

## European birds and aposematic Heteroptera: review of comparative experiments

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### Abstract

The efficiency of defensive mechanisms in 11 European aposematic species of Heteroptera against various passerine predators was analysed. Bird species differed in their reactions to aposematic preys: small insectivorous birds generally avoided aposematic bugs, but granivorous birds as well as large insectivorous birds frequently attacked them. The ability to overcome heteropteran chemical defences appears to be connected with the larger body size of birds and with their food-storing behaviour. From the bird's point of view, various red-and-black aposematic species of Heteroptera form a mimetic complex. However, antipredatory defence properties of individual species differ substantially in their efficiency against bird predators, and the nature of the mimetic complex is rather quasi-Batesian than Müllerian.

**Key words:** antipredatory defences, warning signals, Heteroptera Pentatomomorpha, Passeriformes.

### Introduction

Aposematism is a type of antipredatory strategy, when the prey signals its own unprofitability by a signal understandable to predators (Ruxton *et al.*, 2004). There is considerable evidence that prey defences as well as warning signals may be multimodal, i.e. may consist of visual, behavioural, acoustic, olfactory and gustatory components. Two or more warning signals either reinforce themselves or act synergistically, and the effect may lead to predator's unlearned avoidance or phobia, may accelerate avoidance learning, or enhance memorability of the warning signal (Rowe and Guilford, 1999; Ruxton *et al.*, 2004). With few exceptions (Marples *et al.*, 1994) the evidence is based mainly on experiments with artificial prey items and model predator species (usually domestic chicks). Therefore, the function of multimodal warning signals and defences of real prey species against a variety of their natural predators remains largely unknown.

The Heteroptera possess multimodal antipredatory defences whose main components are (a) visual signals (warning coloration or cryptic coloration), (b) acoustic signals (warning stridulation), and (c) allelochemicals (signalling the unpalatability, or directly repellent to toxic; synthesized by exocrine glands or taken over from hostplants and sequestered). This complex array of antipredatory defences makes the Heteroptera an excellent model group for studying aposematism and mimicry. Our studies were focused mainly on following problems (1) universality of warning function of aposematic signals against different avian predators, (2) abilities of predators to overcome bug defence system, (3) comparison of the efficiency of defensive mechanisms of various heteropteran species, (4) role of individual species in the potential mimetic complex of red-and-black pentatomomorphan true bugs, (5) importance of various components of heteropteran warning signals for learning and discrimination in bird predators.

### Materials and methods

#### Heteroptera

We tested the reactions of birds to adults of the following species: *Pyrrhocoris apterus* (L.), its white, yellow, and orange mutants, and brown-painted individuals; *Pyrrhocoris marginatus* (Kolenati); *Scantius aegyptius* (L.); *Lygaeus equestris* (L.)/*simulans* Deckert; *Spilostethus saxatilis* (Scopoli); *Tropidothorax leucopterus* (Goeze); *Horvathiolus superbus* (Pollich); *Corizus hyoscyami* (L.); *Graphosoma lineatum* (L.); *Eurydema oleraceum* (L.); *Eurydema ornatum* (L.). We included ladybirds *Coccinella septempunctata* L. and *Propylaea quatuordecimpunctata* (L.), and froghopper *Cercopis vulnerata* Rossi as possible non-heteropteran members of mimetic complexes.

#### Birds

Wild-caught passerine birds of the following species were tested: (1) mainly insectivorous species - *Turdus merula* L., *Erithacus rubecula* (L.), *Phoenicurus ochruros* (S. G. Gmelin), *Parus major* L., *Cyanistes caeruleus* (L.), *Periparus ater* (L.), *Lophophanes cristatus* (L.), *Poecile montanus* (Conrad), *Poecile palustris* (L.), *Aegithalos caudatus* (L.), *Sitta europaea* L., *Sylvia atricapilla* (L.); (2) partly granivorous species - *Passer montanus* (L.), *Passer domesticus* (L.), *Fringilla coelebs* L., *Carduelis chloris* (L.), and *Emberiza citrinella* L.. Hand-raised great tits (*P. major*) were used as naive predators in learning, memory, and discrimination experiments.

#### Experimental set-up

Experiments were carried out in the cage equipped with one-way glass, perch, and rotating feeding tray. Cage illumination simulated the full daylight spectrum. Bird's behaviour was scored as a continuous record in Observer Video-Pro (Noldus) and recorded by

videocamera. Each bird was deprived of food about two hours before starting the experiment. Experiment consisted of a sequence of several consecutive five-minute trials, during which the birds were offered individual bugs. The trials followed one after another and alternated with presentations of a standard prey, *Tenebrio molitor* L. larvae, to check bird's foraging motivation.

In the experiments with wild-caught birds, the sequence consisted of 5 trials with heteropterans. In experiments focused on learning in naive hand-reared birds, the sequence of five-minute trials continued until the birds reached the learning criterion. Memory or discrimination tests were performed next day after the learning session. The birds were subjected either to the sequence of trials with the same prey they learned to avoid, or with another prey, or to the preference test with several different types of prey.

## Results and discussion

### Universality of warning function of aposematic signals against different predators

Warning coloration of a certain species of Heteroptera does not have a universal function among passerine predators (Exnerová *et al.*, 2003). Smaller and insectivorous birds (chats, warblers and tits) generally avoided aposematic bugs, but granivorous birds (finches and buntings) as well as larger insectivorous birds (blackbirds and nuthatches) frequently attacked them. Even closely related bird species (family Paridae) can differ in the way they acquire the avoidance; it appears to be innate in some species and learned in others (Exnerová *et al.*, 2007). Surprisingly, firebugs (*P. apterus*) were avoided also by tree sparrows (*P. montanus*), which readily attacked and consumed unpalatable and poisonous ladybirds (*C. septempunctata*) during the experiments.

### Abilities of predators to overcome bug defence system

Generally, it appears that body size (weight) of the bird affects its cautiousness in encounters with potentially noxious aposematic bugs. Out of nine passerine species tested with *P. apterus*, the smallest species (*A. caudatus* and *C. caeruleus*) were most cautious and the largest species (*T. merula*) most prone to attacking and consuming the firebugs (Exnerová *et al.*, 2003). Similar trend was observed in the experiments with *L. equestris/simulans*, *S. saxatilis*, and *G. lineatum*. Some predators may possess physiological or behavioural adaptations to overcome the prey defences (Yosef and Whitman, 1992). Nuthatches (*S. europaea*) and crested tits (*L. cristatus*) frequently used slits in the experimental cage for storing the bugs (*P. apterus*), then checked them time from time, and eventually consumed them, after the repellent secretion vanished. Both species store the food regularly; food-storing behaviour seems to be a general exaptation for consuming noxious prey.

### Importance of heteropteran visual warning signals for learning and discrimination in bird predators

The colour is especially important cue for passerine predators among the various components of the visual warning signal of the true bugs. Majority of wild-caught birds (*P. major*, *C. caeruleus*, *E. rubecula*, *S. atricapilla*) experienced with red-and-black wild type of *P. apterus*, did not recognize its yellow and white colour mutants as the same prey, even though they had the same shape, size, and black pattern (Exnerová *et al.*, 2006). Similarly, naive great tits (*P. major*) primarily attended to colour and not pattern, when they learned to avoid *P. apterus* and its colour mutants. Moreover, their ability to generalize among various aposematic colours seems to be limited – birds generalized their experience with yellow form to the red one but not in the opposite direction. On the contrary, typical warning coloration (red-and-black) did not accelerate the avoidance learning when compared with the non-aposematic (uniformly brown) coloration. However, great tits remembered their experience with red-and-black *P. apterus* even after three months, while their memory for brown-painted form vanished.

### Comparison of the efficiency of defensive mechanisms of various species of Heteroptera

Effectiveness of defensive mechanisms was assessed in the experiments with naive great tits (*P. major*), in which the rate of avoidance learning and memory for the experience with various species of Heteroptera was measured. *L. equestris/simulans* and *S. saxatilis* were strongly defended, the birds usually avoided them after one or two encounters, and even the attacked bugs usually survived. *G. lineatum* appeared similarly well defended, and was protected also by its strongly sclerotised cuticle. *P. apterus* was rather weakly defended; the birds usually took several encounters to develop the avoidance reaction and the encounters were usually lethal for the bug. *Eurydema* species seemed to be weakly defended, and they are probably quasi-Batesian mimics of other heteropterans and ladybirds; this hypothesis may explain their otherwise surprising colour polymorphism.

### Role of individual species in the potential mimetic complex of red-and-black pentatomomorphan true bugs

Majority of red-and-black Pentatomomorpha from Central Europe form a mimetic complex. Wild-caught great tits (with a few exceptions) avoided all the heteropterans studied. Similarly, naive great tits generalized their experience with one of the red-and-black species to any other one, even if it was of quite different appearance. Nevertheless, protection of the individual species does not fit the simple Müllerian concept of all the species sharing the costs and benefits equally. Individual species play rather different roles in the complex: *L. equestris/simulans* and *S. saxatilis* being effective models, and *P. apterus* rather quasi-Batesian mimic. The complex includes also non-heteropteran members – *C. septempunctata* and *C. vulnerata*, which both can play a role of models.

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Original Article

# Different reactions to aposematic prey in 2 geographically distant populations of great tits

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Variation in predator behavior toward aposematic prey was frequently studied at interspecific and individual levels, but interpopulation differences have been neglected. Geographic differences in prey fauna offer an opportunity to test their implications for predator behavior. It can be expected that 1) predator populations inhabiting environments with high diversity of aposematic prey are more neophobic than those living in areas where aposematic prey are scarce, and 2) different levels of neophobia jointly with avoidance learning affect selection on aposematic prey. We compared the behavior of wild-caught great tits (*Parus major*) from Bohemia and Central Finland toward aposematic firebugs (*Pyrrhocoris apterus*), nonaposematic firebugs, novel objects and novel palatable nonaposematic prey. Finnish and Bohemian birds did not differ in their novel-object exploration, but Finnish birds hesitated longer than Bohemian birds before resuming feeding next to a novel object. Latencies to attack novel palatable prey did not differ and were not correlated with the attitude toward novel objects. Tits from the Bohemian population mostly avoided aposematic firebugs and attacked nonaposematic ones. Finnish birds were more likely to attack both firebug color forms, and their attack latencies were correlated with latencies of attacking novel palatable prey. Thus, Bohemian birds avoided the aposematic prey, but were not more neophobic than Finnish birds. These results suggest that differences between Finnish and Bohemian birds in behavior to aposematic prey do not follow differences in exploration strategy and neophobia. The observed differences can be explained by a different experience with local aposematic prey communities.

**Key words:** aposematism, exploration, geographic differences, neophobia, *Parus major*, *Pyrrhocoris apterus*.

## INTRODUCTION

Avoidance of aposematic prey usually involves several cognitive mechanisms that affect the behavior of predators (Ruxton et al. 2004). Reaction toward prey may be influenced by neophobia (e.g., Coppinger 1969, 1970; Exnerová et al. 2010) or dietary conservatism (Marples et al. 1998; Marples and Kelly 1999), and by inherited (Smith 1975; Lindström et al. 1999a) or learned (e.g., Lindström et al. 1999b; Exnerová et al. 2007; Aronsson and Gamberale-Stille 2008; Barnett et al. 2012) aversions against certain warning signals and their combinations (Marples and Roper 1996; Rowe and Guilford 1996; Lindström et al. 2001). The response of a predator to warning signals is affected by associative learning, the degree of which is influenced by memorability,

prey recognition, discrimination, and generalization (Roper and Redston 1987; Gamberale-Stille and Tullberg 1999; Speed 2000; Ham et al. 2006; Svádová et al. 2009). Given the complexity of the cognitive processes, which contribute to the formation of the avoidance of aposematic prey, it is not surprising that there exists a considerable variation in behavior of different predators toward a defended prey species (Brower 1988; Exnerová et al. 2003; Endler and Mappes 2004; Valkonen et al. 2012; Nokelainen et al. 2014).

Predators from different taxa may react differently to a particular prey species, and several mechanisms have been discussed as potential factors responsible for the variation: the energetic requirements of a predator (i.e., body size or hunger level) are important in determining whether or not the predator decides to attack and consume a defended prey (Exnerová et al. 2003; Barnett et al. 2007; Halpin et al. 2014). Also, sensory and cognitive abilities of predators can be highly variable (Hart 2001; Sol et al. 2005) influencing

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their ability to learn to avoid aposematic prey (Endler and Mappes 2004). Closely related species also frequently differ in their reactions to novel environments, objects and food; the neophobia level may be correlated with the degree of habitat and foraging specialization (Greenberg 1989; Mettke-Hofmann et al. 2009, 2012; Tebbich et al. 2009).

Variation in reactions to aposematic prey also exists among conspecific predators, where the initial wariness and rate of avoidance learning may be correlated with personality traits (Exnerová et al. 2010). On the other hand, the rate of incorporation of a novel food item into an individual's diet represents another process: dietary conservatism (Marples et al. 1998; Marples and Kelly 1999), which is independent of personality and not correlated with neophobia (Marples and Mappes 2011). The behavior of predators toward aposematic prey may also be correlated with their age (Lindström et al. 1999a; Exnerová et al. 2006; Langham 2006; Mappes et al. 2014), and due to the importance of learning also highly affected by individual experience (Exnerová et al. 2007; Ihalainen et al. 2008; Barnett et al. 2012; Hotová Svádová et al. 2013).

In contrast to interspecific and individual differences in behavior toward aposematic prey, the potential differences between conspecific populations, namely those living in geographically distant areas and different habitats, have not been studied. Despite being interesting per se, the knowledge of potential geographical differences may be important for the generalization of results based on studies of different populations of a particular species of predator. Individuals from geographically distant populations may react differently to aposematic prey simply because of different individual experience with local aposematic prey. Alternatively, the behavior toward aposematic prey may reflect population-specific differences in neophobia (see Liebl and Martin 2014) and exploration strategies evolved for living in different conditions such as prey diversity and frequency of noxious prey. Individuals from populations living in different conditions (e.g., different predation pressure and environment stability) differ in their exploration of a novel environment, and their reactions to novel objects and novel food (Martin and Fitzgerald 2005; Brydges et al. 2008; Echeverría and Vassallo 2008; Korsten et al. 2010; Liebl and Martin 2014). Likewise, individuals from migratory populations may be more neophobic than their resident conspecifics (Mettke-Hofmann et al. 2013).

In this study, we investigated geographical differences in response to novel stimuli and reaction to aposematic prey in the great tit (*Parus major* L., 1758), a small passerine which is mainly insectivorous during spring and summer, although in autumn and winter, when insect prey become scarcer, adds berries and seeds to its diet (Cramp and Perrins 1993). The great tit is a resident species inhabiting a wide range of woodland habitats in the Palaearctic region, and its distribution covers the whole Europe including the far North (Cramp and Perrins 1993). In recent years, the great tit has become a model species in studies on aposematism and mimicry. Because such studies are based on experiments with birds from various localities across Europe (e.g., Sillén-Tullberg 1985; Lindström et al. 1999a, 1999b; Exnerová et al. 2006), it is worth testing whether birds from different populations behave in the same way. As a model aposematic prey we used the firebug (*Pyrrhocoris apterus*), which is conspicuously red-and-black colored and unpalatable for small passerine birds (Exnerová et al. 2003). The firebug is widespread in the Palaearctic, but it is absent in Britain and most of Northern Europe.

By comparing the behavior of wild-caught great tits from 2 geographically distant areas, Bohemia and Central Finland, we tested

the following hypotheses concerning the reaction of these birds toward aposematic prey: 1) Differences in behavior toward aposematic firebugs follow differences between the 2 populations in their behavior toward novel palatable prey and other types of novel objects, that is, they reflect the levels of individual exploration and neophobia. 2) The 2 populations exhibit specific differences concerning the aposematic firebugs. The Finnish birds are expected to be more willing to attack aposematic firebugs because of their lack of experience with this type of the aposematic prey in their natural environment. 3) Birds from both populations avoid attacking firebugs regardless of their experience. This may happen if the avoidance of aposematic prey has a strong genetic basis or the avoidance learning of prey with a given warning signal is generalized to other prey whose signal is similar enough. Because the local diversity of aposematic prey is important for the interpretation of the behavior of birds, we also analyzed data on the distribution of aposematic and nonaposematic species of Heteroptera between the 2 compared areas.

## MATERIALS AND METHODS

Experiments were carried out at Konnevesi Research Station, University of Jyväskylä (Central Finland) and in Prague at the Faculty of Science, Charles University (Bohemia) during autumn 2012. In order to standardize the phenological differences between the 2 localities, the experiments were conducted during October in Central Finland and during November in Bohemia.

### Comparison of occurrence of aposematic species of Heteroptera in Central Europe and Central Finland

In order to compare the composition of the fauna of true bugs from both areas, we gathered data on the occurrence of heteropterans, particularly of the aposematic species, in Central Europe and Central Finland. Data for Central Europe were obtained combining the whole heteropteran faunas of Germany (species included in Wachmann et al. 2004, 2006, 2007, 2008) and Czech Republic (Štys P, unpublished data). As for Central Finland, we covered species occurring in Finland at the latitude of Konnevesi (62°38'N), or likely to occur there (known to occur slightly more to the South or to the North), as shown by distributional maps (Rintala and Rinne 2010). Purely coastal species were excluded. Taxonomically, we considered only the terrestrial Heteroptera (Leptopodomorpha, Cimicomorpha, and Pentatomomorpha s. lat.). In their adult stage, the dorsum of those species regarded as aposematic is uniformly colored with bright white, yellow, orange or red, or with a combination of any of those and a contrasting dark pattern. For the purpose of this analysis, we also classified as aposematic those species having nonaposematic morphs as well. The dull whitish, yellowish to reddish taxa/morphs were not taken as aposematic (e.g., some Miridae: Phyllinae). All terrestrial true bugs are known to have a chemical defense (Schuh and Slater 1995).

### Predators

Altogether we tested 100 wild-caught great tits, 50 from each of 2 geographically distant populations: 1) Konnevesi in Central Finland (62°38'N, 26°19'E) and 2) Prague in Bohemia (Czech Republic, 50°04'N, 14°26'E). The words "Finnish" and "Finland," whenever used without qualification in the text, always refer to Central Finland at about Konnevesi latitude. The sex and age of both experimental groups were balanced (Central Finland: 30 males and

20 females, 19 yearlings and 31 adults; Bohemia: 29 males and 21 females, 17 yearlings and 33 adults). The birds from both populations had similar body weight (Bohemian birds—mean  $16.5 \text{ g} \pm 0.9 \text{ g}$ ,  $N = 50$ ; Finnish birds—mean  $17.1 \text{ g} \pm 1.0 \text{ g}$ ,  $N = 50$ ).

The habitats around Konnevesi include mainly sparse mixed forest with a low undergrowth of moss, grasses and sedges, herbaceous plants and shrubs; the forest is interspersed with large clearings including also buildings and human settlements; a brook and meadows along lake sides are present as well. The Prague locality consists mainly of large city parks with coniferous and broad-leaved trees; the parks are surrounded by roads and residential houses with small patches of ruderal vegetation interspersed. The major park is an old botanical garden, which represents various Bohemian habitats, and also includes several pools and a small brook. The birds were trapped in the autumn, when overwintering birds typically move around (Cepák et al. 2008). Thus, birds in each locality were likely to be coming from surrounding areas as well.

Birds were caught using food-baited traps (Central Finland, see Ham et al. 2006 for details) or mist nets placed near the feeders (Bohemia) during autumn 2012. They were housed individually in cages (50 cm × 40 cm × 50 cm in Prague; 65 cm × 50 cm × 80 cm in Konnevesi) under natural light conditions and were kept on a diet consisting of mealworms (larvae of *Tenebrio molitor* L., 1758), peanuts, sunflower seeds and water ad libitum. The birds were allowed to habituate to the laboratory conditions for 5–7 days before the experiment. Each bird was used only once in each experiment. After the experiment they were ringed individually and released in the locality of their capture.

## Prey

As aposematic prey we used brachypterous adult firebugs [*Pyrrhocoris apterus* (L., 1758); Heteroptera], which possess a conspicuous red-and-black coloration. The species' defensive secretion from metathoracic glands containing mainly aldehydes (Farine et al. 1992) makes this insect distasteful for small passerine birds including great tits (Exnerová et al. 2003). The firebugs live on the ground and partly also on trees, and feed mainly on seeds of Malvaceae (herbaceous species and linden tree, *Tilia*) and locust tree, *Robinia pseudacacia* (Kristenová et al. 2011; Hotová Svádová et al. 2014). They are widespread in the Palaearctic but absent in most of Northern Europe; their range does not exceed the latitude of 60°N (Aukema and Rieger 2001; Rintala and Rinne 2010).

A nonaposematic variant of the firebugs lacking the red-and-black color pattern was obtained by painting their upper parts with dark brown watercolor dye and chalk. We used these color-manipulated bugs to test the specificity of birds' reaction toward the firebugs' warning coloration, as we needed prey that did not differ from the aposematic prey in any other trait (size, body shape, composition of defensive secretion, and so forth). The dye used to modify the visual part of the firebug warning signal was odorless and nontoxic, and the chemical defense of these artificially made nonaposematic firebugs was unchanged (see Exnerová et al. 2003). The firebugs were collected in Prague (Czech Republic). They were kept at a temperature of  $24 \pm 1 \text{ }^\circ\text{C}$  and a light: dark cycle of 16:8 h, reared on linden seeds (*Tilia cordata*) and provided with water ad libitum.

Mealworms (larvae of *Tenebrio molitor*) were used as a palatable control prey to check the foraging motivation of birds before starting a trial with experimental prey. We used nymphs of Jamaican field crickets [*Gryllus assimilis* (Fabricius, 1775)] carrying a bright-blue paper sticker attached to their dorsal side as a novel, edible,

nonaposematic prey to test the level of birds' food-specific neophobia. The size of crickets offered in experiments matched the average size of tested firebugs (i.e., 10–12 mm). The sticker covered most of the cricket's dorsum, leaving its antennae and legs visible. In a preliminary experiment (involving 2 other groups of 20 wild-caught birds, both from Bohemia), great tits hesitated longer before attacking crickets with a blue sticker (mean  $179.2 \text{ s} \pm 28.2$ ,  $N = 20$ ) than before attacking those without the sticker (mean  $40.9 \text{ s} \pm 12.4$ ,  $N = 20$ ; Mann–Whitney *U*-test:  $Z = -3.92$ ,  $N = 40$ ,  $P < 0.001$ ), which they attacked with similar latencies as familiar mealworms (mean  $24.6 \text{ s} \pm 10.5$ ; Wilcoxon matched pairs test:  $Z = 1.49$ ,  $N = 20$ ,  $P = 0.135$ ). These results indicate that crickets with the blue sticker represent a stimulus sufficiently novel to increase attack latency.

