

Article

Do predator energy demands or previous exposure influence protection by aposematic coloration of prey?

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Abstract

Growing evidence exists that aposematic and toxic prey may be included in a predator's diet when the predator experiences physiological stress. The tree sparrow *Passer montanus* is known to have a significant portion of aposematic and toxic ladybirds in its natural diet. Here, we present experiments testing the attack and eating rate of the tree sparrow toward the invasive aposematic harlequin ladybird *Harmonia axyridis*. We wondered whether the sparrow's ability to prey on native ladybirds predisposes them to also prey on harlequin ladybirds. We compared the attack and eating rates of tree sparrows of particular age and/or experience classes to test for any changes during ontogeny (hand-reared × young wild-caught × adult wild-caught) and with differing perceived levels of physiological stress (summer adult × winter adult). Winter adult tree sparrows commonly attacked and ate the offered ladybirds with no evidence of disgust or metabolic difficulties after ingestion. Naïve and wild immature tree sparrows attacked the ladybirds but hesitated to eat them. Adult tree sparrows caught in the summer avoided attacking the ladybirds. These results suggest that tree sparrows are able to cope with chemicals ingested along with the ladybirds. This pre-adaptation enables them to include ladybirds in their diet; though they commonly do this only in times of shortage in insect availability (winter). Young sparrows showed avoidance toward the chemical protection of the ladybirds.

Key words: chemical protection, toxic prey predation, visual signal, warning signal.

The general theory of aposematism considers that naïve predators need to learn to associate an unpleasant experience with the conspicuous signal of the aposematic prey (Cott 1940; Guilford 1988). At the same time, numerous studies have found that this relationship is also affected by the predator specific characteristics, such as memory (Speed 2000, 2001), experience (Gamberale-Stille and Sillén-Tullberg 1999; Servedio 2000; Veselý and Fuchs 2009), neophobia (Marples and Brakefield 1995; Marples et al. 1998), or dietary conservatism (Marples and Kelly 1999).

There is also evidence showing that bird predators prey on aposematic and toxic prey, because even such chemically defended prey represent a source of nutrients and may be utilized as such. The reasons for choosing this strategy are various. One reason to attack and eat aposematic prey is the relatively low availability of unprotected

prey; though, this situation is rather scarce in nature (Kokko et al. 2003; Lindström et al. 2004). Most often birds include toxic prey in their diet when facing physiological stress, for example, body mass and fat store reduction (Barnett et al. 2007, 2011) or low temperatures in the environment (Chatelain et al. 2013). Predators may also select for toxic prey, when its nutritional value is greater than in unprotected prey (Halpin et al. 2013, 2014; Smith et al. 2014).

There are additional ways that predators can deal with the ingested toxins. Some evidence comes from the tropics (e.g., Chai 1986; Pinheiro 2003), where jacamars *Galbula ruficauda* were shown to prey on *Heliconius* butterflies, which are noxious. The birds usually ate only a limited number of such prey to avoid overdosing with the toxins. A similar strategy was shown in a study with grosbeaks (*Pheucticus melanocephalus*—Brower 1988), which

ceased eating monarch butterflies *Danaus plexippus* when the dose of toxins ingested was too high. Most studies showing the ability to deal with toxins have used quite large-bodied bird species such as the starling (*Sturnus vulgaris*—Wiklund and Järvi 1982; Barnett et al. 2007, 2011, 2014; Chatelain et al. 2013; Halpin et al. 2013, 2014) or blackbird (*Turdus merula*—Exnerová et al. 2003), which probably cope more easily with toxins. Other studies have used birds which are at least partly granivorous or frugivorous, such as the black-headed grosbeak (*Pheucticus melanocephalus*—Brower 1988), chaffinch (*Fringilla coelebs*—Exnerová et al. 2003), green finch (*Chloris chloris*—Exnerová et al. 2003), and yellowhammer (*Emberiza citrinella*—Exnerová et al. 2003). Granivorous and frugivorous birds can be adapted to ingesting some plant toxins and may have developed strategies for dealing with the toxin intake (e.g., eating clay; Brightsmith et al. 2008; Gilardi et al. 1999). The selection pressure of such predators on the aposematic prey may thus remain quite low, because predation events on toxic prey are not very common and kin selection enables much of the genome of individual prey to be passed on even when some individuals are eaten (Guilford 1988).

