Coturnix coturnix*, generalized among three considerably different red-and-black hemipteran species: *Cercopis intermedia* (Cercopidae), *Caenocoris nerii* (Lygaeidae) and *Eurydema decoratum* (Pentatomidae). In contrast, Sillén-Tullberg et al. (1982), using great tits as predators, demonstrated that two warningly coloured prey items must be closely similar to share the protection: the adults of *L. equestris* did not benefit from the predators' experience with its larvae and vice versa. Similarly, wild-caught great tits, blue tits, European robins, *Erithacus rubecula*, and blackcaps, *Sylvia atricapilla*, generalized their experience with red-and-black firebugs only to the most similar orange mutants, but not to the yellow and white ones (Exnerová et al. 2006).

In our experiments hand-reared great tits generalized among differently coloured firebugs, but the generalization was asymmetric: the birds experienced with the yellow form subsequently avoided the red one, whereas the birds experienced with the red form did not avoid the yellow and white forms. An asymmetric generalization has already been reported to occur in the context of avoidance learning. Gamberale & Tullberg (1996) found that domestic chicks experienced with aposematic larvae of *T. leucopterus* of a certain size generalized their avoidance towards the larger (older) instars, but not towards the smaller (younger) ones. Similar results were obtained for the intensity of red coloration (Gamberale-Stille & Tullberg 1999). Chicks experienced with less intensely red larvae of *L. equestris* subsequently avoided more intensely red larvae of *T. leucopterus*, whereas chicks experienced with more intensely red larvae did not avoid those less intensely red. Another trait that may be generalized asymmetrically in avoidance learning is the conspicuousness of prey against the background. Great tits tested in a novel-world experiment shifted their avoidance towards more conspicuous artificial prey items (Lindström et al. 1999b). In contrast, Ham et al. (2006) found that wild-caught great tits generalized their conditioned avoidance of both the yellow and red artificial prey items to novel orange stimuli; the colours were thus generalized symmetrically. The reason for this could be the close similarity of the orange to both the yellow and the red. Birds probably generalize spectrally similar colours easily; then the asymmetry does not appear. Similarly, in our previous experiments with colour mutants of the firebug, wild-caught great tits generalized their experience with the red wild type to novel orange mutants (Exnerová et al. 2006).

The asymmetrical generalization occurring in avoidance learning has been interpreted as the result of a peak shift (Gamberale & Tullberg 1996; Gamberale-Stille & Tullberg 1999) or a process related to it (Lindström et al. 1999b). Whether this interpretation could be valid for our results as well is questionable, and there are at least two alternative explanations (see below).

Peak shift is a consequence of discrimination learning between positive (S^+) and negative (S^-) stimuli, which differ along the same stimulus dimension (Spence 1937; Hanson 1959; Ghirlanda & Enquist 2003; ten Cate & Rowe 2007). The peak of the positive response is then shifted from the positive training stimulus to a stimulus located further away from the negative stimulus. Similarly, the peak of the negative response is shifted further away from the positive stimulus. To identify a peak shift requires testing the birds at several locations along the stimulus dimension, which is difficult to do with live insect prey. Nevertheless, the yellow and red colours represent different points within the light wavelength continuum, which is one of rearrangement stimulus dimensions (that is, dimensions along which the stimuli do not differ in intensity but address different sets of receptors) along which the peak shift is known to occur (Ghirlanda & Enquist 2003; ten Cate & Rowe 2007). Recently, the peak shift has been well documented in domestic chicks trained to discriminate between rewarded and unrewarded stimuli that differed in the yellow-to-red part of the spectrum (Baddeley et al. 2007). The birds in our experiments were trained to discriminate between the palatable prey (mealworm, positive stimulus) and the unpalatable one (firebug of a particular colour, negative stimulus), although the two alternative prey items were not presented simultaneously but in succession. However, discrimination training may involve simultaneous as well as successive presentation of the stimuli to be discriminated (Shettleworth 1998; McFarland 2006), and we cannot a priori rule out the possibility that the birds learned the task this way. Consequently, the asymmetric generalization could be caused by a peak shift, because the birds generalized to the aposematic prey whose colour was further away from that of palatable mealworms (see Fig. 1).

Another explanation could be that the birds generalized towards the prey signal with more contrast against the background. Although all the colour forms of the firebug appeared conspicuous against the background of the wooden feeding tray, the red form was the most contrasting (Fig. 2). This interpretation is consistent with the tendency of domestic chicks to generalize towards a more contrasting pattern (Osorio et al. 1999).

Finally, there is a possibility that the generalization is facilitated in the particular direction by the stronger reflective signal of the red prey than of the yellow prey at all four avian retinal cone sensitivities (Chen & Goldsmith 1986).

Implications for Evolution of Warning Coloration

Whatever cognitive processes are responsible for asymmetric generalization of warning colours, it seems that the red colour represents a more effective warning signal than the yellow, at least for some bird predators. If this is true, we can expect the evolutionary transitions from yellow to red in warningly coloured prey to occur more frequently than in the opposite direction. There are indications of such a scenario in the Pyrrhocoridae: (1) the yellow precedes the red during the process of biosynthesis of pteridine pigments in *P. apterus* (Socha & Němec 1992; Bel et al. 1997); (2) the yellow chromotypes of *P. apterus* occur sporadically in nature but never form stable populations (cf. Exnerová et al. 2006); and (3) there have been three independent origins of red species from their yellow ancestors in the evolution of American *Dysdercus* species, but no reversions (Zrzavý & Nedvěd 1999).

The 'evolutionary transitions from yellow to red' in *P. apterus* and other Pyrrhocoridae indicate successful transitions resulting in chromatically more or less stable species-specific phenotypes (chromotypes) maintained by antiapostatic selection of which the birds are important agents. The mutation that results in the yellow chromotype in *P. apterus* is a deletion of the terminal part of the biochemical pathway of synthesis of the red pigment (erythropterin). Deletions are surely more common than additions, which represent real evolutionary novelties (e.g. yellow to red in our case). However, survival of the convergently arising yellow chromotypes in the field is brief (for discussion see Exnerová et al. 2006), and predation by birds may be the decisive selection factor.

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