## Experimental design and equipment

Experiments were designed to compare the exploration behavior, levels of neophobia, and specific reactions to aposematic and novel palatable prey between 2 populations of great tits. Each bird was tested individually in 4 separate tests in the following order, which was identical for all the birds: 1) exploration test with a novel object, 2) neophobia test with a novel object placed near the food bowl, 3) test of reaction toward novel palatable prey, and 4) test of reaction toward aposematic firebug or its nonaposematic brown-painted variant. This way the recent aversive experience with firebugs did not affect the reactions of birds toward novel objects and palatable prey, and the order per se did not influence the comparison between populations.

### Exploration and neophobia tests

To study exploration behavior and neophobia level we carried out 2 types of novel-object tests: 1) a novel object presented in a neutral location, and 2) a novel object attached to the food bowl. A novel object presented in a neutral location is frequently used to measure exploration behavior and neophilia, because the bird is not forced to approach the novel object, and when it does, it indicates its interest in exploring the object (e.g., Verbeek et al. 1994; Mettke-Hofmann et al. 2002; Drent et al. 2003). The presentation of a novel object close to the food bowl is regarded as a measure of neophobia, because it creates a conflict between foraging motivation and motivation to avoid a novel object; the bird has to overcome the neophobia to come close to the object and feed (Mettke-Hofmann et al. 2002; Feenders et al. 2011; Mettke-Hofmann 2012). Although exploration and neophobia may be correlated as they represent personality traits (van Oers et al. 2004), they are considered to be 2 distinct responses to novel stimuli (Greenberg and Mettke-Hofmann 2001).

In the exploration test, we used a bright-blue pen attached to one of the perches close to the front wall of the home cage. We measured the latency to peck at the novel object. The test lasted 10 min (maximum) and was terminated earlier if the bird pecked at the novel object. Food and water were freely available at all times.

In the neophobia test, we used a pink plastic clothes-peg attached to the food bowl placed on the home-cage floor. The birds were deprived of food for 1 h before the test to increase their foraging motivation. We recorded the latency to feed near the novel object. The test lasted 10 min and was terminated earlier if the bird started to feed in presence of the novel object. To control for potential differences in foraging motivation, we also carried out a control test under the same conditions but with the peg absent.

### Novel and aposematic prey tests

Tests with novel palatable prey and firebugs were performed in experimental cages and followed one after another. Experimental cages used in Bohemia and in Central Finland were of similar size (70 cm × 70 cm × 70 cm in Bohemia and 50 cm × 50 cm × 70 cm in Central Finland); they were made of plywood and a wire mesh, and equipped with a perch and a water bowl. The front wall of cages used in Bohemia was made of 1-way glass; the birds in Central Finland were observed through a small mesh-covered window in the cage wall. Illumination of the cages simulated the natural daylight spectrum (including UV wavelengths). The tested prey was put into the cage in a glass Petri dish placed on a sliding food tray on the cage floor. Despite of difference in the cage sizes, the distance between the food tray and the closest perch was identical (35 cm). All the prey types appeared conspicuous on the light beige background of the plywood food tray. Prior to the experiments, the birds were habituated to the experimental cages, and they were deprived of food for 2 h to increase their foraging motivation. The experiments were video-recorded and the birds' behavior was continuously registered using Observer XT 8.0 software.

### Novel palatable prey test

The novel, palatable, nonaposematic prey was represented by a Jamaican field cricket with a blue-colored paper sticker attached to its dorsal side. The experiment consisted of a sequence of 5-min (maximum duration) trials. At the beginning of the sequence, we offered the bird a mealworm as a control prey to check its foraging motivation. When the bird consumed a mealworm, it was offered a cricket in the subsequent trial. If the bird did not attack the cricket within the time limit, the sequence continued with another mealworm trial followed by another cricket trial up to a maximum of 3 cricket presentations. We measured the latency to attack (touch, peck, or seize) the cricket and recorded whether the cricket was killed and eaten.

### Firebug test

The birds from both Bohemian and Finnish populations were subdivided into 2 experimental groups of 25 birds each, with similar proportion of yearlings and adults, and males and females. One group was tested with aposematic red-and-black firebugs, and the other one with manipulated (brown-painted), nonaposematic (but still unpalatable) firebugs.

The test consisted of a sequence of alternating trials in which the birds were presented either with a control prey (mealworms) as a check of foraging motivation or with a firebug. Each trial lasted 5 min at most, and was terminated earlier if the bird attacked the prey. The sequence always started with a mealworm trial. When the bird consumed a mealworm, it was offered a firebug in the subsequent trial. If the bird did not attack the firebug within the time limit, the sequence continued up to a maximum of 20 firebug presentations. We measured the latency to approach and attack (to touch, peck, or seize) the firebug and recorded whether it was killed, thrown away, or eaten. If the bird attacked one of the firebugs, we kept offering them (alternating with mealworms) until the bird left untouched 3 firebugs in a row, which was considered an avoidance-learning criterion.

### Data analysis

A Cox hazard regression was used to analyze the differences between Bohemian and Finnish great tits in exploration behavior toward a novel object (the latency to peck at the blue pen) and in

neophobia (the latency to feed in presence of a novel object, the pink clothes-peg). Locality of bird origin, and bird's sex and age were used as explanatory variables. In the analysis of neophobia, we included the control (peg absent) latencies in the model as a covariate. The model selection procedure started from the model including all possible 2-way interactions of locality, age and sex, and was subsequently simplified. Model selection was conducted in a hierarchical manner based on the significance of the terms in the model.

A Cox hazard regression was also used to analyze the behavior of birds to novel palatable prey (cricket with blue sticker), aposematic or nonaposematic firebugs, and control familiar prey (mealworm offered at the beginning of the experiment). As a response variable we used latency to attack each prey type (cricket, firebug, or mealworm). Locality, bird's sex and age, and in case of firebugs also their coloration (aposematic, nonaposematic), were used as explanatory variables. The model selection procedure was similar to that used for analyzing reaction to a novel object.

We computed Spearman rank correlations between latency to peck at the novel object in the exploration test, latency to feed in the presence of novel object in the neophobia test, and latencies to attack the novel palatable prey and the firebugs. To check whether the birds considered crickets with blue stickers a novel prey, we compared attack latencies between crickets and mealworms offered in the trial preceding the cricket test; the latencies were compared by Wilcoxon matched pairs test.

We used the number of attacked firebugs before a bird stopped attacking them as an indicator of learning. A generalized linear model with Poisson distribution was used to analyze the data. The model selection was based on significance of the terms in the model. Bird sex and age, as well as locality of bird origin and firebug coloration, and all possible 2-way interaction terms were used as explanatory variables. All analyses were conducted using R 2.11.1 and the lme4 package.

## RESULTS

### Comparison of occurrence of aposematic species of Heteroptera in Central Europe and Central Finland

Fauna of terrestrial Heteroptera of Central Europe comprises 922 species, 766 (83%) of which are nonaposematic, and 156 (17%) aposematic; in Central Finland 262 (90%) out of 292 species of Heteroptera are nonaposematic, whereas 30 (10%) are aposematic. Thus, the less speciose fauna of Central Finland (32% species as compared with Central Europe) has not only a smaller absolute number of aposematic species (19% as compared with Central Europe) but also the proportion of aposematic species in the fauna is 0.59 times smaller than it should be in accordance with an uniform decrease in species diversity with increasing latitude (chi square = 7.75, df = 1, and  $P = 0.006$ ).

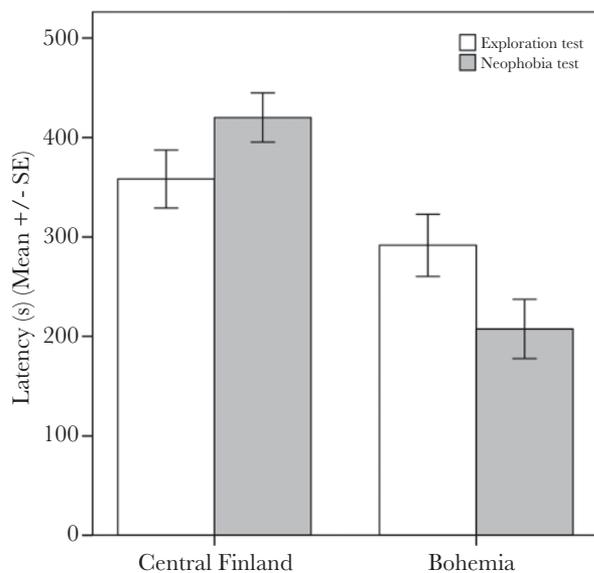
### Exploration and neophobia

We fitted Cox hazard regression model to explain the variation in exploration of a novel object (blue pen). However, no significant differences between the Bohemian and Finnish birds were detected (Table 1, Figure 1). Most birds approached the object and pecked at it within the time limit. The average pecking latency was  $325 \pm 21.5$  s ( $N = 100$ ).

**Table 1**  
Fitting Cox hazard regression describing exploration of a novel object (latency to peck at a blue pen) in Bohemian and Central Finnish great tits

Model	Term removed	df	<i>P</i>
Sex*age + age*locality + sex*locality	Sex*age	1	0.9036
Age*locality + sex*locality	Locality*age	1	0.5712
Age + locality + sex + sex*locality	Sex*locality	1	0.4664
Age + locality + sex	Age	1	0.5506
Locality + sex	Sex	1	0.6572
Locality		1	0.0897

When an interaction is indicated (\*), the main effect of the term is also included in the model. Degrees of freedom and significances are given for the excluded term in the model.



**Figure 1**  
Latency of Bohemian and Central Finnish great tits to peck at a novel object (blue pen; exploration test; open bars) and their latency to start feeding after a novel object was attached to the food bowl (pink peg; neophobia test; gray bars).

Our final Cox hazard regression model explaining the birds' latency to feed in proximity of a novel object (pink peg) included locality, bird age, and the interaction of both (Figure 1, Table 2). In general, Finnish birds hesitated longer compared with those from Bohemia. Among all the birds, Bohemian adults were the least neophobic, which caused the effect of the interaction between locality and age of birds (Figure 1, Table 2).

Latencies measured in exploration and neophobia tests were significantly correlated in birds from both populations (Finnish birds:  $r_s = 0.60$ ,  $t = 5.15$ ,  $df = 48$ , and  $P < 0.05$ ; Bohemian birds:  $r_s = 0.29$ ,  $t = 2.10$ ,  $df = 48$ , and  $P < 0.05$ ).

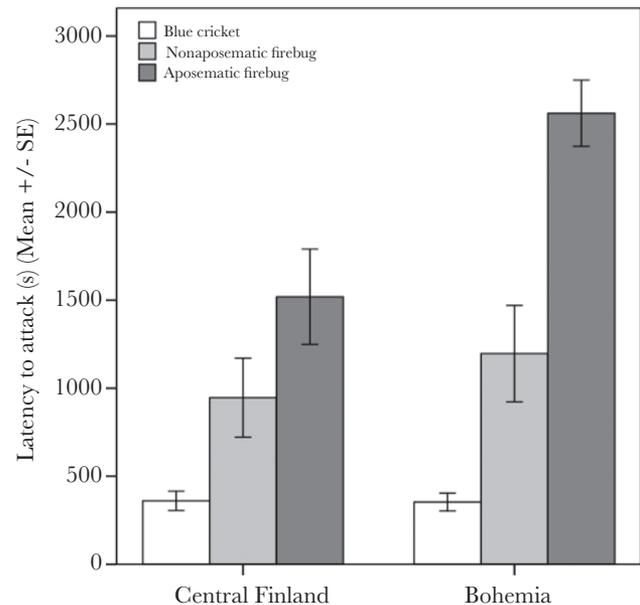
### Reaction to novel palatable prey

Most birds attacked the novel palatable prey (blue cricket) in the first or second trial (mean  $357 \pm 37.2$  s,  $N = 100$ ). The birds from both localities hesitated longer before attacking the blue crickets than before attacking familiar mealworms (Wilcoxon matched pairs test: Finnish birds,  $Z = 5.56$ ,  $N = 50$ , and  $P < 0.01$ ; Bohemian birds,  $Z = 5.23$ ,  $N = 50$ , and  $P < 0.01$ ).

**Table 2**  
Cox regression model explaining latency of Bohemian and Central Finnish great tits to start feeding after a novel object was attached to the food bowl (pink peg; neophobia test)

Source	Coef.	SE	<i>z</i>	<i>P</i>
Locality (Bohemia)	1.336	0.293	4.565	<0.001
Age (juv.)	-0.039	0.377	-0.103	0.918
Sex (males)	-0.002	0.998	-0.010	0.992
Locality (Bohemia): age (juv.)	-0.950	0.519	-1.827	0.068

Interaction terms of the sex and age, and the locality and sex were removed from the model because they were not significant ( $z < \pm 1$ ,  $P > 0.36$ ).



**Figure 2**  
Latency of Bohemian and Central Finnish great tits to attack a novel prey (cricket with a blue sticker; open bars), nonaposematic firebug (brown-painted; light gray bars), and aposomatic red-and-black firebug (wild-type; dark gray bars).

No significant effect of locality of bird origin was found explaining the variation in attack latencies (Figure 2, Table 3). However, nonsignificant trend for adult birds to be less hesitant than yearlings was detected (Table 3). We did not find a significant effect explaining the variation in latency to attack the mealworm offered just before the novel-prey test either (all *P* values of main effects (locality, age, sex) and their interactions were  $> 0.230$ ); this indicates that all the birds entered the test with similar foraging motivation.

Attack latencies did not correlate with the latencies measured in exploration (Finnish birds:  $r_s = 0.04$ ,  $t = 0.27$ ,  $df = 48$ , NS; Bohemian birds:  $r_s = 0.02$ ,  $t = 0.12$ ,  $df = 48$ , NS) and neophobia (Finnish birds:  $r_s = 0.06$ ,  $t = 0.40$ ,  $df = 48$ , NS; Bohemian birds:  $r_s = 0.16$ ,  $t = 1.10$ ,  $df = 48$ , NS) tests.

### Reaction to aposomatic and nonaposematic firebugs

A similar proportion of Finnish and Bohemian birds attacked nonaposematic firebugs (chi square = 1.75,  $df = 1$ , and  $P = 0.185$ ). Aposematic firebugs were mostly attacked by Finnish birds, and mostly avoided by Bohemian birds (chi square = 8.33,  $df = 1$ , and  $P = 0.004$ ).

**Table 3**

**Fitting Cox hazard regression describing latency of Bohemian and Central Finnish great tits to attack a novel palatable prey (blue cricket)**

Model	Term removed	df	<i>P</i>
Sex * age + age * locality + sex * locality	Age * locality	1	0.9049
Sex * age + sex * locality	Sex * locality	1	0.3953
Age + sex + locality + sex * age	Locality	1	0.9588
Age + sex + sex * age	Sex * age	1	0.2273
Age + sex	Sex	1	0.3675
Age		1	0.1283

When an interaction is indicated (\*), the main effect of the term is also included in the model. Degrees of freedom and significances are given for the excluded term in the model.

**Table 4**

**Model fitting of Poisson GLM explaining the number of firebugs (either aposematic or nonaposematic) attacked by Bohemian and Central Finnish great tits**

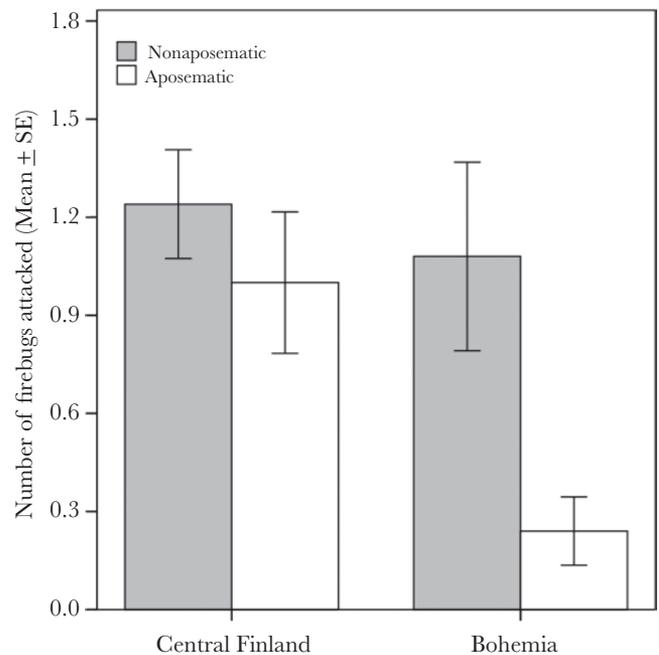
Model	Term removed	df	<i>P</i>
Locality * sex + locality * age + locality * color + age * color + sex * color	Age * color	1	0.9811
Locality * sex + locality * age + age * color + sex * color	Locality * age	1	0.8621
Locality * sex + age * color + sex * color	Locality * sex	1	0.8452
Age + locality + sex + color + locality * color + sex * color	Age	1	0.5427
<b>Locality + sex + color + locality * color + sex * color</b>	None		

If interaction is indicated (\*), the main effect of the term is also included in the model. Degrees of freedom and significances are given for the excluded term in the model. Our final model (see details in Table 5) is highlighted in bold.

Cox hazard regression model explaining the variation in latencies to attack the firebugs revealed a significant interaction between firebug coloration and the locality of bird origin (chi square = 3.95, df = 1, and  $P = 0.047$ ). Other terms included in the model were: locality, age and sex of the bird, and firebug coloration. Bohemian birds were more hesitant to attack aposematic firebugs than Finnish birds, but there was no difference in latencies to attack nonaposematic firebugs (Figure 2).

In Finnish birds, the latencies to attack both aposematic and nonaposematic firebugs correlated with the latencies to attack novel palatable prey (aposematic bugs:  $r_s = 0.46$ ,  $t = 2.46$ , df = 23, and  $P < 0.05$ ; nonaposematic bugs:  $r_s = 0.61$ ,  $t = 3.65$ , df = 23, and  $P < 0.05$ ); for Bohemian birds no such correlation was found (aposematic bugs:  $r_s = 0.10$ ,  $t = 0.49$ , df = 23, NS; nonaposematic bugs:  $r_s = 0.26$ ,  $t = 1.27$ , df = 23, NS).

General linear model explaining the number of attacked firebugs comprised locality of bird origin, bird sex, and firebug coloration and the interactions between locality and firebug coloration, and bird sex and firebug coloration (Table 4, Figure 3). The significant interaction between firebug coloration and locality arose from the fact that Bohemian birds attacked more nonaposematic firebugs than aposematic ones, whereas Finnish birds attacked similar numbers of both color forms (Table 5, Figure 3). Interestingly, in both localities, males tended to attack more aposematic firebugs than females. Individual females usually did not attack more than a single firebug (mean  $1.2 \pm 0.6$ ,  $N = 41$ ), whereas the males frequently attacked 2 and more (up to 4) individuals (mean  $1.6 \pm 0.7$ ,  $N = 59$ ).

**Figure 3**

Difference between Bohemian and Central Finnish great tits in the number of nonaposematic and aposematic firebugs attacked.

**Table 5**

**Poisson GLM explaining number of firebugs attacked by Bohemian and Central Finnish great tits**

Source	Estimate	SE	<i>z</i>	<i>P</i>
Intercept	0.3400	0.2228	1.526	0.1270
Locality	-0.1476	0.2635	-0.560	0.5753
Sex	-0.2353	0.2630	-0.895	0.3710
Color	-0.9606	0.4733	-2.029	0.0424
Locality * color	-1.2795	0.5254	-2.435	0.0149
Sex * color	1.0870	0.5252	2.070	0.0385

Effects of factor levels locality (Central Finland), sex (female), and firebug color (nonaposematic) are included in the intercept.

## DISCUSSION

Environmental factors, particularly food availability and predators, are suggested to be the main drivers selecting for differences in foraging behavior among local bird populations (e.g., Shochat et al. 2004). However, we still have very limited understanding of how such differences arise, and whether certain behavioral traits are selected together or independently. We compared several foraging-related traits in 2 geographically distant populations of great tits. Those populations have presumably experienced different selective environments in terms of habitats, diversity and abundance of noxious prey. Although our experiments are not able to discern whether the observed differences already reflect local adaptations or whether they reflect differences in experience, our results raise some interesting points regarding the nature of differences in the reaction of wild-caught birds to aposematic prey and how they are correlated with exploration behavior and neophobia.

### Exploration and neophobia—comparison of Finnish and Bohemian birds

Finnish and Bohemian great tits did not differ in their tendency to explore a novel object placed in a neutral location in their home

cage. However, Finnish birds appeared more neophobic, and hesitated longer than Bohemian birds before starting to feed next to a novel object attached to their food bowl. Our results suggest that environmental conditions both in Bohemia and Central Finland may, in spite of their differences, favor similar exploration tendencies in great tit populations. Similarly, Miranda et al. (2013) did not find any differences in novel object exploration between 2 populations of European blackbirds (*Turdus merula*) living in different habitats.

The extent of object neophobia has been frequently found to differ between conspecific populations (e.g., Martin and Fitzgerald 2005; Mettke-Hofmann et al. 2013; Miranda et al. 2013, but see Echeverría and Vassallo 2008; Bókony et al. 2012). Several factors may explain the greater object neophobia of Finnish great tits in our experiment. First, Finnish birds may be more cautious to approach novel objects near their feeding place due to greater vigilance caused by higher frequency of potential predators as can be expected in a less populated and more natural landscape. In sticklebacks (*Gasterosteus aculeatus*), individuals living in areas with greater predation pressure are less bold and more neophobic than those inhabiting areas with less predators (Bell 2005; Dingemanse et al. 2007; Brydges et al. 2008). Second, Finnish birds may exhibit greater object neophobia because of a larger proportion of migrating individuals (Cepák et al. 2008). Although the great tit is mostly a resident species, some individuals (mostly yearlings) undergo short-distance autumnal migration (Cramp and Perrins 1993). Greater neophobia of birds from migratory populations than from the resident ones has been found in 2 New World blackbird species (red-winged blackbird *Agelaius phoeniceus* and Brewer's blackbird *Euphagus cyanocephalus*); the difference may have been caused by residents having higher costs of missing new opportunities in the seasonally changing environment (Mettke-Hofmann et al. 2013). Lesser neophobia of Bohemian birds may be also partly explained by their more urbanized locality. Birds from urban populations may be bolder and less neophobic than their rural conspecifics, as is the case in European blackbirds (*Turdus merula*; Miranda et al. 2013), and song sparrows (*Melospiza melodia*; Evans et al. 2010).

### Behavior of Finnish and Bohemian birds toward novel palatable prey

Finnish and Bohemian great tits did not differ in their behavior toward a novel palatable prey, the cricket with a blue sticker attached. They attacked the crickets with similar latencies, and mostly killed and consumed them. This suggests that the hesitation behavior toward novel prey does not reflect any general difference in local prey communities and frequency of potentially dangerous prey.