Ladybirds are generally chemically well-protected insects using alkaloids and pyrazines for their protection (Majerus 1994; Pasteels 2007; Petersson 2012). Previous studies (Marples et al. 1989) have shown that the alkaloids of the 7-spot ladybird *Coccinella septempunctata* may cause significant metabolic difficulties and even death in passerine birds. The harlequin ladybird *H. axyridis* is one of the most toxic ladybirds occurring in Central Europe (Nedvěd et al. 2010). Furthermore, it is an invasive species rather novel to Central Europe. The small-bodied and partially granivorous titmice (Passeriformes, Paridae) have been shown to have high levels of avoidance when presented with the variable visual signal of ladybirds as well as with their various chemical protection (in terms of amount and composition—Marples et al. 1994; Dolenská et al. 2009; Průchová et al. 2014). A strong aversion toward ladybirds may be learned, as naïve great tit nestlings frequently attacked 2 species of ladybirds (*C. septempunctata* and *Scymnus frontalis*; Dolenská et al. 2009).

We aimed to describe the willingness of the tree sparrow *Passer montanus* to include the toxic and warning signalling harlequin ladybird in their diet. The tree sparrow is a small-bodied and partially granivorous bird, having some aposematic ladybirds in its natural diet (Krištín 1984, 1986, 1988; Field et al. 2008). We wondered whether the sparrow's ability to prey on ladybirds predisposes them to also prey on the invasive harlequin ladybird (bigger and possibly more toxic than other central-European ladybirds). In addition, we compared the attack and eating rates of tree sparrows of particular age and/or experience classes to test for any changes during ontogeny (hand-reared × young wild-caught × adult wild-caught) and with differing energy demands for thermoregulation under cold winter conditions (summer adult × winter adult). We tested the separate effect of the visual (preventing the attack) signal (by masking the natural colouration with brown paint) and the chemical signal (preventing eating of the prey) on the ladybird avoidance displayed by tree sparrows. We predicted that: 1) unmodified ladybirds will be attacked less than brown-painted ones (the visual signal is an effective protection); 2) attacked ladybirds will not be eaten (the chemical signal is an effective protection); 3) birds that ate the ladybirds will show discomfort (the chemical protection is effective); 4) naïve sparrows will attack the unmodified ladybirds more than other age and/or experience classes of sparrows (the aversion to ladybirds is not innate); 5) summer adults will attack the unmodified ladybirds less than the winter ones (because the energetic demands associated with

cold winters and a reduction in arthropod prey promote a higher willingness to include toxic prey in the diet). Testing all these predictions should help us to understand how birds cope with ladybird toxicity and how their tolerance varies across bird age classes, and if the tree sparrow can act as a selective force controlling an invasive species of aposematic insect prey.

Materials and Methods

Predator species

The tree sparrow is a small (weighing 18–25 g), partially granivorous passerine occurring throughout the Palaearctic, including the Far East and Japan (Cramp and Perrins 1993). Besides invertebrates, sparrows commonly feed on fruit and grains, especially in the non-breeding season (Krištín 1984; Perkins et al. 2007). During the breeding season (March to August), the diet of adult tree sparrows consists mainly of small invertebrates with, Lepidoptera—Diptera and Coleoptera—larvae being the most abundant (Szlivka 1983; Krištín 1984; Anderson et al. 2002; Orszaghova et al. 2002). In the latter category, carabids and curculionids are the most common; comprising 5%–17% of the overall diet composition (Krištín 1984; Field et al. 2008). Coccinellidae are also found in diet contents; comprising up to 3.5% of the overall adult tree sparrow diet (Krištín 1984; Field et al. 2008). These percentages are much higher than in most insectivorous birds, where ladybirds often do not occur at all (Mizer 1970; Ceryngier et al. 2012). A similar composition was shown to occur in the diet of tree sparrow nestlings (Krištín 1988; Krištín et al. 1995).

In our experiments, we used individuals obtained from the population living on the western border of the town of České Budějovice (South Bohemia, Czech Republic, GPS: North 48.968 to 48.992 and East 14.411 to 14.436). Within this area, nest boxes were provided for breeding and winter feeding places were established. Once collected, birds of all age classes were ringed, measured, and moved into commercially sold, wire mesh birdcages (60 × 45 × 80 cm), where there were 4 perches, water bowl, and 2 feeders. To avoid injury, there was only 1 bird in each cage, but there was more than 1 cage in each room, so that birds had the opportunity to see and hear each other, and so reducing stress from isolation. Birds were fed with mealworms, a mixture of curd cheese, and grated carrots (with minerals and vitamins), and a partially germinated mixture of grains (sunflower, colza, millet, and flax). They had no access to other insect prey. We divided the test subjects into four categories according to the level of actual, recent experience with natural prey.