Surprisingly, we did not find any correlation between the behavior of birds toward novel prey and their reaction to novel objects (exploration and neophobia) in the 2 populations studied. In juvenile Dutch great tits coming from lines selected for opposed personality traits (Drent et al. 2003), the generally more neophobic “slow explorers” hesitated longer before attacking novel prey (red-and-black firebugs) than the less neophobic “fast explorers” (Exnerová et al. 2010). There are several mutually nonexclusive factors possibly responsible for the difference between the results of our present and previous studies (Exnerová et al. 2010): 1) The inherited correlation between food neophobia and object neophobia may be prominent in naive juvenile birds. In contrast, adult wild-caught individuals may not show such a correlation due to their experience with various types of both palatable and unpalatable prey, as

well as experience with food shortage periods. In wild-caught tits living outside the breeding season in small flocks, the food neophobia may also be influenced by the position of an individual in the flock hierarchy (Farine et al. 2015). In black-capped chickadees (*Poecile atricapillus*), for instance, subordinate individuals are less neophobic than dominant ones (An et al. 2011). 2) The correlation may be more apparent in birds coming from the lines selected for the opposed personalities (Drent et al. 2003), than in birds from natural populations with possibly less extreme values of personality traits. 3) Food neophobia may be correlated with other personality traits in some populations (Dutch; Exnerová et al. 2010), but not in others (Finnish and Bohemian, this study). Bókony et al. (2012) found that food neophobia in house sparrows (*Passer domesticus*) correlated with activity, risk-taking, and object neophobia only in 1 of 4 Hungarian populations studied. In Kenya none of the 8 populations of house sparrows tested showed any relationship between exploration of a novel object and consumption of novel food (Liebl and Martin 2014).

### Reactions of Finnish and Bohemian birds toward firebugs

The behavior of birds toward firebugs was affected by the firebug coloration. In contrast to behavior toward novel palatable prey, the Finnish birds partly differed from the Bohemian birds in their reactions. Nonaposematic firebugs were attacked in similar proportions by Finnish and Bohemian birds. The birds from both populations also hesitated about the same time before attacking nonaposematic firebugs, and learned to avoid them at a similar rate. In Finnish birds, the attack latencies for nonaposematic firebugs correlated with those for novel palatable prey, whereas in Bohemian birds the latencies were not correlated. These results indicate that nonaposematic, brown-painted firebugs were novel for Finnish birds, and that the reaction to them followed the general behavior of birds toward a novel prey. Noncorrelated latencies of Bohemian birds suggest that these birds may be experienced with some similar, nonaposematic but unpalatable true bugs from the wild (e.g., some species of Rhyparochromidae), and that they partly generalized their experience. On the other hand, the Bohemian birds attacked nonaposematic firebugs more frequently than the aposematic ones, which confirms the results of previous studies (Exnerová et al. 2003, 2006) suggesting that the characteristic red-and-black color pattern of firebugs facilitates their recognition by avian predators.

The Finnish and Bohemian great tits significantly differed in their behavior toward aposematic firebugs. Most Bohemian birds avoided them on sight, whereas most Finnish birds attacked at least 1 individual. Finnish birds generally behaved toward aposematic firebugs in a similar way as to the nonaposematic ones, and their initial reactions (before the first contact with firebug defense chemicals) were correlated with their reactions to palatable prey of novel appearance (blue crickets). The differences in behavior toward the aposematic firebugs did not follow the differences between the 2 populations in exploration and neophobia. Birds from both populations behaved similarly in response to a novel object and to a novel palatable prey, and the only difference—hesitation to feed in presence of a novel object—does not correspond with behavior toward the aposematic prey, because the Finnish birds were more neophobic. Therefore, the difference in avoidance between the wild-caught Finnish and Bohemian great tits is likely to be the result of individual learning and their different experiences in the wild.

Although the Finnish birds attacked aposematic firebugs more frequently than the Bohemian birds, it is interesting that the

Finnish birds stopped attacking them on average after only 1 trial. This means that the Finnish wild-caught birds learned to avoid the novel aposematic firebugs considerably faster than the conspecific naive hand-reared birds from Bohemia, which attacked on average 5 firebugs (Svádová et al. 2009) before learning to avoid them. This difference suggests that the Finnish wild-caught birds may have generalized their previous experience with some unpalatable defended prey, even of a different appearance than firebugs, and this may have increased their avoidance learning rate.

Alternatively, the difference may suggest an innate bias against conspicuous aposematic prey (Lindström et al. 1999a), which can speed up avoidance learning. Avoidance learning against an aposematic prey that is evolutionarily novel needs typically several unpleasant experiences before the novel prey is learned to be avoided (e.g., Mappes and Alatalo 1997; Lindström et al. 1999b). In previous studies, naive juvenile great tits did not hesitate longer before attacking aposematic than nonaposematic prey (Svádová et al. 2009), but they showed an innate bias against aposematic prey, when the nonaposematic (Lindström et al. 1999a) or less conspicuous (Fabricant et al. 2014) alternative prey was present. These 2 alternatives are not mutually exclusive, and the reactions observed in wild-caught birds are likely the result of an interaction between an innate bias and individual experience. Assessing the effect of both processes would, however, require further experiments with naive predators and the use of the prey novel for both populations.

For the nonaposematic firebugs, the learning rates were similar in Bohemian and Finnish birds, indicating that the populations do not differ in their ability to learn avoiding unpalatable prey. The Finnish birds, for which both firebug color forms were novel, learned to avoid red-and-black and brown-painted firebugs at a similar rate. This agrees with previous studies with great tits and defended conspicuous prey where learning rate between “typical” warning colors versus gray or brown did not differ (Ham et al. 2006; Svádová et al. 2009). Because our experiment was designed mainly to test the attack willingness toward the aposematic prey, rather than learning abilities, we are unable to make any strong conclusions about the general differences in learning abilities between populations. Furthermore, we compared an experienced population to a naive one. In the future, it would be interesting to compare learning abilities between populations by using a completely novel aposematic prey and/or naive birds.

Our results indicate a stronger avoidance of aposematic bugs by female great tits than by males. Similar results were obtained in a study where female bobwhites (*Colinus virginianus*) showed more aversion than males toward red- and orange-dyed food (Mastrota and Mench 1994). A different study with the same species however, found no differences between the sexes in color aversion, presumably because the individuals used in both experiments differed in age (Mastrota and Mench 1995). Color aversion in females, but not in males, seems to increase with age (Mastrota and Mench 1994). A possible explanation is that females consume more insects than males, and they teach chicks to avoid toxic prey. Whether this is the case for great tits is a matter of future research.

### Aposematic Heteroptera and other insects of Central Finland as potential models

Wild-caught great tits are potentially experienced with an unknown number of both palatable and unpalatable species of insects, both in Central Finland (around the 62°N latitude) and Bohemia. Unfortunately, there are no data available to compare the abundance and diversity of potential insect prey between the 2 areas.

However, because the reactions of the birds toward the firebug were specific, we consider the evaluation of heteropteran faunas informative, as it compares the numbers of potential models the birds may be experienced with from the wild.

We have documented that the fauna of aposematic terrestrial Heteroptera of Central Finland is much less diverse than that of Central Europe both in absolute and relative number of species (30 vs. 156 species, 10% vs. 17% of total faunas, respectively). The Bohemian great tits are certainly experienced with *Pyrrhocoris apterus* due to the frequent occurrence of the bug, its aggregations and its ubiquitous host plants (Exnerová et al. 2006); these birds can be potentially experienced with other chemically protected red-and-black true bug species as well (Hotová Svádová et al. 2010). Although *P. apterus* does not occur in Central Finland (Rintala and Rinne 2010), we cannot exclude a priori the local occurrence of insect species that the birds would generalize with. However, the other Finnish similarly colored aposematic insects are either too rare (*Corizus hyoscyami*, Rhopalidae), or look too different (plant bugs, Miridae; burnet moths, Zygaenidae; ladybird beetles, Coccinellidae) to function as models for generalization with red-and-black firebugs. Moreover, recent analysis of Finnish Lepidoptera showed that only less than 5% of caterpillars are aposematic (Mappes et al. 2014). It is still possible that the negative experience with some of the above taxa may have played a role in the generalization of the firebug color patterns as great tits have been shown to generalize their learned avoidance among colors (red, yellow, and orange; Ham et al. 2006), among aposematic species (Hotová Svádová et al. 2013) and between bi-chromatic symbols (Ihalainen et al. 2008).

## CONCLUSIONS

Even though a broader generalization of our results is limited by the study of only 2 populations, we have shown that conspecific birds from 2 geographically distant populations may express similar reactions to a prey which is novel for both of them. In our case, food neophobia was low in both populations, whereas the avoidance of aposematic prey was different between the populations, and could be explained by differences in experience. Bohemian birds have plenty of opportunities to meet firebugs in their natural environment and learn about their unpalatability; Finnish birds do not have such opportunity. Our results emphasize that naive birds' tendency to attack novel aposematic prey may have important implications for range extensions of prey species. We can not tell whether the difference in avoidance was a result of Finnish birds' lower experience with any aposematic prey, or whether the avoidance learning is prey specific. Our results indicate, however, that studies on aposematism and mimicry based on geographically distant conspecific populations can be compared and generalized.

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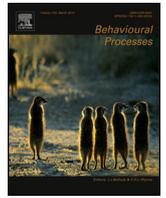
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# Through experience to boldness? Deactivation of neophobia towards novel and aposematic prey in three European species of tits (Paridae)

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## ABSTRACT

European tits (Paridae) exhibit species-specific levels of initial wariness towards aposematic prey. This wariness may be caused by neophobia, dietary conservatism or innate bias against particular prey traits. We assessed the contribution of these three mechanisms to the behaviour of juvenile tits towards novel palatable prey and novel aposematic prey. We compared levels of initial wariness in great tits (*Parus major*), blue tits (*Cyanistes caeruleus*) and coal tits (*Periparus ater*), and tested how the wariness can be deactivated by experience with a palatable prey. One group of birds was pre-trained to attack familiar naturally coloured mealworms the other one, novel red-painted mealworms. Then all the birds were offered a novel palatable prey of different colour and shape: cricket (*Acheta domestica*) with blue sticker, and then a novel aposematic firebug (*Pyrrhocoris apterus*). The three species of tits differed in how the experience with a novel palatable prey affected their behaviour towards another novel prey. Great tits and coal tits from experienced groups significantly decreased their neophobia towards both palatable prey and aposematic prey while blue tits did not change their strongly neophobic reactions. The interspecific differences may be explained by differences in body size, geographic range, and habitat specialisation.

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## 1. Introduction

Aposematic prey signal their noxiousness or unprofitability to potential predators by a conspicuous warning signal (reviewed in Ruxton et al., 2004). Bird predators usually learn to avoid aposematic prey, and many experimental studies have focused on factors affecting the mechanisms of avoidance learning, memory and generalisation (e.g. Sillén-Tullberg, 1985; Roper and Redston, 1987; Gamberale and Tullberg, 1996; Lindström et al., 1999a; Riipi et al., 2001; Gamberale-Stille and Guilford, 2003; Ham et al., 2006; Exnerová et al., 2010; Aronsson and Gamberale-Stille, 2008, 2012; Svádová et al., 2009; Barnett et al., 2012). The avoidance of aposematic prey may also have an inherited component, which causes even inexperienced individuals to avoid aposematic prey or at least to hesitate longer before attacking them (Smith, 1975, 1977; Lindström et al., 1999b; Exnerová et al., 2007), and is important for the evolution of aposematic prey (Marples et al., 2005; Marples and Mappes, 2011). Recent studies on the phenomenon of innate wariness have shown that it may vary between bird species (Exnerová et al., 2007) and individuals (Exnerová et al., 2010), may often

include hidden biases that are manifested only when a combination of visual and chemical signals of a prey are involved (Rowe and Guilford, 1996, 1999a; Jetz et al., 2001; Lindström et al., 2001; Kelly and Marples, 2004; Rowe and Skelhorn, 2005), and that the wariness may in fact be a complex of several, partly independent mechanisms (Marples et al., 1998; Marples and Kelly, 1999; Exnerová et al., 2003; Marples and Mappes, 2011). Innate wariness towards aposematic prey may include three mutually not exclusive processes: (1) food neophobia, (2) dietary conservatism and (3) specific innate biases against warning signals of aposematic prey. All these processes may contribute to some degree to the predator's response and, in a natural situation, it is difficult to distinguish them.

Neophobia, usually defined as a tendency to avoid novel objects and situations (Barrows, 2011), is a widespread phenomenon first described in rats tested with novel objects (Barnett, 1958). It has since been observed in many animal taxa also as a response to novel food (Honey, 1990; Galef, 1993; reviews in Kelly and Marples, 2004; Mappes et al., 2005; Marples et al., 2005). Food neophobia has been measured as a hesitation to approach a new food and come into a physical contact with it, which lasts usually only several minutes, and is followed by investigation of the novel food (Marples and Kelly, 1999). In some bird species, food neophobia may be correlated with object neophobia (e.g. blacked-capped chickadees

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(*Poecile atricapillus*)—An et al., 2011), whereas in others the correlation may be absent or present only in some populations (e.g. great tits (*Parus major*)—Exnerová et al., 2010, 2015; house sparrows (*Passer domesticus*)—Bókony et al., 2012). After the first contact with novel food, birds may no longer show neophobia, but they may still refuse to consume the novel food. This reaction was termed dietary conservatism, and is usually measured as the time from initial contact with the novel food item to its incorporation into the diet (Marples et al., 1998; Kelly and Marples, 2004). Dietary conservatism (DC) has been defined as a relatively long lasting refusal (persisting for days to months) of some individuals to accept novel food into their diet (Marples et al., 1998; Thomas et al., 2003; Thomas et al., 2004). Individual variation in dietary conservatism has been shown to have a genetic basis (Marples and Brakefield, 1995). Neophobia and dietary conservatism together are usually referred to as dietary wariness (Mappes et al., 2005; Marples et al., 2005; Marples et al., 2007) and the evidence that neophobia but not dietary conservatism can be reduced through experience with food of various colours in chicks (Jones, 1986; Marples et al., 1998) and turkeys (Lecuelle et al., 2011) supports the idea of the existence of two distinct processes.

The third process contributing to innate wariness of aposematic prey is a specific innate bias against particular warning signals and their combinations. This type of inherited avoidance of warning signals has been found in birds from several taxa (Galliformes, Momotidae and Passeriformes) and is usually associated with conspicuous aposematic colour patterns. Naive domestic chicks (*Gallus gallus domesticus*) avoid red painted mealworms (larvae of *Tenebrio molitor*) (Roper and Cook, 1989; Roper, 1990) and mealworms with black-and-yellow stripes (Schuler and Hesse, 1985); naive northern bobwhites (*Colinus virginianus*) avoid red-and-yellow pinheads (Mastrota and Mench, 1995); naive hand-reared turquoise-browed motmots (*Eumomota superciliosa*) and great kiskadees (*Pitangus sulphuratus*) avoid the coral snake pattern (Smith, 1975, 1977); and hand-reared juveniles of great tits (*Parus major*) avoid black-and-yellow striped mealworms (Lindström et al., 1999b). Furthermore, warning signals are often multimodal and innate biases may emerge only when visual signals are combined with olfactory, gustatory or acoustic cues (Rowe and Guilford, 1999a). These hidden biases have been intensively studied mainly in domestic chicks, in which a combination of warning coloration and smell (Rowe and Guilford, 1996, 1999b; Jetz et al., 2001), taste (Rowe and Skelhorn, 2005; Skelhorn et al., 2008) or sound (Rowe and Guilford, 1999a; but see Siddall and Marples, 2011) triggers manifestation of innate biases.

The degree of innate wariness may differ even between closely related bird species, and also the mechanisms responsible for the wariness may be species specific (Exnerová et al., 2007). Moreover, predator species may differ not only in their initial degree of innate wariness, but also in how easily it is modified by further experience with palatable or unpalatable prey. Considerable variation in innate wariness exists among European species of tits (Paridae). While naive hand-reared great tits (*Parus major*) and crested tits (*Lophophanes cristatus*) readily attack aposematic red-and-black firebugs, *Pyrrhocoris apterus*, naive hand-reared coal tits (*Periparus ater*) and blue tits (*Cyanistes caeruleus*) avoid them (Exnerová et al., 2007). The fact that naive coal tits and blue tits avoided not only aposematic firebugs, but also non-aposematic brown-painted ones, suggests an effect of neophobia. However, the potential role of dietary conservatism and specific biases against warning colours remain to be studied.

In this study we compared the degree of innate wariness towards novel prey in three European species of tits and attempted to assess the roles of neophobia, dietary conservatism and specific bias against aposematic coloration in an overall wariness. Specifically, we compared the reactions of hand-reared juveniles of great

tits (*Parus major*), coal tits (*Periparus ater*) and blue tits (*Cyanistes caeruleus*) towards two types of novel prey: (1) novel palatable prey (a cricket *Acheta domestica* with a blue sticker) and (2) novel aposematic prey (the red-and-black firebug *Pyrrhocoris apterus*). Within each species, we tested whether the previous positive experience with another novel palatable prey (red-painted mealworms) affects willingness of the birds to attack and consume the two novel prey types.

## 2. Material and methods

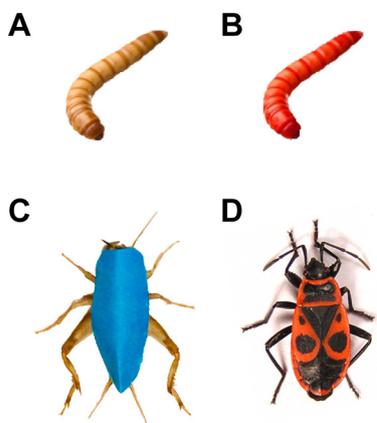
### 2.1. Birds

Hand-reared juveniles of three European tit species were tested: 85 great tits (*Parus major* L. 1758), 65 coal tits (*Periparus ater* (L. 1758)) and 85 blue tits (*Cyanistes caeruleus* (L. 1758)). All three species are sedentary, and their diet includes mainly small arthropods, supplemented with seeds and berries (Cramp and Perrins, 1993). Preferred prey length of all the three tit species is around 1 cm, with coal tits preferring slightly smaller prey than the other two species (Cramp and Perrins, 1993). Coal tits cache their food regularly, whereas blue tits and great tits do not (Štorchová et al., 2010). Great tits and coal tits are widespread thorough the whole Palaearctic (Cramp and Perrins, 1993). Great tits inhabit a wide range of woodland habitats including urban and suburban areas, whereas coal tits are restricted to coniferous and mixed woodlands (del Hoyo et al., 2007). Blue tits are endemic to the West Palaearctic (Harrap and Quinn, 1996), inhabiting mostly lowland and submontane deciduous woodlands (Cramp and Perrins, 1993; del Hoyo et al., 2007). The great tit is the largest of the three species (weight: 14.0–22.0 g, body length: 140 mm), followed by blue tit (weight: 7.5–14.7 g, body length: 115 mm) and coal tit (weight: 7.2–12.0 g, body length: 115 mm) (Cramp and Perrins, 1993; del Hoyo et al., 2007). The weight of the juveniles tested in our experiment ranged 14–18 g in great tit (mean = 16.2 ± 0.8 g), 9.5–13 g in blue tits (mean = 11.1 ± 0.8 g) and 8.5–10.5 g in coal tits (mean = 9.5 ± 0.5 g).

The nestlings were taken from nest boxes at the age of 12–16 days, when they had only very limited visual experience with prey brought to the nest by their parents. The nest boxes were placed in large parks at the outskirts of Prague (50°04'N, 14°26'E) and in mixed woods near Hradec Králové (50°12'N, 15°50'E). Not more than two nestlings were taken from a single brood. Nestlings were kept in artificial nests and were fed every two hours from 6 AM to 10 PM for several days, until they were able to feed themselves. Their diet consisted of mealworms, boiled eggs, handmix (Orlux), egg mixture Oké-bird (Versele-Laga), mixtures for insectivorous birds Uni patee (Orlux), Nutribird (Versele-Laga), Insect patee (Orlux) and vitamins Roboran (Unisvit), Vitamin plus V (Sera) and Activ plus W (Sera). After fledging, the birds were housed individually in plastic home cages (50 × 40 × 40 cm) with wire-mesh front wall. Each cage was equipped with three perches, two water bowls with drinking and bathing water *ad libitum*, and two feeders placed on the bottom of the cage. Birds were kept under natural light conditions (16:8 h light/dark) and were daily provided with fresh water and food. All birds were tested when they were fully independent, most of them at the age of 38–65 days (minimum 35 days and maximum 73 days).

### 2.2. Prey

Naturally coloured mealworms (larvae of *Tenebrio molitor*, Fig. 1), which were familiar to the birds, were used to check their foraging motivation during the experiment and for training the birds from non-experienced experimental group (see below). We



**Fig. 1.** Types of prey used in the experiments: (a) naturally coloured mealworm, (b) red mealworm, (c) blue cricket, (d) aposematic firebug. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used three types of novel prey (Fig. 1): (1) red-painted mealworms, which were dyed with non-toxic finger paints (Jovi S.A. Barcelona, red); the dye covered the whole mealworm; (2) late-instar larvae of house cricket, *Acheta domestica*, with head and body covered with an oval-shaped bright blue-coloured (Faber-Castell, textliner 1548 blue) paper sticker attached to their dorsal side; and (3) adults of the red-and-black aposematic firebug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Unlike mealworms and crickets, the firebugs are chemically defended, which makes them unpalatable for small passerine birds (Exnerová et al., 2003, 2007; Svádová et al., 2009). The defensive secretion is produced in the metathoracic glands and contains mostly short-chained aldehydes (Farine et al., 1992). Firebugs are widespread in various habitats of the Palaearctic region, mainly in deciduous woodlands, parks and gardens. They feed on seeds of linden trees (*Tilia* spp.), various herbaceous Malvaceae, and locust tree (*Robinia pseudacacia*). The firebugs were collected on linden trees at several localities in Prague. Cricket nymphs and adult firebugs were of similar body length (10–12 mm), mealworms were about 15 mm long. All prey used in the experiments were live.

### 2.3. Experimental cages

Experiments were carried out in wooden cages (70 × 70 × 70 cm) with wire-mesh walls and front wall made of one-way glass. Cages were equipped with a wooden perch, a water bowl and a rotating feeding tray with six cups (see Exnerová et al., 2003 for details). The perch was placed 30 cm from the feeding tray. Cage illumination (Biolux Combi 18W, Osram) simulated the daylight spectrum. Birds were trained to search for mealworms in one of the cups of the feeding tray and then deprived of food for two hours before the experiment.

### 2.4. Experimental design

Within each species, we divided the birds into two experimental groups. Birds from the first group were trained to attack red-painted mealworms (experienced group henceforth), while birds from the second (control) group were trained to attack naturally coloured mealworms (non-experienced group henceforth). The training consisted of a sequence of five-minute trials; the bird was offered a single mealworm in each trial. For the experienced group the red mealworms were alternating with naturally coloured mealworms to check the foraging motivation of the birds. Birds from the non-experienced group were offered a sequence of natu-

rally coloured mealworms. Birds from the experienced group had to attack and consume one red mealworm to be considered having a positive experience with the novel prey. After this experience, which was the same in all individuals, the training was stopped. If the bird did not eat the first red mealworm, the training continued up to the maximum of 30 five-minute trials (15 red mealworms and 15 naturally coloured mealworms). Ten birds from the experienced group (4 great tits and 6 blue tits) did not meet our criterion during the entire training session. These birds were not included in the analyses. The non-experienced group had the same training setup, but with naturally coloured mealworms only (Table 1).

To test whether the experience with the red mealworms affects the behaviour of birds towards a prey of another novel colour and unfamiliar body shape, all birds were offered a blue cricket in a single five-minute trial immediately after the training phase. To check the foraging motivation of birds, we offered a naturally coloured mealworm before the blue-cricket trial (Table 1). We measured the latency to approach and attack (touch, peck or seize) the blue cricket and recorded whether the cricket was killed and eaten. If the bird did not attack the blue cricket within the five minutes, it was assigned a maximum latency of 300 s.

In the subsequent five-minute trial all birds were offered the aposematic firebug. In this trial we tested whether the reaction of birds to red-and-black aposematic prey can be affected by their positive experience with palatable red-coloured prey of different body shape (Table 1). Again, the latency to approach and attack (touch, peck or seize) the firebug and whether it was killed and eaten was recorded. If the bird did not attack the firebug within five minutes it was assigned a maximum latency of 300 s.

All trials were recorded using Observer XT 8.0 ©Noldus and a digital videocamera.

### 2.5. Ethical note

We obtained permissions for taking juvenile tits from nest boxes in Prague (MHMP-043585/2009/OOP-V-26/R-8/Pra) and Hradec Králové (MMHK/10568/2009/ŽP/Han17386), and for laboratory experiments with the birds (29532/2006-30, 150/99 and CZ01059) issued by Central Commission for Animal Welfare of the Czech Republic. The permissions cover all the necessary methodological details, i.e. housing and feeding conditions, experimental design, and release after the experiment. All birds were individually ringed (licence No. 1087, Czech Ringing Centre, Praha) and released in healthy condition on days of fair weather at their origin. The releasing sites were equipped with feeders and provided with supplementary food for several days after releasing.

### 2.6. Statistical analyses

A Cox hazard regression was used to analyse the interspecific differences and an effect of experience with red mealworms on latencies to attack (a) novel palatable blue crickets, and (b) aposematic firebugs. Treatment (training group), species, and their interaction were used as explanatory variables in both analyses.

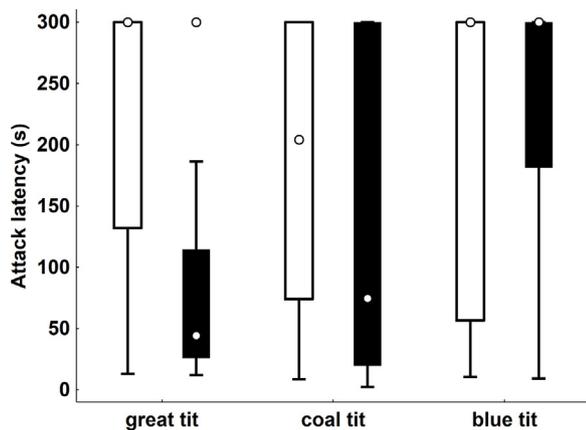
Using a generalised linear model (GLM ANOVA) with binomial distribution and logit link function we tested whether the probability to consume (a) novel palatable blue crickets, and (b) aposematic firebugs differed between the tit species and was affected by the previous experience with palatable red mealworms. Treatment (training group), species, and their interaction were used as explanatory variables in both analyses.

Since the behaviour of birds towards the firebugs may also be influenced by experience with the crickets, we have included the result of the cricket test (whether the cricket was consumed or not) as a factor into the analyses of both attack latencies and prey

**Table 1**

Sample size of tested birds, which met the criteria in training session (consuming of red mealworm) and were used in next experiments with novel prey (blue cricket) and aposematic prey (red-and-black firebug).

Non-experienced			Experienced		
Great tits	Coal tits	Blue tits	Great tits	Coal tits	Blue tits
40	20	40	41	45	39
naturally coloured mealworms	naturally coloured mealworms	naturally coloured mealworms	red mealworms	red mealworms	red mealworms
↓	↓	↓	↓	↓	↓
blue cricket	blue cricket	blue cricket	blue cricket	blue cricket	blue cricket
↓	↓	↓	↓	↓	↓
aposematic firebug	aposematic firebug	aposematic firebug	aposematic firebug	aposematic firebug	aposematic firebug



**Fig. 2.** Latency to attack novel prey (cricket with blue sticker) by tits with (black bars) and without (white bars) experience with red coloured mealworms. Circles denote the median, boxes denote lower and upper quartile, whiskers denote non-outlier range, \*denote outliers.

consuming in the firebug test. All calculations were made in S-Plus 4.0 (MathSoft, 1997).

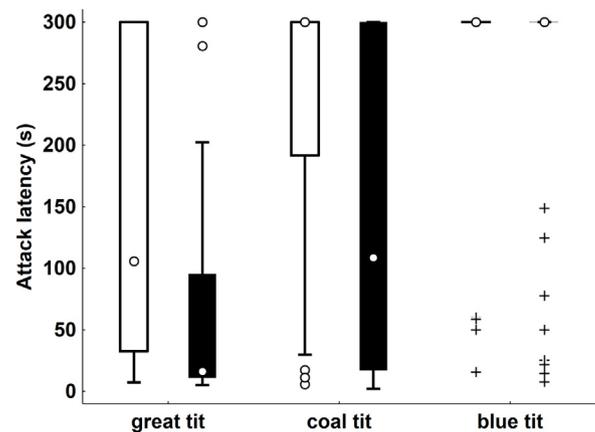
### 3. Results

#### 3.1. Reaction to blue crickets (*Acheta domestica*)

The latency to attack the blue cricket was affected by treatment (Cox hazard regression model:  $Z = 4.91$ ,  $p < 0.001$ ). The species had no effect ( $Z = 0.91$ ,  $p = 0.36$ ), but there was a significant effect of interaction between the species and treatment ( $Z = -3.95$ ,  $p < 0.001$ , Fig. 2).

The biggest intraspecific difference in reactions to blue crickets was observed in great tits. The latencies to attack blue crickets were significantly shorter in birds from the group experienced with palatable red mealworms than in the non-experienced group ( $Z = -4.74$ ,  $p < 0.001$ , Fig. 2). In coal tits, the positive experience with red mealworms also influenced the latencies to attack blue crickets. Coal tits from the experienced group attacked blue crickets significantly faster than the birds from the non-experienced group ( $Z = -3.21$ ,  $p = 0.02$ , Fig. 2). In contrast to great tits and coal tits, positive experience with palatable red mealworms did not affect the reactions of blue tits; latencies to attack blue crickets did not differ between experienced and non-experienced groups ( $Z = -1.13$ ,  $p = 0.26$ , Fig. 2).

Probability of consuming the blue crickets differed between the tit species (GLM ANOVA: Chi-square = 15.68,  $df = 2222$ ,  $p < 0.001$ ), but was not affected by the previous experience with red



**Fig. 3.** Latency to attack aposematic prey (red-and-black firebug) by tits with (black bars) and without (white bars) experience with red coloured mealworms. Circles denote median, boxes denote lower and upper quartile, whiskers denote non-outlier range, \*denote outliers, +denote extremes.

mealworms (Chi-square = 0.02,  $df = 1221$ ,  $p = 0.89$ ); the effect of interaction of both factors was marginal (Chi-square = 5.33,  $df = 2219$ ,  $p = 0.07$ ).

In great tits, there was a non-significant tendency to consume blue crickets more frequently among the birds experienced with palatable red mealworms (Chi-square = 2.87,  $df = 1,79$ ,  $p = 0.09$ ) than among the birds from the non-experienced group. The probability of consuming blue crickets was not affected by the previous experience with red mealworms in coal tits (Chi-square = 0.27,  $df = 1,63$ ,  $p = 0.61$ ) and in blue tits (Chi-square = 2.20,  $df = 1,77$ ,  $p = 0.14$ ).

#### 3.2. Reaction to aposematic firebugs (*Pyrrhocoris apterus*)

The latency to attack the firebugs was affected by species (Cox hazard regression model:  $Z = -3.75$ ,  $p < 0.001$ ), treatment ( $Z = 2.31$ ,  $p = 0.02$ ), and the interaction between species and treatment ( $Z = 2.46$ ,  $p = 0.02$ , Fig. 3). Neither consuming the blue cricket ( $Z = 1.21$ ,  $p = 0.23$ ) nor any interaction of this and other factors had a significant effect ( $p > 0.1$ ).

The positive experience with palatable red mealworms resulted in significantly shorter attack latencies of great tits from experienced group than from non-experienced group (Cox hazard regression model:  $Z = 2.71$ ,  $p < 0.01$ , Fig. 3). Consuming the blue cricket had a marginal effect on shortening the latency to attack the firebug ( $Z = 1.85$ ,  $p = 0.06$ ); the interaction between the two factors was not significant ( $Z = 0.42$ ,  $p = 0.67$ ). The experience with red mealworms shortened latencies to attack the firebugs also in coal

tits (Cox hazard regression model:  $Z = 2.15$ ,  $p = 0.03$ , Fig. 3); neither consuming the blue cricket ( $Z = 0.60$ ,  $p = 0.55$ ) nor the interaction between the two factors had a significant effect ( $Z = -0.84$ ,  $p = 0.40$ ). Blue tits showed strong initial wariness of the firebugs, and their attack latencies were affected neither by experience with palatable red mealworms (Cox hazard regression model:  $Z = 1.15$ ,  $p = 0.25$ , Fig. 3) nor by consuming the blue cricket ( $Z = 0.13$ ,  $p = 0.90$ ) nor the factors interaction ( $Z = -0.02$ ,  $p = 0.98$ ).

Probability of consuming the aposematic firebugs differed between the three species (GLM ANOVA: Chi-square = 52.80,  $df = 2222$ ,  $p < 0.001$ ) and was also affected by previous experience with red mealworms (Chi-square = 8.01,  $df = 1221$ ,  $p < 0.01$ ). Whether the bird consumed the blue cricket had a marginal effect on increasing the probability of consuming the firebug (Chi-square = 3.33,  $df = 1220$ ,  $p = 0.07$ ). None of the interactions between the factors had a significant effect ( $p > 0.1$ ).

The proportion of great tits that consumed the firebug was higher in the group experienced with palatable red mealworms than in the non-experienced group (GLM ANOVA: chi-square = 4.61,  $df = 1.79$ ,  $p = 0.03$ ). Consuming the blue cricket had a marginal effect on increasing the probability of consuming the firebug (Chi-square = 3.40,  $df = 1.78$ ,  $p = 0.07$ ); the interaction between the two factors was not significant (Chi-square = 1.84,  $df = 1.77$ ,  $p = 0.16$ ). Although coal tits remained cautious about consuming the firebugs, we observed an increasing trend in the willingness to consume them in the group experienced with palatable red mealworms (GLM ANOVA: chi-square = 2.84,  $df = 1.63$ ,  $p = 0.09$ ). Neither consuming the blue cricket (Chi-square = 0.19,  $df = 1.62$ ,  $p = 0.66$ ) nor the interaction between the two factors had a significant effect (Chi-square = 1.47,  $df = 1.61$ ,  $p = 0.23$ ). Only one coal tit from the non-experienced group consumed the aposematic firebug, as opposed to nine coal tits from the group experienced with palatable red mealworms. Additionally, four coal tits which consumed the firebug cached it into the slits in experimental cage before consuming. The number of blue tits that consumed aposematic firebugs did not differ between the groups experienced and non-experienced with palatable red mealworms (GLM ANOVA: chi-square = 1.42,  $df = 1.77$ ,  $p = 0.23$ ), and was not affected either by consuming the blue cricket (Chi-square = 0.22,  $df = 1.76$ ,  $p = 0.64$ ) or the factors interaction (Chi-square = 0.01,  $df = 1.75$ ,  $p = 0.98$ ). Only one of the experienced blue tits consumed the firebug, and blue tits from the non-experienced group neither killed nor ate a single firebug.

#### 4. Discussion

The fear of novelty may be seen as an adaptive strategy for avoiding potentially dangerous and toxic food (Barnett, 1958; Heinrich, 1988). However, especially when the food becomes scarce, overcoming neophobia and sampling new types of food can be advantageous (Greenberg, 1990; Greenberg and Mettke-hofmann, 2001). Reducing neophobia may be particularly important for resident bird species, which are confronted with food supply changes throughout the year, and frequently explore new sources of food and invent new foraging methods (Sol et al., 2005). We tested whether positive experience with a novel palatable prey can increase the willingness of juveniles of three tit species to attack and consume another type of novel prey.

##### 4.1. Reaction towards novel palatable prey

Great tits, coal tits, and blue tits differed in how the experience with a novel palatable prey (red mealworms) affected their behaviour towards another palatable novel prey (blue crickets). In great tits and coal tits the latencies to attack novel palatable

prey were significantly shorter in those birds that had the positive experience than in those that lacked it. These results are in accordance with previous studies of blue jays (*Cyanocitta cristata*, Schlenoff, 1984), domestic chicks (Jones, 1986; Marples et al., 2007) and turkeys (Lecuelle et al., 2011), which have shown that the positive experience with food of a novel colour was sufficient for the deactivation of neophobia towards another novel-coloured food. Moreover, our results show that the positive experience with food differing from the familiar one in a single trait (colour) may deactivate or decrease neophobia towards food novel also in other characters (shape, way of movement).

Our results on great tits and coal tits support the results of previous studies of domestic chicks showing (Marples and Kelly, 1999; Marples et al., 2007) that food neophobia may be deactivated by just a single experience with a novel palatable food. Marples et al. (2007) offered two alternative explanations. Either the chicks use novelty itself as a cue and generalise their experience to other types of novel food, or they first form a mental representation of a palatable food (i.e. chick crumb) and after the experience with novel-coloured chick crumbs they disregard the colour as an important trait of a palatable food (Marples et al., 2007). Because the neophobia in great tits and coal tits decreased towards the food that was novel not only in colour but also in shape and way of movement, it is likely that the birds used the novelty itself as a cue for recognizing palatable food.

In blue tits, however, the hesitation to attack a novel palatable prey was not affected by their previous positive experience with another type of novel prey. This indicates that the food neophobia in blue tits is stronger and more persistent than in the other two species. Juvenile blue tits remain cautious when attacking a prey of novel colour and shape regardless of their previous experience.

In contrast to its effect on attack latencies, the positive experience from training with novel palatable prey did not affect the willingness to consume another novel palatable prey (blue cricket) in any of the three tit species tested. Although the birds from experienced groups were more likely to attack the blue crickets and shortened their attack latencies significantly, their willingness to consume the prey was the same as that of the birds from non-experienced groups. Consequently, overcoming neophobia towards novel and fully palatable prey does not necessarily involve the acceptance of such prey into the diet. These results suggest that the birds made decisions about the food in two rather independent steps, one concerning attacking and the other consuming the prey. This is consistent with the hypothesis that neophobia and dietary conservatism are two distinct processes (Marples and Kelly, 1999). In tits, only neophobia, and not dietary conservatism, was deactivated by the positive experience with the novel prey during training. In contrast, domestic chicks deactivate both neophobia and dietary conservatism through the exposure to a coloured palatable prey during training (Marples et al., 2007). There are three possible explanations for this difference between the results of Marples et al. (2007) and the present study: (a) This difference may have resulted from different durations of exposures to the novel food during training. Marples et al. (2007) showed that while neophobia in domestic chicks was significantly reduced after one-minute exposure to novel food, deactivation of both components of dietary wariness required longer time (25–40 min). In our experiments the exposure usually took 15–30 min, and it is possible that longer experience could deactivate both components of dietary wariness; (b) It may be easier for the birds to overcome dietary conservatism towards novel food differing from the training food only in colour (chicken crumbs in Marples et al., 2007) than towards food differing also in shape and way of movement (red mealworms versus blue crickets in our experiment); (c) There may be an overall difference in strength and duration of dietary conservatism between domestic gallinaceous and passerine birds,

as Marples et al. (1998) observed the dietary conservatism in robins (*Erithacus rubecula*) and blackbirds (*Turdus merula*) to last for weeks or even for months. Moreover, the strength of dietary conservatism in domestic chicks might have been reduced by the artificial selection for rapid weight gain of the breeds used for the experiments (Marples and Kelly, 1999).

#### 4.2. Reaction towards aposematic prey

Predator species may differ in their innate wariness against aposematic prey, and these differences may concern specific biases against warning coloration (Smith, 1975, 1977) or general degree of food neophobia (Exnerová et al., 2007). Initial innate wariness may, however, be modified by experience, and even species with similar degree of innate wariness may differ in how much they are susceptible to further changes. Several studies have shown that innate wariness may be decreased or deactivated by a positive experience with novel palatable food (Coppinger, 1970; Marples et al., 2007), but comparative data are still lacking. On the other hand, the wariness may also be increased or reactivated by a negative experience with unpalatable food (Schlenoff, 1984; Marples et al., 2007). Among the European species of Paridae, juvenile blue tits and coal tits exhibit high degree of food neophobia and innate wariness against aposematic firebugs (Exnerová et al., 2007). In this study, we have found that whereas in coal tits the positive experience with red-coloured palatable mealworms significantly reduced initial wariness, in blue tits the wariness persisted irrespectively of experience. In coal tits (and similarly in great tits, the least wary species) the decreased wariness may be caused either by deactivation of neophobia or by broad generalisation between red mealworms and red-and-black patterned firebugs. Whatever the case, none of these processes appeared to take place in blue tits. To overcome their stronger innate wariness, blue tits may require repeated positive experience with novel palatable prey, because adult (wild-caught) blue tits are less neophobic than the juveniles (Adamová-Ježová, unpublished).

In contrast to blue tits and coal tits, the naive juvenile great tits usually do not show any strong wariness against aposematic prey (Exnerová et al., 2007; Svádová et al., 2009; Hotová Svádová et al., 2013; but see Lindström et al., 1999b). Individual birds, however, differ in their degree of hesitancy to attack novel aposematic prey, and the differences are associated with differences in personality (Exnerová et al., 2010). A positive experience with red-coloured palatable mealworms farther decreased the hesitancy of great tits to attack red-and-black firebugs, and in contrast to the reactions towards blue crickets, the experience also made the birds more willing to consume the aposematic firebugs. A similar though not significant trend was found also in coal tits. The explanation may be in deactivated wariness of novel prey (both neophobia and dietary conservatism) by repeated positive experience with palatable food of various colours and shapes, similarly as in the domestic chicks (Marples et al., 2007). Alternatively, birds may have generalised their experience with palatable red-coloured mealworms to the red-and-black firebugs. Colour is for the birds the most salient visual stimulus in associative and discrimination learning about prey palatability (Aronsson and Gamberale-Stille, 2008, 2012; Kazemi et al., 2014), and the birds from the experienced group may have associated the red colour with palatability. Even if we cannot assure whether the experience with the red colour alone was responsible for making the birds more willing to eat the firebugs or it was combined experience with prey of a similar colour (red mealworms) and shape (blue crickets), it is evident that experience with palatable prey of merely the shape similar to the firebugs had considerably smaller effect.

#### 4.3. Comparison between species

There are several factors that may have influenced the inter-specific differences in reactions to novel palatable prey and novel aposematic prey.

- (a) Size. We may expect the larger species to be less neophobic towards the food than smaller ones, because the larger body size would contribute to easier physiological overcoming the adverse effects of noxious food (Lamanna and Hart, 1968; Anderson and Weber, 1975; Smith and Phillips, 2006). Larger passerine species also attack aposematic prey more frequently than smaller ones (Exnerová et al., 2003). This assumption would explain the differences between the large great tit and much smaller blue tit (and partly also between great tit and the smallest species, the coal tit) but not the coal tit's lesser hesitancy to attack the novel prey in comparison with the blue tit.
- (b) Dominance in mixed winter flocks. Kawamori and Matsushima (2012) studied the risk-taking in three sympatric tit species in Japan. The varied tit (*Poecile varius*), which is the largest and dominant over two smaller species (marsh tit, *Poecile palustris* and great tit, *Parus major*), was also most neophobic. Smaller and submissive marsh tits and great tits have probably been driven by the dominant species to exploit variable and risky food. Our results, however, do not support this hypothesis, because the far less neophobic species in our study was the great tit, which is the largest of the three species, and is also dominant in winter foraging flocks (Cramp and Perrins, 1993; Morse, 1978). The difference between the results of Kawamori and Matsushima (2012) and our results may simply be due to the different species studied. According to the nowadays mostly accepted taxonomy (Kvist et al., 2007; Päckert et al., 2005; Kvist and Rytken, 2006), the Japanese great tits belong to the Eastern-Palaeartic species *Parus minor* (Temminck and Schlegel, 1848). Moreover, while Kawamori and Matsushima (2012) tested wild-caught adults, we have tested naive juvenile birds, and the innate degree of neophobia may be modified by experience, especially during the first winter period.
- (c) Geographic range. The range is another explanatory factor though its delimitation depends on the taxonomy accepted (see Kvist et al., 1996; Gill et al., 2005). The blue tit (*sensu stricto*; Salzburger et al., 2002; Kvist et al., 2004) inhabits the Western Palaeartic, an area with largely moderate climate without regular temperature excesses (particularly without too low temperatures in winter). On the other hand, the ranges of great tits (regardless whether conceived in restricted or broad taxonomic sense) and coal tits include an extensive part of Siberia (Cramp and Perrins, 1993; del Hoyo et al., 2007; Tietze and Borthakur, 2012) in which harsh climate and scarcity of food occur regularly. This may explain the “affordable” neophobia in blue tit and “enforced” neophilia in great and coal tit enhanced by long-lasting postglacial selection. More exact development of this hypothesis would need phylogeographic assessments of postglacial biogeographical histories of the species concerned.
- (d) Habitat. The great tit and the blue tit are habitat generalists, and encounter a wide range of different (and potentially noxious) insect prey; this may have enhanced the neophobia of the small-sized blue tit. On the other hand, the coal tit is a habitat specialist preferring coniferous woods (Cramp and Perrins, 1993; del Hoyo et al., 2007) with more monotonous food supply and smaller chance to encounter aposematic prey, including *Pyrrhocoris apterus*. Therefore, even with the smallest body size, coal tits can be less neophobic than blue tits, which have greater chance to encounter a novel noxious prey.

- (e) Diet composition. This factor is closely related with habitat. All the three species feed in the spring and summer (when our experiments were carried out) on various invertebrates, and a large proportion of their diet consists of small spiders and caterpillars (Cramp and Perrins, 1993). Unlike the other two species, coal tits also include into their diet large proportion of aphids (Hemiptera: Sternorrhyncha; Cramp and Perrins, 1993; Krištín, 1992), which are usually not chemically defended (Gullan and Cranston, 2014) and represent a safe and abundant source of food. This diet preferences might allow for the less neophobic reactions of coal tits in comparison with blue tits, which are similarly small but have more varied and potentially noxious diet.
- (f) Food caching. Of the three species, only coal tits cache the food. This behaviour can be associated with greater food neophobia, since the food-caching species may be at lesser immediate risk of starvation than the non-caching species. On the other hand, some food caching bird species are known to cache toxic prey and return to it later, when the toxins have degraded (Yosef and Whitman, 1992; Exnerová et al., 2008), a strategy that may lead to lesser hesitation to attack the novel prey and partly explains the difference between the coal tits and the blue tits. In our experiments we observed this behaviour in coal tits. They used slits in experimental cage for storing the firebugs and after several minutes, when the repellent secretion has probably degraded, they occasionally consumed them.

## 5. Conclusions

The three species of tits differed in how the experience with a novel palatable prey (red mealworm) affected their behaviour towards another novel prey. Great tits and coal tits which had a positive experience were more willing to attack another novel palatable prey (blue cricket) and a novel aposematic prey (red-and-black firebug). On the contrary, blue tits remained highly aversive towards the both types of novel prey irrespective of their experience. We have considered the factors potentially important for the differences between the three tit species studied. The great tit is in all respects the boldest of the three species and this is most likely associated with its large size. Size itself, however, does not explain all the differences. High degree of neophobia in the blue tit as compared with the coal tit is in accordance with the more restricted geographic distribution of the blue tit and with habitat specialisation to coniferous forests in the coal tit. The potential role of food caching in the preferences of coal tits remains unclear.

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1 **Growing out of personality? Reactions of adult great tits towards an aposematic firebug**

2

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10

11 **ABSTRACT**

12 Variation in the reaction to novel and aposematic prey is common among conspecific

13 individuals of avian predators. In wild-caught adults this variation may be caused by

14 individual experience with various types of prey, but similar variation is present also among

15 naive hand-reared juveniles. This variation may be explained by the effect of personality – a

16 complex of correlated, heritable behavioural traits consistent across contexts. Two extreme

17 personality types were defined in great tits (*Parus major*): the "fast explorers" are bold,

18 aggressive and routine-forming; the "slow explorers" are shy, non-aggressive and innovative.

19 We have tested the influence of personality type on innate wariness towards aposematic prey

20 in adult hand-reared great tits from two opposite lines selected for exploration ("fast" against

21 "slow"). The birds were offered aposematic firebugs (*Pyrrhocoris apterus*) in a sequence of

22 trials in two following days. Birds from both personality types showed similar innate wariness

23 towards the firebugs on the first day, but on the second day the "fast" birds approached

24 firebugs significantly faster and more frequently than "slow" birds. Whether a bird attacked

25 the firebug was also dependent on its personality. On the second day, half of the "fast" birds

26 approached and also attacked the firebugs. While the "slow" birds showed the same level of  
27 wariness towards aposematic prey during the whole experiment, the initial wariness of "fast"  
28 birds decreased. However, the overall proportion of birds that attacked aposematic firebugs  
29 was much smaller than in the previous study of similarly reared hand-reared juveniles. The  
30 personality-related individual differences in reactions of great tits towards the aposematic  
31 prey were consistent across time, but the overall willingness to attack such a prey has  
32 decreased in older birds. We suggest that laboratory conditions with unlimited food supply  
33 and restricted variety of food types might have enhanced the food neophobia in "slow" birds,  
34 leading at the same time to fixation on the familiar food in routine-prone "fast" birds.

35

## 36 **KEYWORDS**

37 Age, aposematism, naive adult predators, *Parus major*, personality, *Pyrrhocoris apterus*

38

## 39 **INTRODUCTION**

40 Aposematic prey advertises its unprofitability and noxiousness by the conspicuous coloration  
41 (for review see Ruxton et al. 2004). The initial reactions of bird predators towards aposematic  
42 prey may vary among species (Brower 1988; Exnerová et al. 2003; Endler and Mappes 2004;  
43 Valkonen et al. 2012; Nokelainen et al. 2014) as well as among the conspecific individuals  
44 (Exnerová et al. 2007, 2010, 2015). Encountering novel aposematic prey can elicit neophobic  
45 reaction, which differ individually in wild birds with previous experience with a variety of  
46 food types (Marples et al. 1998, 2005; Exnerová et al. 2015), but also in naive hand-reared  
47 juveniles (Sillén-Tullberg 1985; Marples and Brakefield 1995; Exnerová et al. 2007, 2010;  
48 Svádová et al. 2009). As has been shown in previous studies, hand-reared juveniles of great  
49 tits (*Parus major*) do not exhibit any strong innate bias against aposematic prey (Sillén-  
50 Tullberg 1985; Exnerová et al. 2007, 2010; Svádová et al. 2009; Hotová Svádová et al. 2013;

51 Fabricant et al. 2014), but there is a variation in neophobia, rate of avoidance learning, and  
52 memory for aposematic signals among the individuals (Svádová et al. 2009; Exnerová et al.  
53 2010; Hotová Svádová et al. 2013). This individual variability can be correlated with  
54 personality types (Exnerová et al. 2010). Personality is recognized in a variety of animal  
55 species (Gosling 2001) and it is described as “a complex of correlated behavioural traits that  
56 are consistent across time and ecological situations” (Benus et al. 1990; Sih et al. 2004). In  
57 this study we tested great tits (*Parus major*) coming from two lines selected for the opposite  
58 types of personality (for more see Verbeek et al. 1994; Drent et al. 2003). ”Slow” individuals  
59 are cautious, innovative, less aggressive and more thorough explorers, ”Fast” individuals are  
60 more aggressive, more prone to risk-taking behaviour, are superficial explorers and routine  
61 formers (Verbeek et al. 1994; Drent et al. 2003; van Oers et al. 2004; Groothuis and Carere,  
62 2005). In our previous study (Exnerová et al. 2010), the "slow" juvenile great tits were more  
63 neophobic and more cautious in attacking aposematic firebugs (*Pyrrhocoris apterus*), and  
64 were more successful in avoidance learning than the "fast" individuals. But whether these  
65 differences are consistent across time has not yet been studied.

66 Neophobia was shown to be non-consistent during the life of an individual (Vince 1960;  
67 Greenberg 1992; Heinrich 1995; Fox and Millam 2004; Biondi et al. 2010, 2013). Low  
68 neophobia in young birds makes this period important in shaping their foraging niche and is  
69 mainly restricted to the juvenile period, the phase when individuals show the greatest  
70 plasticity associated with object exploration (Greenberg 2003; Biondi et al. 2010). Neophobia  
71 may increase during the life (Greenberg and Mettke-Hofmann, 2001). Heinrich (1995)  
72 showed that juvenile common ravens (*Corvus corax*) are highly explorative and curious, but  
73 at the age of one and half year they started to be more hesitant in contacting novel items.  
74 Hand-reared juveniles of Orange-winged amazon parrot (*Amazona amazonica*) exhibited  
75 lower avoidance of novel objects at the age of 7 months than at 12 months (Fox and Millam

76 2004), and young wild-caught Chimangos Caracara (*Milvago chimango*) were more  
77 explorative and less neophobic than their adult conspecifics (Biondi et al. 2010, 2013).  
78 Nevertheless the correlation between neophobia to novel objects and neophobia to novel food  
79 is still ambiguous. While some studies found strong correlation between exploration of novel  
80 objects (Exnerová et al. 2010) or neophobia (An et al. 2011) and acceptance of novel food,  
81 others did not show such a relationship (Bókonyi et al. 2012; Exnerová et al. 2015).

82 The aim of our study was to test whether the personality type of naive hand-reared adults  
83 of great tits (*Parus major*) affects their reaction to a novel aposematic prey - the firebug  
84 (*Pyrrhocoris apterus*). In our previous study (Exnerová et al. 2010) we found that naive hand-  
85 reared juveniles from lines selected for opposite personalities ("slow" versus "fast") showed  
86 different responses to aposematic prey. The juvenile birds were tested between 64 – 90 days  
87 of age, and almost all of them attacked the aposematic firebugs. Nevertheless, "slow" birds  
88 showed a greater degree of unlearned wariness and learned to avoid the firebugs faster than  
89 did the "fast" birds (Exnerová et al. 2010). In the present study we tested naive hand-reared  
90 adults (1,5 – 5,5 years old) from the same two personality lines, and compared their  
91 reactions towards aposematic firebugs with the behaviour of juvenile birds (Exnerová et al.  
92 2010) to find out , whether the personality-related differences are consistent across time.

93

## 94 **MATERIAL AND METHODS**

95 The experiments were carried out in the Netherlands Institute of Ecology, Heteren.

96

### 97 (a) **Birds**

98 We tested hand-reared juveniles of great tits (*Parus major*) from the F4 generation of artificial  
99 selection for two distinct personality lines: fast and slow explorers (Drent et al. 2003). Birds  
100 were tested at the age of 35 days for early exploratory behaviour by standard tests (reaction to

101 novel objects and behaviour in novel environment (for details see Verbeek et al. 1994; Drent  
102 et al. 2003). Birds were housed individually in plastic home cages (90 x 40 x 50 cm) with  
103 front wire mesh wall, three perches, three feeders and water bowl with *ad libitum* drinking  
104 and bathing water. Birds were daily provided with mixture of seeds, dried egg yolk, dried beef  
105 heart and vitamins, supplemented with mealworms every second day. Mealworms (*Tenebrio*  
106 *molitor* larvae) were used in our experiments as a control familiar prey. We tested altogether  
107 40 individuals, balanced in terms of personality and sex - 18 fast explorers (8 females and 10  
108 males) and 22 slow explorers (12 females and 10 males). Within fast group we had six pairs  
109 of siblings and in slow group five pairs of siblings (2 birds from one nest, in one case we had  
110 3 birds from one nest). Birds had no previous experience with aposematic or any other  
111 noxious prey and were between 1,5 and 5,5 years old when the experiments with aposematic  
112 firebug were carried out. After the experiments all birds were returned to breeding facilities.

113

#### 114 **(b) Prey**

115 Adult brachypterous firebugs (*Pyrrhocoris apterus*; Heteroptera: Pyrrhocoridae) were used as  
116 novel aposematic prey. Firebugs are 7 – 12 mm long and possess red-and-black coloration  
117 with contrasting pattern. They produce defensive secretions from methathoracic glands which  
118 contain, beside others, short-chained aldehydes (Farine et al. 1992). Previous studies showed  
119 that firebugs are unpalatable for small passerine birds and their consuming may result in  
120 nausea and vomiting in some birds (Exnerová et al. 2003, 2007; Svádová et al. 2009).  
121 Firebugs are widespread in Palaearctic region and can be found all over the Europe (excluding  
122 Ireland, Norway and Finland) (Aukema and Rieger 2001). The firebugs feed mainly on seeds  
123 of *Tiliaceae*, *Malvaceae* and *Robinia pseudoacacia* (Socha 1993). We have collected adult  
124 firebugs on linden trees at two localities in Czech Republic (Prague, 50°04'N, 14°26'E and

125 Uherské Hradiště, 49°04'N, 17°26'E) and kept them under a long-day photoperiod (18:6h) at a  
126 temperature of  $24 \pm 1^\circ\text{C}$ . The firebugs were provided with linden seeds and water *ad libitum*.

127

### 128 **(c) *Experimental design***

129 The experimental cages were made of plywood and wire-mesh with one-way glass front wall  
130 (70 x 70 x 70cm). They were equipped with water bowl, beige wooden rotating feeding tray  
131 containing 6 glass dishes, 1 and a perch placed 30cm from the feeding tray. The simulation of  
132 daylight condition was achieved by the lamps Biolux Combi, Osram (see Exnerová et al.  
133 2003, 2010 for details). The birds were deprived of food for 2 hours before an experiment and  
134 trained to search for mealworms in one of the dishes.

135 The experiment consisted of (1) avoidance learning session and (2) memory test. (1) The first  
136 day birds learned to avoid firebugs in a sequence of 5 min trials, in which the control  
137 palatable mealworms were alternating with aposematic firebugs. The learning session was  
138 considered successful when the bird refused to attack three firebugs in a row. If the bird did  
139 not attack the first firebug, the sequence continued until a maximum of 10 firebugs was  
140 presented, and the learning session was repeated next day. (2) If the bird attacked the firebugs,  
141 and then learned to avoid them, the memory test was carried out on the following day. The  
142 memory test consisted of a sequence of 5 firebug trials. We recorded whether the firebug was  
143 approached, attacked, killed and consumed as well as the approach and attack latency.

144 Behaviour of birds was recorded in Observer Video-Pro © Noldus and on video recorder.

145

### 146 **(d) *Data analysis***

147 We compared latencies to attack the mealworms offered at the beginning of each-day sessions  
148 to check that birds from both personality groups entered the experiment with similar foraging  
149 motivation and habituation to the experimental cage. The latencies were log-transformed, and

150 then analysed using ANOVA with personality type as a factor. Latencies of the first approach  
151 to the firebug were log-transformed and analysed by ANCOVA with personality as fixed  
152 factor, bird's sex as random factor, and age as covariate. Numbers of approaches per trial were  
153 square-root transformed and tested by ANCOVA with personality as fixed factor, bird's sex as  
154 random factor, and age as covariate. Within-group changes in approach latencies between day  
155 1 and day 2 were tested by t-tests for dependent samples. We also compared counts of birds  
156 that attacked at least one firebug during the experiment using GLM ANCOVA model for  
157 binomial distribution and logit link function, with personality as fixed factor, bird's sex as  
158 random factor, and age as covariate. The calculations were made using Statistica 6.1 (StatSoft  
159 Inc.).

160

## 161 **RESULTS**

### 162 **(a) *Reaction to familiar prey***

163 There was no difference between the two personality groups in latencies to attack the  
164 mealworm offered in the first trial of avoidance learning (ANOVA:  $F_{1,38} = 2.121$ ,  $p = 0.154$ ),  
165 as well as in latencies to attack the first mealworm in the memory test (ANOVA:  $F_{1,38} =$   
166  $0.961$ ,  $p = 0.333$ ). All birds killed and ate the mealworms they were offered.

167

### 168 **(b) *Reaction to aposematic prey***

169 All the birds but five (three slow explorers and two fast explorers) approached the firebugs  
170 and inspected them from a close distance at least once during the first-day learning session.  
171 The latencies of the first approach to the firebug and the numbers of approaches per trial were  
172 not influenced by personality (ANCOVA: latency -  $F_{1,31} = 0.003$ ,  $p = 0.960$ ; number:  $F_{1,31} =$   
173  $0.024$ ,  $p = 0.879$ ), sex (ANCOVA: latency -  $F_{1,30} = 3.149$ ,  $p = 0.086$ ; number:  $F_{1,30} = 0.091$ ,  $p$

174 = 0.765) and age of the birds (ANCOVA: latency -  $F_{1,29} = 0.851$ ,  $p = 0.364$ ; number:  $F_{1,29} =$   
175 0.239,  $p = 0.629$ ).

176 In the second-day test, there was a difference between the two personality groups in both  
177 the latencies of approaching the firebugs (ANCOVA:  $F_{1,31} = 18.394$ ,  $p < 0.001$ ) and in the  
178 number of approaches per trial (ANCOVA:  $F_{1,31} = 10.430$ ,  $p = 0.003$ ). Fast birds hesitated  
179 shorter before first approaching the firebugs, and also inspected the firebugs at close distance  
180 more often than slow birds. The sex and age of the bird affected neither the latencies  
181 (ANCOVA: effect of sex -  $F_{1,30} = 2.561$ ,  $p = 0.120$ ; effect of age -  $F_{1,29} = 2.727$ ,  $p = 0.109$ ) nor  
182 the number of approaches (ANCOVA: effect of sex -  $F_{1,30} = 0.183$ ,  $p = 0.672$ ; effect of age -  
183  $F_{1,29} = 1.377$ ,  $p = 0.250$ ). Fast birds shortened their latencies to approach the firebugs from  
184 the first-day to the second-day session (t-test:  $t = 2.283$ ,  $df = 13$ ,  $p = 0.040$ ), whereas the  
185 approach latencies of slow birds remained the same (t-test:  $t = -1.214$ ,  $df = 18$ ,  $p = 0.240$ ).

186 Although the birds repeatedly approached the firebugs and inspected them from close  
187 distance, most of them refused to attack any firebug during both days of learning sessions.  
188 Nevertheless, whether the birds attacked at least one firebug was influenced by their  
189 personality (GLM ANCOVA: Chi-square = 6.390,  $df = 1$ , 38,  $p = 0.011$ ); half of the fast birds  
190 and only three of the slow birds attacked the firebugs. There was no effect of sex and age of  
191 the birds on the probability of attacking the firebugs (GLM ANCOVA: effect of sex - Chi-  
192 square = 0.076,  $df = 1$ , 37,  $p = 0.783$ ; effect of age - Chi-square = 0.442,  $df = 1$ , 36,  $p =$   
193 0.506). The birds learned to avoid the firebugs very quickly, attacking mostly only one  
194 individual during the process of learning. They usually only slightly pecked the firebug or  
195 seized it by the bill and dropped it. The firebugs almost always survived the attacks. Only  
196 two fast birds attacked, killed, and consumed several firebugs before reaching the learning  
197 criterion. None of the birds that learned to avoid the firebugs attacked any firebug during the  
198 memory test.

199

## 200 **DISCUSSION**

### 201 **(a) *Effect of age***

202 There is a considerable difference in behaviour towards aposematic prey between naive hand-  
203 reared juvenile great tits and their experienced wild-caught conspecifics (Exnerová et al.  
204 2007). Since the previous studies did not show any strong innate wariness of aposematic prey  
205 in hand-reared great tits (Exnerová et al. 2007, 2010; Svádová et al. 2009; Hotová Svádová et  
206 al. 2013), it is surprising that in our experiment hand-reared adults frequently avoided the  
207 aposematic prey. Birds in this study rarely attacked, pecked or seized the aposematic firebugs  
208 (*Pyrrhocoris apterus*), and they mostly only came to a close proximity and inspected the prey  
209 by sight. In the previous study (Exnerová et al. 2010), juvenile great tits reared in the same  
210 conditions and same location as birds tested in this study exhibited greater willingness to  
211 attack, kill and consume the firebugs. The difference between behaviour of juvenile and adult  
212 birds might have been caused by changes in neophobia or dietary conservatism during the life.

213 Neophobia is a process which protects animals from toxic and dangerous food, but on the  
214 other hand, the exploration of novel resources is important for young individuals when their  
215 foraging behaviour is being shaped (Greenberg and Mettke-Hofmann 2001). Several studies  
216 have shown that birds are more willing to explore novel stimuli, when they are young (Vince  
217 1960; Heinrich 1995; Fox and Millam 2004; Biondi et al. 2010). Whereas Coppinger (1970)  
218 found innate avoidance of novel aposematic butterflies (Nymphalinae) in hand-reared blue  
219 jays (*Cyanocitta cristata*) and red-winged blackbirds (*Agelaius phoeniceus*) tested at the age  
220 of 9 – 10 months, no such avoidance was recorded by Smith (1980) testing hand-reared  
221 juveniles of the same bird species with novel and warningly coloured stimuli when they were  
222 40 days old. Mastrota and Mench (1994) observed greater aversion of female bobwhites  
223 (*Colinus virginianus*) towards red- and orange-dyed food, when tested in 64 – 67 weeks, than

224 in younger, 31 weeks old females (Mastrota and Mench 1995). No such an effect was  
225 observed in the conspecific males. The aversion of novel-coloured food in female bobwhites  
226 thus appears to increase with age, and a possible explanation may be a different role of sexes  
227 in parental care in bobwhites, where the females teach their chicks to avoid toxic prey.  
228 Considering the role of both parents in post-fledging care in great tits, we may expect the  
229 same level of wariness in both sexes.

230 The hypothesis of increasing neophobia with age was not supported by the study of  
231 Langham (2006), who found older individuals of wild-caught rufous-tailed jacamars (*Galbula*  
232 *ruficauda*) to be more willing to attack novel aposematic butterflies (*Heliconius* sp.) than  
233 their younger (and also wild-caught) conspecifics. It is therefore likely that wild-caught  
234 experienced adult birds are bolder to attack novel prey, because they have already learned to  
235 avoid unpalatable food, whereas wild-caught younger birds may be more cautious due to a  
236 recent experience with unpalatable or toxic prey. This hypothesis is supported by Lindström  
237 et al. (1999), who compared reactions towards warningly coloured yellow-and-black prey in  
238 three age categories of great tits: hand-reared juveniles, wild-caught yearlings and wild-  
239 caught adults. While both hand-reared juveniles and wild-caught adults readily attacked  
240 warningly coloured prey, wild-caught yearlings appeared to be the most neophobic age group  
241 (Lindström et al. 1999). Similarly, wild-caught yearlings of great tits attacked and killed more  
242 individuals of novel colour forms of the firebug (*Pyrrhocoris apterus*) than did the wild-  
243 caught adults (Exnerová et al. 2006).

244 Even though the great tits in this experiment were inexperienced with aposematic prey and  
245 1,5 – 5,5 years old at the time of testing, they were very cautious to attack unfamiliar  
246 aposematic firebugs. Contrastingly, wild-caught great tits from the Finnish population, which  
247 lack any experience with aposematic firebugs (due to their absence in Finland), showed no  
248 hesitation to attack them. There might be another factor possibly responsible for the cautious

249 behaviour of birds in the present study. It is likely that the environment and rearing conditions  
250 play an important role in shaping the reactions towards novelty in birds (Jones 1986; Meehan  
251 and Mench 2002; Fox and Millam 2004, 2007). Studies of domestic chicks (*Gallus gallus*)  
252 and orange-winged Amazon parrots (*Amazona amazonica*) demonstrated the importance of  
253 enrichment of the home environment. Enriched environment significantly reduced the fear of  
254 novel objects in orange-winged Amazon parrots (Meehan and Mench 2002; Fox and Millam  
255 2004, 2007), and in chicks, the home environment with assorted novel objects even increased  
256 the acceptance of novel food into their diet (Jones 1986).

257 The experience with variety of food reduced neophobia and increased acceptance of novel  
258 food in chicks (Jones 1986; Marples et al. 2007) and turkeys (Lecuelle et al. 2011, but see  
259 Mastrota and Mench 1995). Because the majority of birds in our study did not explore the  
260 firebugs by tactile contact, we can not decide, whether their avoidance of novel prey was a  
261 result of neophobia or dietary conservatism (Marples and Kelly 1999). However, food  
262 neophobia is described as a short-time response lasting usually only a few minutes and  
263 followed by a tactile contact with the novel food (Marples and Kelly 1999), whereas the birds  
264 in our study showed a relatively long-lasting refusal of novel prey. The rearing conditions  
265 with a limited variety of food types and an unlimited supply of them could make the birds  
266 unwilling to explore a novel prey. This would correspond with the relationship between  
267 generalist-specialist behaviour and exploration, where the food or habitat specialists exhibit  
268 greater neophobia than the species that exploit broader variety of foods and habitats  
269 (Greenberg 1984, 1990; Mettke-Hofmann et al. 2002; Webster and Lefebvre 2001; Tebbich et  
270 al. 2009).

271

272 **(b) Effect of personality**

273 Although the birds of both personality types showed similar aversion towards the aposematic  
274 firebugs on the first day, their behaviour on the second day considerably differed. On the  
275 second day, the "fast" birds approached the firebugs significantly earlier and more often than  
276 the "slow" ones. Whether the birds attacked the firebugs was also dependent on personality  
277 types. On the second day, half of the "fast" birds not only approached and inspected the  
278 firebugs from a close distance, but also seized and pecked it. Nevertheless, only two fast birds  
279 consumed several firebugs during the experiment. Long-lasting process of refusing to  
280 consume novel food was termed dietary conservatism (Marples et al. 1998; Marples and Kelly  
281 1999). In the wild-caught great tits, the dietary conservatism has been shown to be  
282 independent on personality, because the dietary conservative birds were not found to be  
283 slower explorers (Marples and Mappes 2011). However, in Japanese quails (*Coturnix coturnix*  
284 *japonicus*), selected accordingly to their speed of recruitment of aposematic non-toxic two-  
285 spot ladybirds (*Adalia bipunctata*) into their diet, the variation in acceptance of novel prey  
286 had a genetic basis, suggesting an effect of dietary conservatism (Marples and Brakefield  
287 1995). Whether the dietary conservatism is connected with personality traits is still a question  
288 for future research.

289 The relationship between exploration/object neophobia and neophobia towards novel food  
290 is also still ambiguous. Only a few studies dealt with the correlation between food neophobia  
291 and other personality traits (Exnerová et al. 2010, 2015; An et al. 2011; Bókony et al. 2012;  
292 Liebl and Martin 2014). Exnerová et al. (2010) showed a positive correlation between  
293 exploration and reaction towards novel aposematic prey in juvenile great tits. Similarly, An et  
294 al. (2011) have found positive correlation between latency to approach and feeding in the  
295 presence of novel object and approach and pecking novel type of food in wild caught blacked-  
296 capped chickadees (*Poecile atricapillus*). However, food neophobia correlated with object  
297 neophobia only in one of the four Hungarian populations of house sparrows (*Passer*

298 *domesticus*), and in none of the eight populations studied in Kenya (Bókony et al. 2012; Liebl  
299 and Martin 2014). The correlations seem to be population-specific, not only in house  
300 sparrows, but also in great tits. While Dutch population showed strong correlation between  
301 personality traits and behaviour to novel food, no such correlation was found in Finnish and  
302 Bohemian populations of great tits (Exnerová et al. 2010, 2015).

303         In great tits, the slow explorers tend to be more successful in avoidance learning than  
304 the fast explorers, presumably because they are more neophobic and sensitive to negative  
305 stimuli (Exnerová et al. 2010). In our study, all birds that have overcome their neophobia and  
306 attacked the aposematic firebugs learned to avoid them very quickly, attacking mostly only  
307 one firebug during the learning session. The firebugs were mostly only pecked or seized and  
308 dropped, and only occasionally consumed. Such a behavioural response might indicate an  
309 effect of dietary conservatism, but considering the unpalatability of the firebug, it also may be  
310 a result of a rapid avoidance learning. Similarly, wild-caught adult great tits from Finnish  
311 population that were inexperienced with the aposematic firebugs, learned to avoid them  
312 considerably faster than their naive hand-reared conspecifics (Svádová et al. 2009; Exnerová  
313 et al. 2010). Interestingly, the Finnish wild-caught adults were much less cautious in attacking  
314 aposematic prey (Exnerová et al. 2015) than the hand-reared captive adults in our study.

315

## 316 **CONCLUSIONS**

317 On the first day of the experiment, the fast birds hesitated as long as the slow birds in  
318 approaching the aposematic firebugs. However, the type of personality influenced the  
319 approach latencies on the second day, when the slow birds hesitated longer than the fast birds.  
320 The slow birds were also approaching the firebugs less frequently than the fast birds. While  
321 the slow birds showed the same level of innate wariness towards aposematic prey during the  
322 whole experiment, the level of innate wariness of the fast birds significantly decreased. We

323 suggest that the laboratory rearing conditions with unlimited food supply and restricted  
324 variety of food types might have enhanced the innate wariness in slow birds, whereas in fast  
325 birds the same conditions might have lead to developing a routine behaviour of feeding  
326 exclusively on a familiar food . Even though hand-reared juveniles from the same selection  
327 lines (Exnerová et al. 2010) reacted differently towards aposematic firebugs than hand-reared  
328 adults tested in this study, we were still able to observe significant differences in reactions of  
329 birds of different personality types. We can therefore conclude that the individual differences  
330 in reactions of great tits towards aposematic prey are consistent across time, but the particular  
331 way of reactions and their intensity changes during their life.

332

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475

## 476 **FIGURES AND LEGENDS**

477 Figure 1. Approach latencies of naive adult great tits of the two personality types (S-slow, F-  
478 fast) measured from the beginning of the first firebug trial to first approach to the firebug in  
479 the first (DAY 1) and second day (DAY 2) of firebug presentation. Square, mean; box, mean  
480  $\pm$  s.e.; whiskers, confidence intervals (0.95); circles, outliers; stars, extremes.

481

482 Figure 2. Number of approaches to firebugs per trial by naive adult great tits of two  
483 personality types (S-slow, F-fast) in the first (DAY 1) and second day (DAY 2) of firebug  
484 presentation. Square, mean; box, mean  $\pm$  s.e.; whiskers, confidence intervals (0.95); circles,  
485 outliers; stars, extremes.

486

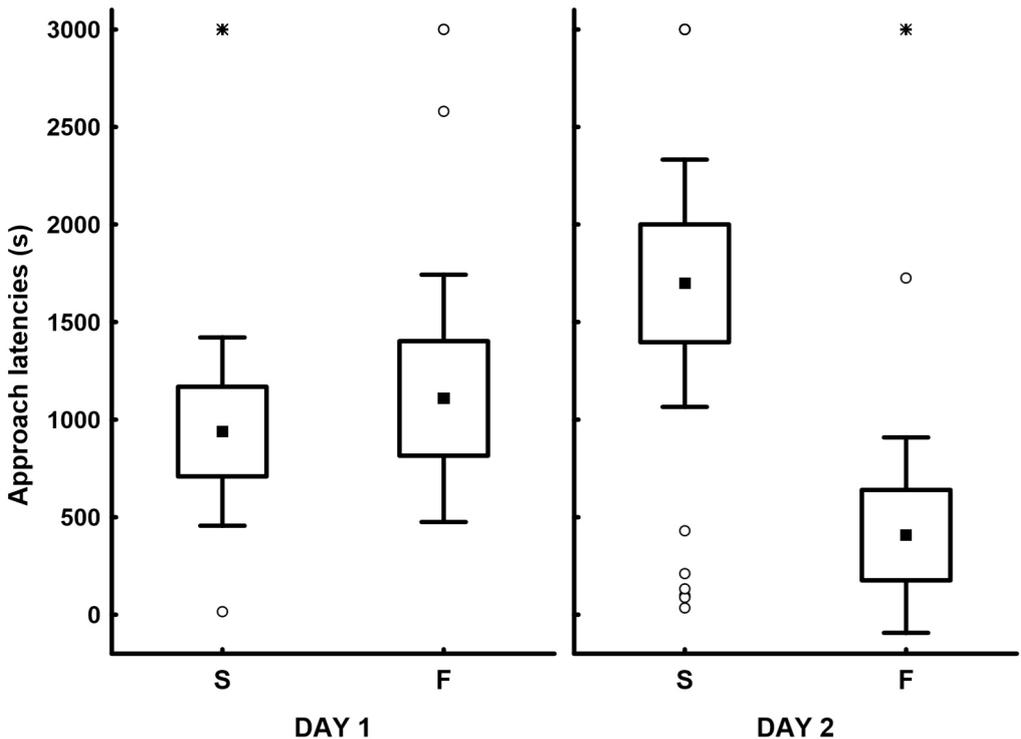
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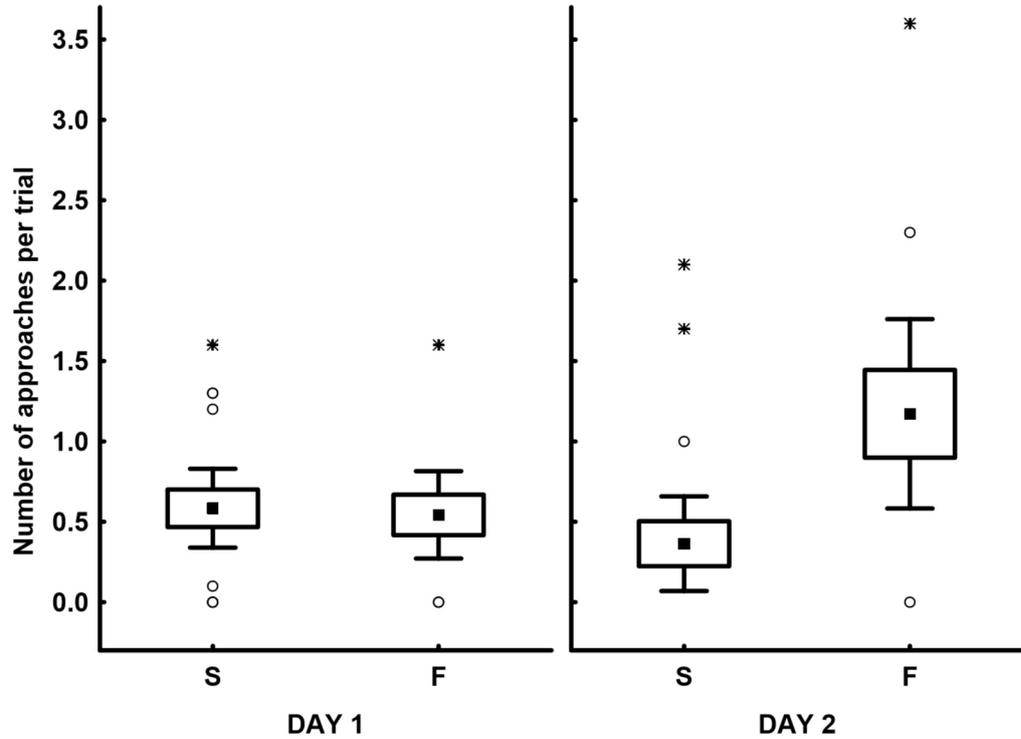
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493 Figure 1.

494



495

496 Figure 2.

497



## Scared by shiny? The value of iridescence in aposematic signalling of the hibiscus harlequin bug



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Studies on aposematism have generally focused on the benefits of red or yellow coloration, occasionally in contrast with green or brown, but rarely blue or orange. Furthermore, almost no studies have explicitly studied the utility of iridescent coloration in aposematism. To evaluate the survival benefit of iridescent coloration, we tested the ability of the natural colour extremes of *Tectocoris diophthalmus* jewelbugs to induce initial avoidance, learned avoidance, discrimination from palatable alternatives and broad generalization against avian predators: naïve hand-reared and experienced wild-caught great tits, *Parus major*. Artificial baits were created by hollowing out bugs and inserting pieces of mealworm. Preference tests presented iridescent and orange baits simultaneously, then birds were divided into training groups and sequentially exposed to palatable black baits alternated with iridescent or orange baits made unpalatable by soaking mealworms in quinine solution. This was followed by simultaneous black/coloured discrimination tests, then a generalization test with both previously experienced and novel baits (all palatable). All groups showed a preference for orange baits over ones with iridescent patches. For wild-caught birds, attack latencies of iridescent and orange training groups were statistically indistinguishable, although only orange caused increased attack latency over the sequence. Hand-reared birds showed no change in attack rate/latency towards iridescent bugs over the sequence. In postlearning discrimination tests, all groups showed equally strong preference for palatable black baits and their unpalatable training baits. In generalization tests, hand-reared birds were most averse towards trained baits, but increased avoidance of iridescent-and-black baits suggests iridescence alone can contribute to aposematism. Wild-caught birds showed strong aversion to iridescent and novel orange-and-black baits regardless of training, suggesting birds may be broadly generalizing experience from local red-and-black aposematic bugs. Results suggest iridescent coloration and patterning can be an effective aposematic signal, especially in the presence of alternative palatable prey and/or other aposematic species.

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Aposematism is the phenomenon of prey advertising their unprofitability to potential predators through conspicuous and memorable signals that are often visual in nature (reviewed in Ruxton, Sherratt, & Speed, 2004). There is a rich literature on how predators, particularly birds, respond to visual aposematic signals and their individual components. Several studies have demonstrated that birds attend mostly to colour when learning to avoid aposematic prey (Exnerová et al., 2006; Ham, Ihalainen, Lindström, & Mappes, 2006; Svádová et al., 2009). Other studies have demonstrated the relative value of background contrast (Gamberale-Stille, 2001; Gamberale-Stille & Guilford, 2003), the relative value of internal contrast and patterning (Aronsson &

Gamberale-Stille, 2008, 2009; Hegna, Saporito, Gerow, & Donnelly, 2011), the importance of pattern regularity/symmetry (Forsman & Merilaita, 1999; Stevens, Castor-Perry, & Price, 2009) and the breadth/direction of generalization (Gamberale-Stille & Tullberg, 1999; Svádová et al., 2009) for aposematic signals. Theory and empirical studies also suggest that aposematic patterns tend towards signal uniformity (Greenwood, Cotton, & Wilson, 1989; Lindström, Alatalo, Lyytinen, & Mappes, 2001), and maximal conspicuousness or distinctiveness (Roper & Redston, 1987; Sherratt & Beatty, 2003), but instances of intraspecific variation and/or low conspicuousness are common (Stevens & Ruxton, 2012). Understanding the selection pressures upon these ‘non-textbook’ aposematic systems is currently an active area of research (Blount, Speed, Ruxton, & Stephens, 2009; Endler & Mappes, 2004; Lindstedt, Talsma, Ihalainen, Lindström, & Mappes, 2010; Speed & Ruxton, 2007).

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However, despite the breadth of the literature, some areas have received considerably less attention. For example, many authors cite the prevalence of red and yellow in aposematic signals (Endler & Mappes, 2004; Ruxton et al., 2004; Théry & Gomez, 2010), but no research has quantified this prevalence. Few authors have experimented with other colours. One paper showed ultraviolet cues are not effective in avoidance learning of great tits, *Parus major* (Lyytinen, Alatalo, Lindström, & Mappes, 2001). While there are few potentially aposematic species with blue components in their signals, seemingly none have been tested empirically (reviewed in Umbers, 2013), although artificial blue/cyan prey have been used successfully by Gamberale-Stille and Guilford (2003) and Aronsson and Gamberale-Stille (2008). The efficacy of green as a colour in avoidance learning is difficult to establish, as green or brown is often used as an 'inconspicuous' control in experiments. However, exposure to pyrazine odours (Rowe & Guilford, 1996) or the bitter taste of quinine (Rowe & Skelhorn, 2005) can cause unlearned biases against yellow or red but not green. However, there is at least one study showing green food as being more resistant to overcoming dietary conservatism than yellow or red (Marples, Roper, & Harper, 1998). Better understanding of the learning value of less common 'warning' colours such as green or blue requires more study.

Similarly to short wavelengths, iridescent colours in aposematism are an understudied phenomenon. By definition, the hue of iridescent colours changes with viewing angle, owing to the arrangement of multiple reflecting layers creating a coherent Bragg mirror (Seago, Brady, Vigneron, & Schultz, 2009). Additionally, small variations in the layer spacing can result in a large change in hue or saturation (Kurachi, Takaku, Komiya, & Hariyama, 2002). These aspects of iridescent coloration may affect their efficacy in providing a reliable aposematic signal. However, iridescent and other structural colours can be very bright (Seago et al., 2009), and are capable of creating short-wavelength hues such as blues and ultraviolets uncommon in pigments (Doucet & Meadows, 2009; Umbers, 2013). Paired with pigmentary colours, this ability to create chromatic and/or luminance contrast may enhance the conspicuous and distinctiveness of the aposematic signals (Doucet & Meadows, 2009; Endler, 1992). Therefore, there is great value in studying the role of iridescence in aposematic learning.

There are scattered studies documenting a role for iridescent colours in aposematic signals in beetles (Schultz, 2001) and butterflies (Bowers & Larin, 1989; Pegram, Lillo, & Rutowski, 2013; Rutowski, Nahm, & Macedonia, 2010), but none for true bugs. Iridescence is relatively common in some families of true bugs (Heteroptera), particularly in the assassin bugs (Reduviidae), plant bugs (Miridae), leaf-footed bugs (Coreidae), burrower bugs (Cydnidae), shield bugs (Pentatomidae and Scutelleridae) and some minor families (P. Štys, unpublished data). In many of them, iridescence is combined with yellow, orange or red pigment coloration producing a potentially aposematic effect. This type of coloration is sometimes limited only to larvae for poorly understood reasons (P. Štys, unpublished data).

*Tectocoris diophthalmus* (Heteroptera: Scutelleridae), the hibiscus harlequin bug, is an ideal study system to engage with questions of intraspecific variation and learning value of iridescent coloration. Rather than employing the more 'typical' aposematic colour scheme of red or yellow with black markings, *T. diophthalmus* bugs display a matte orange background overlaid with bright metallic blue-green iridescent patches. These patches are highly variable between individuals, ranging in size from almost covering the dorsal surface to being entirely absent (Fabricant, Kemp, Krajčiček, Bosáková, & Herberstein, 2013). Like other true bugs, *T. diophthalmus* produces defensive secretions (Staddon, Thorne, & Knight, 1987), and has been demonstrated to be capable of inducing avoidance learning in

chickens (Fabricant & Smith, 2014). The species lives in Australia and Malesian and Melanesian islands, and is arboreal and mainly phytophagous, feeding on *Hibiscus*, *Gossypium* and *Lagunaria* species (Malvaceae; Cassis & Gross, 2002).

The aim of this study was to test the efficacy of the iridescent coloration of *T. diophthalmus* in inducing initial aversion, avoidance learning and generalization. We tested this bug, a native to Australia, against European great tits, an allopatric species which has no experience with *T. diophthalmus* or any other iridescent species of true bug. We tested both wild-caught and hand-reared birds; hand-reared great tits are completely naïve to noxious prey, and have been shown in experiments to lack innate bias against red-and-black coloured bugs (Exnerová et al., 2007; Svádová et al., 2009), but wild-caught birds are likely to have experience with noniridescent true bugs, including local red-and-black aposematic species (Hotová Svádová, Exnerová, Kopečková, & Štys, 2010). We compared wild-caught and hand-reared birds with respect to their initial wariness towards palatable iridescent and noniridescent (orange) bugs, and in their behaviour towards unpalatable iridescent bugs across a learning sequence, discrimination tests and generalization tests using novel bugs sharing features of conditioned bugs. Because we could not control the previous experience of wild-caught birds, we also compared the behaviour of wild-caught birds exposed to unpalatable iridescent bugs with that of birds exposed to unpalatable orange bugs that lacked iridescent patches.

Our specific questions were as follows. (1) Do the iridescent patches of *T. diophthalmus* increase initial avoidance? (2) Are iridescent patches efficient in inducing avoidance learning and discrimination from palatable alternatives? (3) Do birds generalize broadly among different colour phenotypes of the bugs, including to more 'typical' orange-and-black bugs? (4) Do experienced and naïve birds differ in their patterns of initial avoidance, learning to avoid unpalatable iridescent prey, and generalization of novel bug phenotypes? By comparing the results of these experiments to previous studies, we aimed to elucidate what components of the visual warning signals of *T. diophthalmus* are most salient to birds, and how variation may affect their survival.

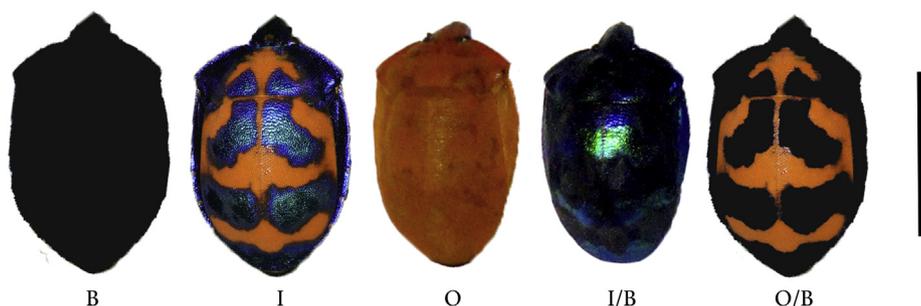
## METHODS

### Avian Predators

Great tits are small, predominantly insectivorous passerine birds inhabiting mostly woodlands, parks and gardens (Cramp & Perrins, 1993). They are frequently used as model predators in studies of warning signals and mimicry (Lindström, Alatalo, & Mappes, 1997; Sillén-Tullberg, Wiklund, & Järvi, 1982; Svádová et al., 2009) and are suitable for studies involving novel prey, because they are less neophobic than other European species of Paridae, and their avoidance of aposematic insects is mostly based on individual learning (Exnerová et al., 2007).

### Hand-reared Birds

We used 20 hand-reared great tits as naïve predators, and all were trained only with iridescent bugs. Juvenile birds were taken from their nestboxes in spring 2012 when 12–16 days old. At this age the nestlings have no visual experience with their food. The nestboxes were placed in parks and orchards in the outskirts of Prague, Czech Republic. No more than two nestlings were taken from the same brood. The nestlings were transported to the laboratory in the same artificial nests they were housed in (plastic boxes for keeping small animals with textile and tissue-paper lining for insulation and comfort). The length of transport did not exceed 30 min. Nestlings were



**Figure 1.** Examples of bug baits: black (B), iridescent (I), orange (O), iridescent-and-black (I/B) and orange-and-black (O/B). Black bar is 1 cm.

kept in artificial nests until fledging, and then housed individually in plastic cages (40 × 50 cm and ×40 cm high) with a wire-mesh front wall. The home cages were equipped with three wooden perches, two water bowls with drinking and bathing water provided ad libitum and with two feeders situated on the bottom of the cage. The birds were housed at 22–25 °C. The photoperiod was set the same as outdoors (16:8 h light/dark). The birds were hand-reared with a standard food for passerines (Handmix, Orlux) along with mealworms. When they started to feed independently, they received food mixtures for insectivorous passerines (egg mixtures Oké-bird and Nutribird, Versele-Laga; Uni patee and Insect patee, Orlux). They were tested when they were fully independent at the age of 50–60 days. Individuals were released after experiments in the third week of July, on days without rain or wind, in the same locality they were taken, as specified by our permits. They were first given a veterinary check-up to ensure they were at normal weight and in good condition, their wing and tarsus lengths were measured, and they were individually banded. They were provided with supplementary food (mealworms, sunflower seeds and the commercial mixture for insectivorous birds) in the feeders placed at the locality for several days.

#### Wild-caught Birds

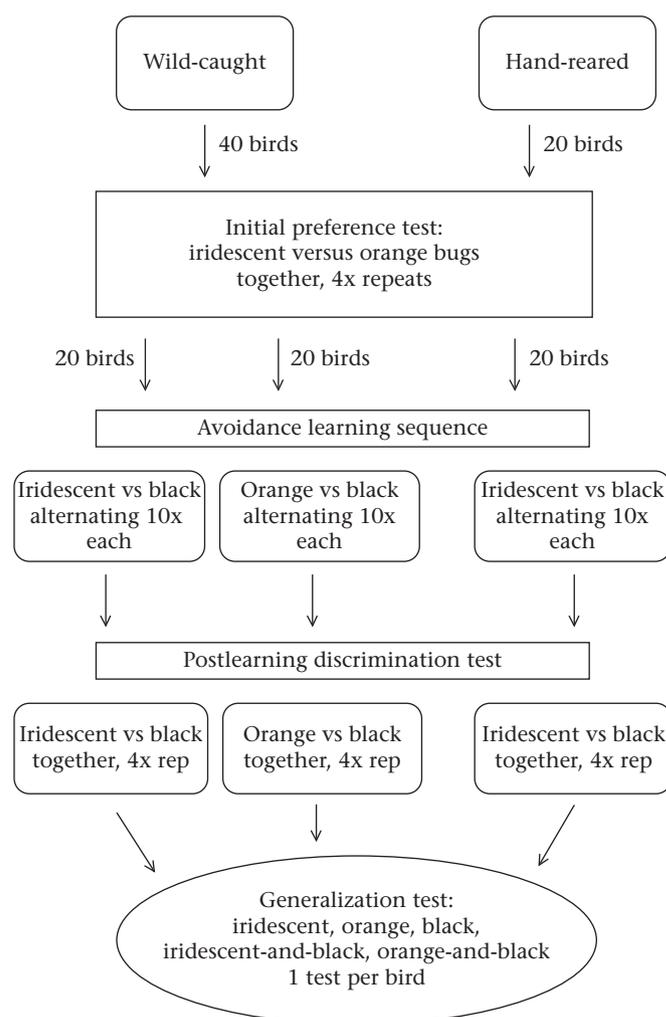
Forty adult great tits (20 males and 20 females; 18 yearlings and 22 older birds) were caught in mist nets in September 2012 in the Botanical Garden of Charles University in Prague. The birds were housed individually in the same cages, at the same temperature and photoperiod, and fed with the same food as hand-reared juveniles. After the experiments, they were released after testing in the third week of September, in the same location they were caught as specified by our permits, on days without rain or wind. As for the hand-reared birds, the wild-caught birds were given a veterinary check-up, measured and banded and provided with supplementary food on release. While no detailed monitoring took place afterwards, regular catching and banding of birds at this location suggests a number of the birds survived and bred in the same locale for multiple years.

Prior to the experiment, the wild-caught birds were allocated to two experimental groups, one assigned for the avoidance learning with iridescent bugs and the other with orange bugs. The composition of both groups was balanced with respect to sex (iridescent group: 10 males/ 10 females; orange group: 11 males/ 9 females) and age (iridescent group: 10 yearlings/ 10 adults; orange group: 10 yearlings/ 10 adults) to maximize validity of between-group comparisons.

#### Tectocoris Baits

Adult *T. diophthalmus* were hand picked off *Lagunaria patersonia* trees in a suburban population in Narrabeen, New South Wales, Australia. Bugs were killed by freezing, and then dried completely in a fume cupboard. Their ventral abdominal cuticle, remnants of

internal organs, antennae and legs were then removed, creating a hollow dome consisting of the insect's head, thorax, wings and body length scutellum (abdominal shield). The bug retained its natural shape and colour patterns when viewed from above (see Johansen et al., 2010 for details). Inside the hollow body cavity we placed half a mealworm, *Tenebrio molitor* (henceforth referred to as 'mealworm'), serving as an unconditioned stimulus. The mealworms were attached to the ventral side of bugs and were not visible until the birds seized or turned over the baits.



**Figure 2.** Flowchart of the experimental design. Following the arrows down shows the series of experiments that each training group underwent, as well as their sample size and the number of repetitions of each experiment. Pretraining regimes, identical for all groups, are not included in this flowchart.

We used five types of bait (Fig. 1) differing in their coloration: (1) iridescent bugs with approximately 50% of their dorsal surface covered by iridescent blue-green patches and the rest remaining orange; (2) naturally orange bugs with only orange coloration; (3) black bugs, individuals painted all black with nontoxic Tim & Tess poster colour (shade 105 – carbon black); (4) iridescent-and-black bugs with their orange parts painted black; and (5) orange-and-black bugs with their iridescent parts painted black. Both iridescent-and-black and orange-and-black bugs had approximately half of their dorsal surface painted black and the other half orange or iridescent, respectively.

#### Experimental Set-up

Experiments were carried out in wooden cages (70 × 70 × 70 cm) with wire-mesh walls and the front wall made of one-way glass. Each cage was equipped with a wooden perch, water bowl and rotating circular feeding tray with six cups. The perch was 20 cm from the feeding tray. The cups had a white lining on which all types of baits appeared highly conspicuous. The cage was illuminated by daylight-simulating bulbs (Biolum Combi 18 W, Osram). A continuous record of the bird's behaviour in the experiment was made using the Observer XT 8.0, and the behaviour was also video-recorded. Experiments were spread across 3 days and were divided into several successive steps (Fig. 2). Before the experiment, the birds were caught by hand in their home cages, put into a textile bag and transported to the experimental cages; they were transported to their home cages in the same manner after the experiments. The birds were allowed to acclimate to the experimental cage and to search for food in one of the cups of the feeding tray before the experiment. Each day before starting the respective part of the experiment, the birds were deprived of food for 2 h. They showed no behavioural markers of stress during or after the experiment.

#### Pretraining (Days 1 and 2)

To minimize the number of bugs destroyed during the experiment we used black *Tectocoris*-shaped silhouettes cut from cardboard with a mealworm glued to their lower side by nontoxic glue (Kores glue stick) to train the birds to handle the baits and extract the edible piece of mealworm from the inedible shell. The birds had to pass three successive stages of the pretraining: (1) handling the upside-down bait with the mealworm on the top; (2) handling the bait with the mealworm beneath the paper silhouette, but with part sticking out; (3) handling the bait with the mealworm hidden completely beneath the paper silhouette. The birds had to handle successfully two baits with a hidden mealworm in a row to participate in the experiment. At the beginning of the second-day session, the pretraining was repeated with black-painted *Tectocoris* baits to check whether the birds remembered their experience and generalized it to the real *Tectocoris* baits. Identical pretraining was performed for both hand-reared and wild-caught birds.

#### Initial Preference Test (Day 2)

To determine whether iridescent coloration increases initial avoidance, we tested whether the birds had any preference for either of the two natural forms of *Tectocoris* bugs used in the experiment. The birds were offered two baits, one iridescent and one orange, both with a palatable piece of mealworm hidden inside, together in the same dish. This process was repeated a total of four times. We recorded the order in which baits were chosen, and measured the latency to attack each bait. The latency to attack the second bait was adjusted to account for the handling and feeding time of the first bait. After the bird finished handling the first bait, it

had 3 min (180 s) to handle the other bait; otherwise the trial was stopped and a nonattack was recorded. The initial preference testing procedure was identical for both hand-reared and wild-caught birds.

#### Avoidance Learning Sequence (Day 3)

The next phase involved training birds against a specific colour morph of bug, to determine the efficiency of avoidance learning. Wild-caught birds were divided into two experimental groups of 20 birds: one group were trained to avoid iridescent bugs, the other to avoid orange bugs. All 20 hand-reared birds were trained to avoid iridescent bugs. Aposematic baits were made unpalatable by soaking the mealworm in 6% quinine (chloroquine phosphate) solution, while the mealworms for palatable baits were soaked in water. Baits were presented in alternating sequence; in each trial only one bait (either black/palatable or coloured/unpalatable) was offered. Although this sequential discrimination task is likely to be more difficult than simultaneous presentation (Beatty & Franks, 2012), we argue that it reflects better a natural situation. Sequences always started with black/palatable, and baits were reused in a sequence unless the birds inflicted damage on them. A trial was terminated after 3 min (180 s) if no attack had occurred, and a nonattack was recorded. Birds were exposed to 10 trials of presentation of each alternating bait type, for a total of 20 trials.

#### Postlearning Discrimination Test (Day 3)

To assess whether there was a difference in how well the birds learned discrimination between palatable and unpalatable baits, we offered birds a simultaneous presentation of the two bait types used in the preceding learning sequences. Those birds trained with iridescent baits were now offered black and iridescent baits side by side in one dish, and likewise with birds trained on orange baits. This test was repeated a total of four times, and unpalatability of the aposematic baits was maintained during tests. We recorded the order of baits attacked as a binary choice (yes/no palatable first).

#### Generalization Test (Day 3)

Finally, trained birds were given a generalization test to determine whether training stimulus colour or previous experience in the wild influenced patterns of generalization. In a single trial, birds were offered five baits, each of a different 'phenotype': iridescent (I), orange (O), black (B), iridescent-and-black (I/B) and orange-and-black (O/B) (Fig. 1). The test was carried out under extinction conditions (all baits palatable) to prevent further learning from interfering with the birds' previous experience. The baits were presented in a rectangular dish (17 × 8 cm), aligned in a row parallel to the enclosure's perch. The order of baits in the row was randomly generated for each bird. We recorded the order of attack and the latency to attack for each bird. The latency was adjusted for the handling time of the preceding baits. There was no time limit; the trial was stopped after the bird had attacked all five baits, although no trial lasted longer than 1000 s. The generalization test procedure was identical for all birds.

#### Statistical Analyses

Differences in the total number of each bait type attacked by each bird in the initial preference test were compared using a Wilcoxon signed-ranks test. The repeated binary choices of the initial preference tests and postlearning discrimination tests, and attack decisions of the initial preference tests and learning sequences, were analysed using generalized linear mixed models,

GLMMs (logistic regression with random intercepts for individual birds). Attack latencies in the initial avoidance tests and the learning sequences were analysed using Cox regression frailty models with random intercept and slopes for birds, a form of survival analysis. The models were built and tested in R3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria, <http://www.r-project.org>), with packages ‘lme4’ (for GLMM) and ‘coxme’ (for survival analysis). Models were fitted by maximum likelihood, with Laplace approximations in GLMMs. Coefficients are given as estimates plus 95% confidence interval, and their significance is tested using Wald’s  $Z$  test, as recommended in Bolker et al. (2009). However, log-likelihood tests result in qualitatively identical results. The generalization tests were analysed using Friedman’s tests with post hoc analysis as implemented in SPSS v20 (SPSS Inc., Chicago, IL, U.S.A.). The training groups were analysed separately. The post hoc test  $P$  values were adjusted using the false discovery rate (Benjamini & Hochberg, 1995), a stepwise procedure wherein  $P$  values are ordered lowest to highest ( $P_{(1)} \dots P_{(i)} \dots P_{(m)}$ ), and all  $P$  values that satisfy the inequality  $P_i \leq (i/m)\alpha$  have their null hypotheses rejected. As this correction was performed on a per experiment basis, the post hoc correction was  $P_i \leq (i/10)0.05$ .

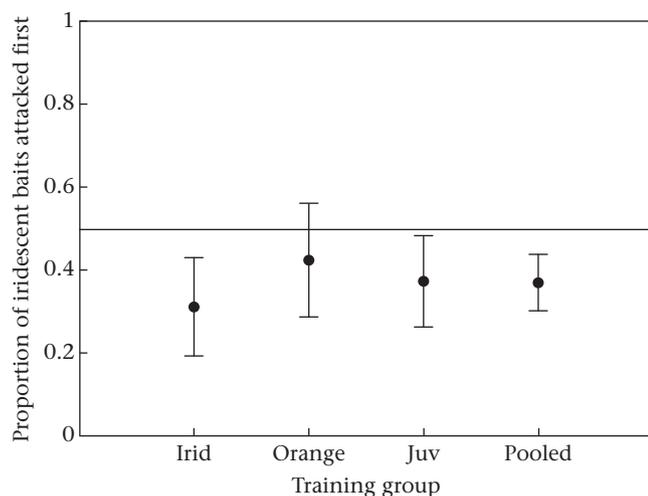
### Ethical Note

We obtained Czech government permission for catching the adult birds (MHMP-154521/04/OOP-V-25/R-40/09/Pra), for taking juvenile great tits from nestboxes placed in parks at the outskirts of Prague (S-MHMP-043585/2009/OOP-V-26/R-8/Pra), and for laboratory experimentation with birds (No. 150/99 and 29532/2006-30). The collection of *T. diopthalmus* from council land in Narrabeen, New South Wales is permitted under Australian law, and transfer of dried *Tectocoris* between two CITES-listed institutions for research purposes is also permitted under CITES rules.

## RESULTS

### Initial Preference Test

In simultaneous presentations, birds showed a preference to attack the orange baits before those with iridescent patches (Fig. 3). Despite repeated exposure, there was no effect of trial number



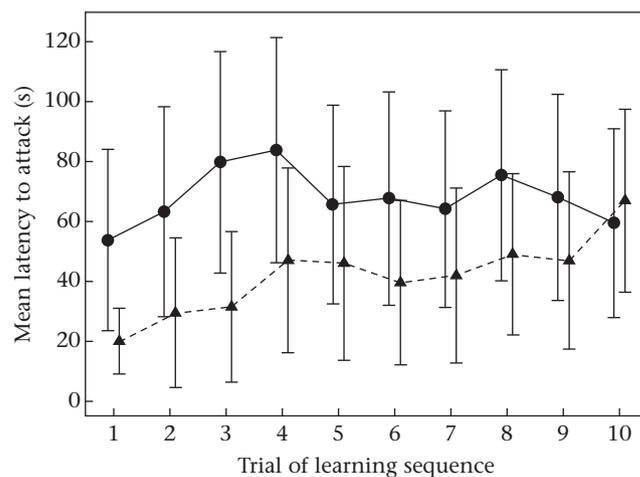
**Figure 3.** Mean proportion (and 95% CI) of times a bird attacked an iridescent bait first for training groups in the preference test. ‘Irid’ is wild-caught iridescent-trained birds, ‘Orange’ is wild-caught orange-trained birds, ‘Juv’ is hand-reared iridescent-trained birds and ‘Pooled’ is all birds combined. Data from each bird were summed across the four trials and normalized to account for repetition.

(Wald test:  $Z = -0.648$ ,  $P = 0.49$ ), and there was no difference in the bias pattern between wild-caught and hand-reared birds (Wald test:  $Z = 0.095$ ,  $P = 0.92$ ). Within wild-caught birds, there was no statistically significant difference between the initial preferences for the iridescent and orange training groups (Wald test:  $Z = 1.365$ ,  $P = 0.17$ ). However, the 95% confidence interval for the orange training group includes parity (50/50 odds), so this group did not share the bias of the other training groups despite not being statistically different from them (Fig. 3). When we pooled all groups, the odds of choosing an orange bait was 1.75 (95% CI 1.31–2.32; Wald test:  $Z = 3.791$ ,  $P = 0.0002$ ), or an average probability of 64%.

The hand-reared birds did not show a significant difference in latency in attacking orange versus iridescent baits (Wald test:  $Z = -1.34$ ,  $P = 0.18$ ). Hand-reared birds also attacked virtually all baits offered, with only two nonattacks recorded, in different birds and against different bait types. Wild-caught birds showed a bias in attack, as their preference for orange baits was accompanied by a small but statistically significant reluctance to attack iridescent baits during the observation period (mean baits attacked  $\pm$  SD: iridescent =  $3.38 \pm 1.03$ , orange =  $3.78 \pm 0.53$ ; Wilcoxon signed-ranks test:  $V = 19$ ,  $P = 0.034$ ). Overall, 33 of 40 birds sampled three or more iridescent baits, whereas 38 of 40 birds sampled three or more orange baits. This attack bias was also accompanied by a significantly greater latency to attack iridescent baits (hazard ratio = 0.65, 95% CI 0.51–0.83; Wald test:  $Z = -3.43$ ,  $P = 0.0006$ ). Neither attack rate (Wald test:  $Z = 0.684$ ,  $P = 0.494$ ) nor latency (Wald test:  $Z = 1.56$ ,  $P = 0.12$ ) changed between trials.

### Avoidance Learning Sequence

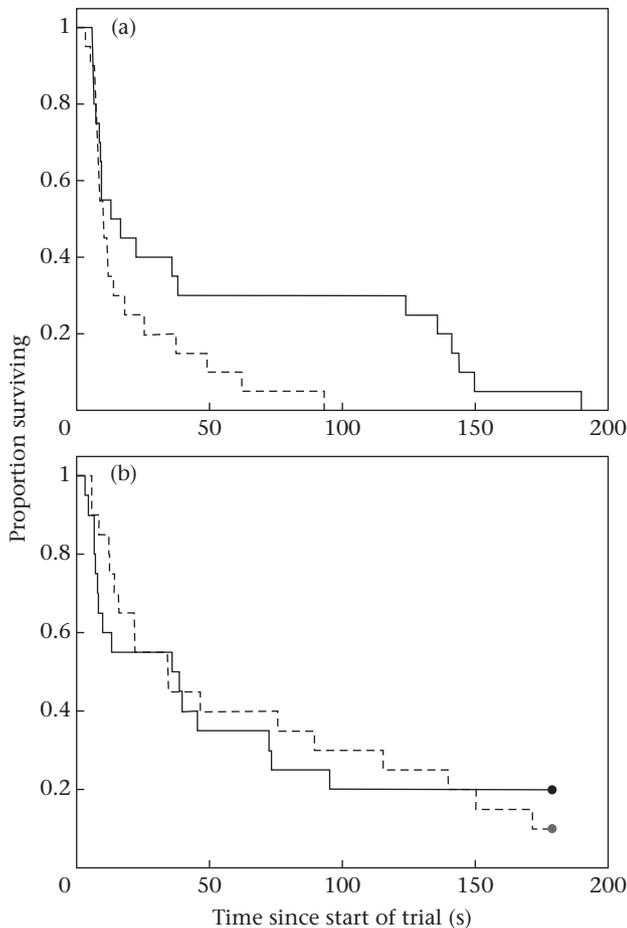
To test the efficiency of avoidance learning, latency to attack was measured over a series of alternating presentations of palatable black and unpalatable coloured baits. There was no effect of bait type on latency for wild-caught birds (Wald test:  $Z = 1.35$ ,  $P = 0.18$ ). With bait types pooled, there was an increase in attack latencies over the course of the learning sequence (hazard ratio = 0.928, 95% CI 0.872–0.989; Wald test:  $Z = -2.34$ ,  $P = 0.019$ ), translating into a 7.7% reduced instantaneous risk of attack in each subsequent trial (Fig. 4). When analysed within groups, a significant increase in attack latencies over the course of the training sequence was observed in the orange bait training group (hazard ratio = 0.916, 95% CI 0.286–0.967; Wald test:  $Z = -3.17$ ,  $P = 0.002$ ), but not the



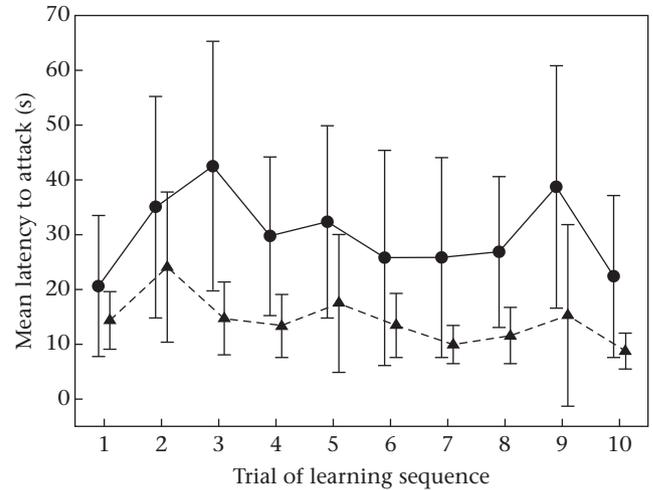
**Figure 4.** Mean attack latency (and 95% CI) for unpalatable coloured baits at each trial of the learning sequence of wild-caught birds. Each point is the group mean for that trial of the learning sequence. Circles connected by solid lines are iridescent baits; triangles connected by dashed lines are orange baits.

iridescent bait training group (Wald test:  $Z = -1.31$ ,  $P = 0.19$ ) or hand-reared birds trained to iridescent baits (Wald test:  $Z = 1.67$ ,  $P = 0.094$ ; Fig. 5). Overall, wild-caught birds trained to iridescent baits had significantly higher latencies than their hand-reared counterparts, with a 3.2 times lower instantaneous risk of attack across the sequence (hazard ratio = 0.301, 95% CI 0.120–0.804; Wald test:  $Z = -2.41$ ,  $P = 0.016$ ). Prior to first exposure to quinine, the wild-caught birds displayed a nonsignificant trend towards greater latency to attack iridescent bugs (hazard ratio = 0.518, 95% CI 0.259–1.039; Wald test:  $Z = -1.85$ ,  $P = 0.06$ ). This trend disappeared by the end of the learning sequence as the hazard ratio approached unity (Wald test:  $Z = 0.066$ ,  $P = 0.95$ ; Fig. 5).

Wild-caught birds also showed differences between training colours in how they handled the palatable control black baits (Fig. 6). Both groups showed a sharp increase in latency for the first one or two trials after initial exposure to baits containing quinine-laced mealworm pieces. Despite this, there was an overall small but significant reduction in latency to attack black baits over the learning sequence (hazard ratio = 1.099, 95% CI 1.034–1.167, Wald test:  $Z = 3.03$ ,  $P = 0.002$ ). Over the course of the trials there was a comparatively greater latency to attack black bugs for wild-caught birds trained to iridescent bugs, compared to wild-caught birds trained to orange bugs (hazard ratio = 0.421, 95% CI 0.205–0.862; Wald test:  $Z = -2.37$ ,  $P = 0.018$ ). However, this difference was not present on first presentation of black baits, before any exposure to



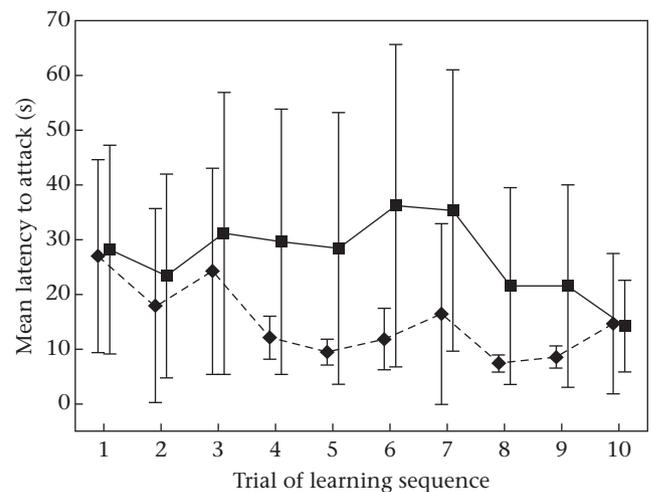
**Figure 5.** Survival curves (proportion surviving) for unpalatable baits as attacked by wild-caught birds. (a) The first trial of the learning sequence (before exposure to quinine). (b) The last trial of the learning sequence. Solid lines are iridescent baits; dashed lines are orange baits. Trials were terminated after 180 s, so any baits not attacked are considered having 'survived'.



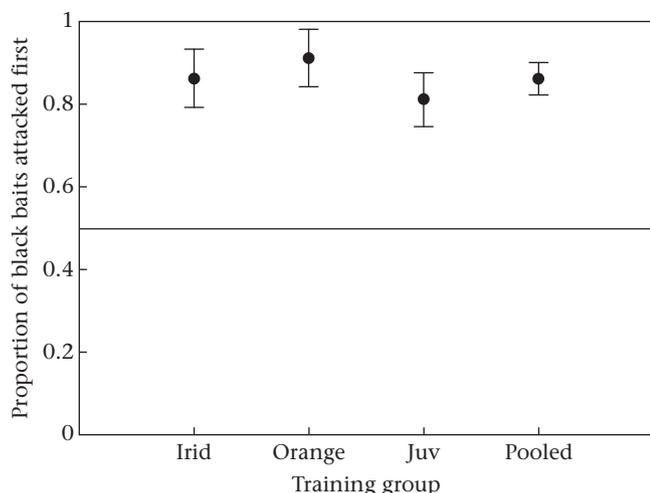
**Figure 6.** Mean attack latency (and 95% CI) for palatable black bugs at each trial in the wild-caught birds' learning sequences. Circles connected by solid lines are the black bait attack latencies of birds in the iridescent training group. Triangles connected by dashed lines are the black bait attack latencies of birds in the orange training group.

quinine (Wald test:  $Z = -0.728$ ,  $P = 0.47$ ). Despite not showing a significant increase in latency to attack iridescent bugs over the course of the trials, hand-reared birds showed significantly higher latency to attack iridescent bugs compared to black bugs over the entire sequence (hazard ratio = 0.621, 95% CI 0.501–0.768; Wald test:  $Z = -4.37$ ,  $P < 0.001$ ; Fig. 7).

Despite a longer latency towards attacking unpalatable baits compared to black baits, few birds learned to abstain from attacking the unpalatable baits. In the wild-caught birds, four of 20 birds trained to iridescent baits abstained from attacking on at least half of the presentations, whereas only two of 20 birds trained to orange and only one of 20 hand-reared birds did. Within wild-caught birds, there was no difference between patterns of nonattack between iridescent- and orange-trained birds (Wald test:  $Z = 1.315$ ,  $P = 0.19$ ), nor any significant change over the course of the learning sequence (Wald test:  $Z = -1.333$ ,  $P = 0.18$ ). There was a nonsignificant trend towards wild-caught birds trained against iridescent abstaining from attack more often than their hand-reared



**Figure 7.** Mean attack latency (and 95% CI) for hand-reared birds at each trial in the learning sequence. Squares connected by solid lines are mean attack latencies at each trial for the unpalatable baits. Diamonds connected by dashed lines are the mean attack latencies at each trial for the palatable black baits.



**Figure 8.** Mean proportion (and 95% CI) of times a bird attacked a black (palatable) bait first for training groups in the postlearning discrimination tests. 'Irid' is wild-caught iridescent-trained birds, 'Orange' is wild-caught orange-trained birds, 'Juv' is hand-reared iridescent-trained birds and 'Pooled' is all birds combined. Each bird was summed across the four trials and normalized to account for repetition.

counterparts (odds ratio = 8.78, 95% CI 0.84–81.26; Wald test:  $Z = -1.87$ ,  $P = 0.07$ ). All black baits were attacked by all birds on all presentations.

#### Postlearning Discrimination Test

For all training groups, there was a strong preference for the palatable black baits over their respective unpalatable coloured baits in side-by-side preference tests, suggesting clear discrimination learning for all training groups (Fig. 8). Over a series of four trials, there were no effects of trial order (Wald test:  $Z = -1.251$ ,  $P = 0.21$ ), or between wild-caught and hand-reared birds (Wald test:  $Z = -1.581$ ,  $P = 0.11$ ). Within the wild-caught birds, there was no difference between being trained against orange or iridescent baits (Wald test:  $Z = 0.993$ ,  $P = 0.32$ ). The pooled odds ratio of attacking a black bait first is 6.27 (95% CI 4.35–9.05; Wald test:  $Z = 9.796$ ,  $P < 0.0001$ ). Therefore the mean probability of attacking a palatable black bait over the trained unpalatable stimulus is 86%, regardless of the prior experience of birds or bug colour morph.

#### Generalization Test

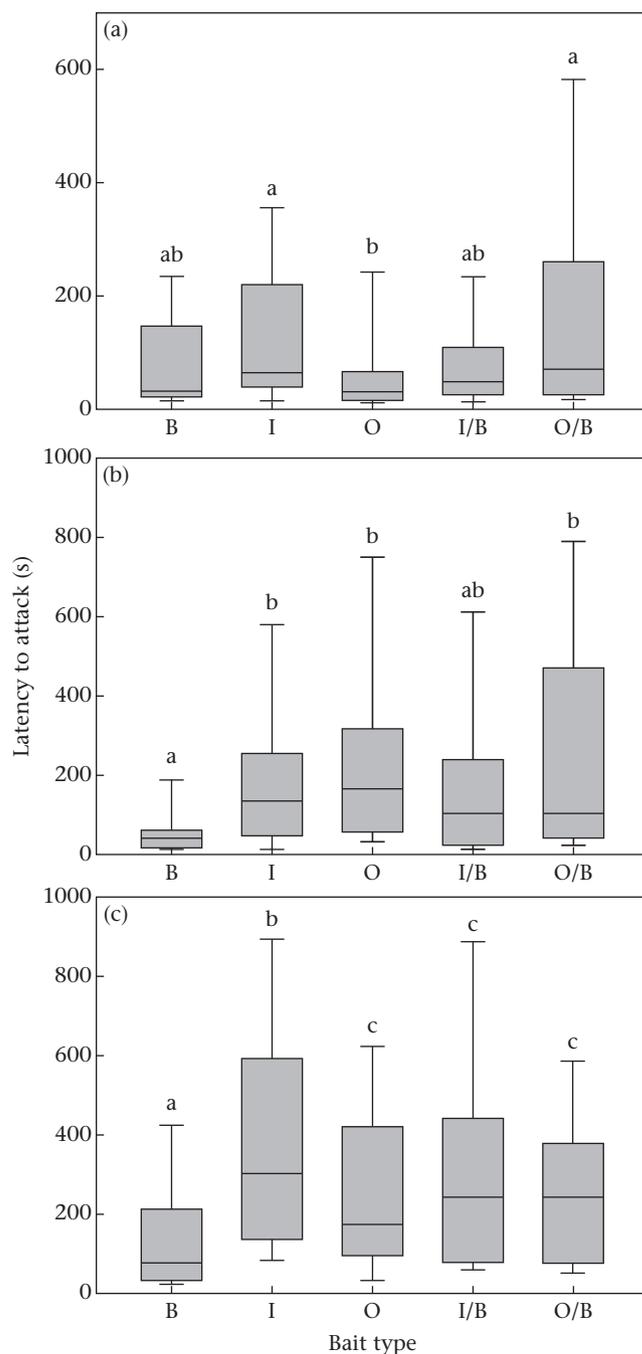
All three training groups of birds had distinctly different results in the generalization tests, suggesting effects of training colour and between wild-caught and hand-reared birds (Table 1, Fig. 9). Within-group Friedman tests comparing the five stimuli (three familiar, two novel) were all significant (wild-caught orange-trained:  $Q_4 = 13.56$ ,  $P = 0.009$ ; wild-caught iridescent-trained:  $Q_4 = 11.48$ ,  $P = 0.02$ ; hand-reared iridescent-trained:  $Q_4 = 41.28$ ,

**Table 1**  
Mean rank of each bait type in the generalization tests

Group	Black	Irdescent	Orange	Irdescent/Black	Orange/Black
Orange	1.90	3.15	3.55	3.00	3.40
Irdescent	2.58	3.50	2.22	3.08	3.62
Juvenile (Irdescent)	1.50	4.65	2.95	3.25	2.65

Scores denote the average rank (out of 5) that each bait type was chosen, so a bait type with a lower mean rank is chosen on average before one with a higher rank. Mean rank values are the result of a within-group Friedman test, so each mean rank is relative to the other values in its respective row.

$P < 0.001$ ). For wild-caught birds trained to orange baits, there were significant pairwise differences between black baits and orange baits, but also between black baits and iridescent baits, and between black baits and orange-and-black baits. For wild-caught birds trained to iridescent, there were significant pairwise differences between orange baits and iridescent baits, and between orange baits and orange-and-black baits. Neither wild-caught



**Figure 9.** Latency (s) for birds to attack each bait, with handling times of previous baits subtracted, in the generalization tests. (a) Wild-caught birds trained against iridescent baits, (b) wild-caught birds trained against orange baits, and (c) hand-reared birds trained against iridescent baits. Bait types are black (B), iridescent (I), orange (O), iridescent-and-black (I/B) and orange-and-black (O/B). Box plots include median, interquartile range and range. Within each plot, significantly different groups (as judged by a Friedman test post hoc analysis with  $P$  values adjusted for the false discovery rate) are marked with different letters. Boxes marked 'ab' are therefore not significantly different from 'a' or 'b'.

training group showed a significant difference between iridescent baits and orange-and-black baits with the shared pattern.

Most pairwise differences in hand-reared birds were significant (Fig. 9). Of particular note is that orange and orange-and-black baits were not significantly different. Additionally, iridescent-and-black baits were significantly higher ranked than black baits, but significantly lower ranked than iridescent baits; these distinctions are not made by either training group in the wild-caught birds (Table 1).

## DISCUSSION

These results permitted an evaluation of the question of whether iridescent coloration is effective in inducing initial avoidance, efficient avoidance learning and discrimination, and broad generalization. Iridescent patches (when paired with quinine defences) were effective in inducing greater initial avoidance and broad generalization. For wild-caught birds, attack latencies and rates of nonattack were not statistically distinguishable between birds trained to avoid iridescent or orange baits. All training groups showed equivalent strong preference for baits associated with palatability after their respective training sequences. Hand-reared and wild-caught birds trained against iridescent baits showed group differences in generalization behaviour that were probably related to previous experience, but iridescence was an effective discrimination cue for both groups.

### Initial Preference Tests

There was a significant initial bias against the iridescent baits, and this preference was the same for wild-caught and hand-reared birds. In hand-reared birds this bias was likely to be innate, since they had no previous experience with any aposematic prey. This result is surprising, since in previous studies naïve hand-reared great tits did not show any innate avoidance of aposematic prey (Exnerová et al., 2007; Hotová Svádová, Exnerová, Kopečková, & Štys, 2013; Svádová et al., 2009; but see Lindström, Alatalo, & Mappes, 1999). It is possible that the innate bias in great tits is more a question of preference than a strong avoidance, and that it can be observed only when the birds are given a choice between the alternative prey types (Lindström et al., 1999) and not when they encounter only a single prey item at a time (Exnerová et al., 2007; Hotová Svádová et al., 2013; Svádová et al., 2009).

The question arises: what specific stimulus is responsible for the preference for orange baits over the iridescent ones? Several studies (Hauglund, Hagen, & Lampe, 2006; Mastrota & Mench, 1995; Schuler & Hesse, 1985) have demonstrated that blue and green are chosen by birds preferentially over yellow and red, so it is unlikely that in this experiment, the great tits were being driven off by blue or green coloration per se. It is possible that bias against iridescent bugs was caused by higher luminance contrast of the iridescence (Sandre, Stevens, & Mappes, 2010), or by the presence of a contrasting pattern itself (Hauglund et al., 2006; Roper & Cook, 1989), both enhancing the prey's conspicuousness, although for hand-reared naïve birds, the bias is unlikely to be caused by aversion to patterning per se (Aronsson & Gamberale-Stille, 2008, 2009; Osorio, Jones, & Vorobyev, 1999). Conversely, the birds may have had an innate preference for the orange stimulus and preferred the prey with more of an orange colour, but preference for red over green has only been found when the birds recognize the food as berries and not insects (Schmidt & Schaefer, 2004). In other studies, birds did not show any preference for red berries over green ones, but always preferred green insects to red ones (Gamberale-Stille, Hall, & Tullberg, 2007; Gamberale-Stille & Tullberg, 2001). Future research should perform preference tests using the range of baits

used in the generalization tests on naïve hand-reared birds, to verify that iridescence per se is the aversive trait.

The wild-caught birds appeared more cautious, with greater latencies to attack overall in the preference tests. They also showed a significant difference in attack latencies between orange and iridescent bugs, something not seen with the hand-reared juveniles. Since both colour morphs were novel to the great tits, this could be due to increased neophobia or dietary conservatism (Marples et al., 1998; Marples & Kelly, 1999) of older birds. However, in other experiments, adult wild-caught great tits frequently attacked novel colour morphs of prey (Exnerová et al., 2006), and adult individuals were even less cautious than yearlings (Lindström et al., 1999). It is therefore more likely that wild-caught birds partly generalized their previous experience with local red-and-black aposematic true bugs (Exnerová et al., 2006; Hotová Svádová et al., 2010) to the *Tectocoris* baits, especially as the great tits generalize easily from red to orange prey (Exnerová et al., 2006; Ham et al., 2006).

### Avoidance Learning Sequence

The results suggest that over a learning sequence of this duration, iridescent and orange baits were equally effective for wild-caught birds. There was no statistically significant difference between the two groups, although this may be an artefact of the high variance in latency. When analysed separately, birds trained on iridescent baits did not show an increase in latency over the learning sequence, whereas those trained to orange baits did show an increase. This, however, is likely to be due to the demonstrated initial aversion towards iridescence (see above), and may potentially be influenced by birds choosing to sample fewer iridescent baits during the preference tests. It is worth noting that while there was a nonsignificant trend for higher latency against iridescent baits at the beginning of the learning sequence, this difference evaporated by the last trial as latency against orange baits increased. It is unclear whether latency against orange would have continued to increase to greater levels or whether the two groups would have remained equally aversive over a long period of exposure. It is also unclear whether more birds would have abstained from attack in a longer learning sequence or with stronger chemical defences, although the natural chemical defences of *T. diophthalmus* are rather weak (Staddon et al., 1987).

Hand-reared juveniles, like their wild-caught counterparts, did not show an increase in attack latency or change in attack rate over the course of the learning sequence. There was, however, a large difference in latency between hand-reared and wild-caught birds in how they treated iridescent bugs. Therefore, previous experience with more noxious prey may be essential to increase attack latency. Odorous secretions, which live *T. diophthalmus* produces but quinine baits do not, may also be important in promoting avoidance (Jetz, Rowe, & Guilford, 2001; Rowe & Guilford, 1996; Siddall & Marples, 2008). Conversely, juvenile birds may simply be more hungry and/or more tolerant of weak defences, which may influence their eagerness to attack (Alcock, 1973; Barnett, Bateson, & Rowe, 2007; Sandre et al., 2010). Despite a lack of observed increase in attack latency, hand-reared birds had significantly higher attack latencies overall against iridescent unpalatable baits than palatable black baits, suggesting discrimination was occurring.

One surprising result from the learning trials was the increased latency for attacking the black control baits in the iridescent-trained group, compared to the orange-trained group. Perhaps from an oblique viewing angle the iridescent patches appeared dark to the birds. It has been suggested that luminance contrast is not as relevant as colour in bird learning (Osorio et al., 1999), which may explain this unusual finding. Sandre et al. (2010) suggested

that luminance contrast influences initial wariness, but this may not extend to a prolonged learning sequence. Studies of the aposematic effect of luminance contrast alone are rare in bird research, although it is discussed by Stevens (2007) and luminance contrast is effective in teaching mantids to avoid emetic seed bugs (Prudic, Skemp, & Papaj, 2007). The importance of chromatic versus achromatic contrast in aposematism is a ripe area for further research.

#### Postlearning Discrimination Test

Both colour morphs were shown to be equally effective cues in a postlearning discrimination test with simultaneous presentation of palatable alternative prey. Age and experience of birds did not affect this outcome, as wild-caught adults and hand-reared juveniles were equally keen to pick the black control baits over their respective unpalatable baits. The presence of alternative prey may therefore be very important to the harlequin bug's survival, especially if this weakly defended stinkbug (Staddon et al., 1987) is capitalizing on imperfect Müllerian or quasi-Batesian mimicry to other sympatric noniridescent noxious bugs (Kokko, Mappes, & Lindström, 2003; Lindström, Alatalo, Lyytinen, & Mappes, 2004). The different performances of birds at the end of the learning sequence and in the postlearning discrimination test also support the idea that the sequential discrimination task may be more difficult than the simultaneous one (Beatty & Franks, 2012), or at least that sequential discrimination learning can in some cases be almost behaviourally silent and become evident only when the two stimuli are presented simultaneously.

#### Generalization Test

The generalization trials exposed the major differences between the wild-caught birds and the hand-reared birds, distinguished patterns of generalization between iridescent- and orange-trained wild-caught birds, and allowed predictions of how aposematic signals utilizing iridescence may fare in the wild. Regardless of training colour morph, the orange-and-black baits were highly aversive to wild-caught birds. Furthermore, the iridescent baits remained repellent to the birds trained to avoid orange baits, despite these birds only experiencing the iridescent colour morph as a palatable food item. The reverse was not true for birds trained to avoid iridescent baits: orange baits remained the most preferred food choice, even more so than the black baits. Despite its pattern being fairly distinct from local red and black Heteroptera (Exnerová et al., 2008), the harlequin bugs appear to be benefiting from experience with the local Müllerian/quasi-Batesian mimetic complex (Hotová Svádová et al., 2010; Hotová Svádová et al., 2013; but see Veselý, Veselá, & Fuchs, 2013). Although bird species do vary in how they respond to aposematic prey (Exnerová et al., 2003; Exnerová et al., 2007), these findings are likely to be relevant to the harlequin bug's survival against avian predators in its home range.

For the wild-caught birds, the presence of a contrasting pattern was necessary for generalizing unpalatability, as birds trained to iridescent baits did not show elevated avoidance of orange baits despite iridescent colour morphs containing small orange patches. This is surprising since several studies have doubted the importance of contrasting patterns for learning to recognize aposematic prey (Aronsson & Gamberale-Stille, 2008, 2009; Exnerová et al., 2006; Svádová et al., 2009; but see Aronsson & Gamberale-Stille, 2013). However, the attention that predators give to the pattern may depend on the amount of their previous experience, which agrees with findings that experienced predators usually select for a closer mimetic resemblance than naïve ones (Ihalainen, Lindström,

Mappes, & Puolakkainen, 2008; Lindström, Lyytinen, Mappes, & Ojala, 2006; Rowe, Lindström, & Lyytinen, 2004). Iridescent-and-black baits, which test iridescence as a signal without the contribution of contrasting orange, were not a strong signal to wild-caught birds. To birds trained on orange baits, they were much less aversive than the other stimuli (except black), possibly because of the colour or luminance contrast. For birds trained to iridescent baits, black and iridescent-and-black baits were statistically even, supporting the notion that the iridescent patches are harder to distinguish from black than orange is. Behaviour of wild-caught birds thus seemed to be affected by their experience with red-and-black aposematic bugs in the wild, since they attended mostly to orange colour and contrasting dark pattern (either black or iridescent) when learning to avoid unpalatable baits. This pattern of generalization may be overridden with extensive training, as Pegram et al. (2013) found no difference between how birds responded to selective blacking-out of blue or orange components of the warning signal of *Battus philenor* (Papilionidae) butterflies.

The hand-reared, formerly naïve birds, on the other hand, performed exactly as would be expected from being conditioned that iridescent-and-orange bugs are unpalatable. Their most aversive stimulus was the one to which they were trained, while the black baits were most preferred. Both novel phenotypes (iridescent-and-black and orange-and-black) were less aversive than the trained unpalatable bait, because they were imperfect matches but also possibly because they shared part of their pattern (black colour) with the positive stimulus (the palatable bait). The iridescent-and-black baits were the second most aversive stimuli, suggesting that naïve birds can associate iridescent coloration with unpalatability, and that iridescence alone, without taking advantage of a mimetic complex, can function as an aposematic signal. This result is unlikely to be due to neophobia since the other novel stimulus, the orange-and-black baits, was the second-least aversive stimuli, supporting the idea that aposematic patterns must be learned in great tits and are not innately aversive (Exnerová et al., 2007; Hotová Svádová et al., 2013; Sillén-Tullberg, 1985). The surprise here was that the orange baits, which had previously only been experienced as palatable, were significantly more aversive than black ones. This could be evidence for biased generalization or peak shift (Gamberale & Tullberg, 1996; Gamberale-Stille & Tullberg, 1999; ten Cate & Rowe, 2007), as the training stimulus had both iridescent and orange patches, so very large (in this case, whole body) orange patches could also increase avoidance (Rommel & Tammaru, 2011). It is also worth noting the greater latency against iridescent baits, even after correcting for the handling time of other baits, further supporting the possible role of alternative prey in promoting survival in *T. diophthalmus*.

#### Conclusion

It appears as though the iridescent patches of *T. diophthalmus* can act as an aposematic signal. This may be through either the innate avoidance of high luminance contrast (Sandre et al., 2010) or avoidance learning. The presence of iridescent patches creating a contrasting pattern was important for initial avoidance and generalization in wild-caught birds. However, this benefit appears to be greatly increased by the presence of alternative palatable prey. The large differences we observed between wild-caught and hand-reared birds may reflect behavioural differences by age (Lindström et al., 1999), or less cautiousness and discrimination of hand-reared birds (Exnerová et al., 2007), but above all else they appear to reflect experience. The wild-caught birds probably had experience with local red-and-black Heteroptera (Hotová Svádová et al., 2010), and their performance supports the idea of broad

generalization in colour and pattern (Aronsson & Gamberale-Stille, 2009, 2013; Exnerová et al., 2006; Ham et al., 2006). Iridescent bugs may thus benefit from experience of bird predators with other similar red or orange prey with contrasting but noniridescent patterns.

Our results suggest that orange is not superior to iridescence (with small orange patches) for learning to avoid aposematic prey. This result is surprising, given the noted prevalence of long-wavelength colours in aposematic signalling (Ruxton et al., 2004). This result also supports previous findings that internal contrast is not more important than colour in avoidance learning (Aronsson & Gamberale-Stille, 2009; Roper & Cook, 1989), although internal contrast may be beneficial in unlearned avoidance (Sandre et al., 2010). Despite within-group differences in the learning sequence, both morphs were equally efficient discrimination cues compared with palatable alternative prey. Together, these observations support the idea that the iridescent patches may be selectively advantageous despite not being maximally aposematic. Furthermore, the equivalent survival of the extreme morphs suggests that, at least in a one-predator system, variation in aposematic signals may be evolutionarily stable.

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