1) Naïve birds ($N = 20$) were collected from nest boxes at the age of 10–12 days, when the nestlings are developed enough to adapt to laboratory conditions, but still fully dependent on parental care and unfamiliar with their wider natural surroundings, except for what parents had brought them as food. There is evidence that the diet of tree sparrow nestlings includes ladybirds as well (Krištín 1988; Krištín et al. 1995), so nestlings may not have been completely naïve. When collecting the birds, only one sparrow nestling was taken from each nest to avoid sibling bias (tree sparrows have usually 2–4 nestlings in their broods—Cramp and Perrins 1993). After they had learned to forage independently, (attacking a mealworm when offered, aged 20–30 days) they were ready for the experiment. Experiments with this age class were conducted during the summer of the years 2013 and 2014. When the experiments were finished, the birds were trained to fly in the aviary, to prey on a rich insect diet (netted insects), and, after 2–3 weeks, they were

released in the surroundings of their nest box. Thus, birds spent at maximum 2 months in the lab.

2) Immature birds ($N = 31$) were obtained by mist-net capture at suitable localities during the summer of the years 2013 and 2014. These birds had their juvenile plumage (light brown-ruff head cap, gray cheek spots, and bib), which usually lasts in tree sparrows until the age of 4 months (Cramp and Perrins 1993). Besides coloration, the level of skull ossification was checked, birds older than 4 months have completely ossified skulls (Svensson 1992). In the Czech Republic, the first tree sparrows fledge (leave the nest) at the beginning of April (their juvenile features thus persist until the end of July), and the last fledglings from the third breeding leave the nest in August (their juvenile features may thus persist until December in these birds—Hudec and Štátný 2011). Birds of this age class had the opportunity to encounter various insect prey in their surroundings and sense their chemical as well as visual signals (contrary to the naïve ones). They could immediately enter the experiment (after the capture and acclimatization to the laboratory conditions and food), and after the experiment, they could be immediately released at the place of capture. They, therefore, spent 2–3 days in the lab.

3) Summer adult birds ($N = 13$) were caught by mist-netting at suitable localities during the summer of the years 2013 and 2014. These birds possessed adult plumage (dark brown cap, black cheek spots, and bib) and were at least 1 year old. To avoid misidentification with already moulted yearlings, we captured both classes (immatures and summer adults) during June and July, because immature birds first moult at the beginning of August (completely) and cannot then be distinguished from adults. They were assumed to have broad recent experience with various insect prey, as well as long-term experience at least from 2 summer seasons, and not usually to have faced any physiological stress caused by low temperatures. These birds were subjected to the same laboratory procedure as the immature ones.

4) Winter adult birds ($N = 42$) were caught by mist-netting at the winter feeders during winter in the years 2010–2013. They had adult plumage and were thus at least 4 months old. The average winter (January, February, and March) temperatures for these years in České Budějovice were below zero (2010: -1.8 , 2011: -1.3 , 2012: -1.0 , 2013: 0.2). This prevents insect activity and as sparrows usually do not forage for insects in their hibernacula (Cramp and Perrins 1993) they were assumed to have less recent experience with aposematic and probably also other insects. Perhaps more importantly, these temperatures increase energy demands for thermoregulation at a time when the food supply is reduced. These birds were subjected to the same laboratory procedure as the immature ones.

All sparrows were weighted before the experiment using 50 g scales (PESOLA AG, Switzerland). Sparrows of all age/experience classes were of the same body mass (ANOVA, $F_{3, 105} = 1.254$, $P = 0.854$). All birds were individually marked and no individual was used twice.

Prey species

The harlequin ladybird is an invasive alien species in Europe and many other parts of the world, with a natural area of distribution in eastern Asia (Brown et al. 2011). This species has been reported in the Czech Republic since 2006 (Brown et al. 2008). It has several colour forms that can be either genetically determined or thermally induced (Tan 1946; Michie et al. 2010; Knapp and Nedvěd 2013). All tested ladybirds were collected during the autumn migration (late October of each year) in České Budějovice and were kept at

5°C. They were provided with moist cotton wool and half a grapevine berry as a water and energy supply.

In our experiments, we used the succinea color form, which is orange-red with 19 black spots and is the most common in the Czech population (Knapp and Nedvěd 2013). It shows a higher level of chemical protection (Nedvěd et al. 2010) than the 7-spot ladybird that has been proven to elicit high avoidance in birds (Marples et al. 1989). The protection of the harlequin ladybird against predators is realized by the presence of 2 alkaloids in their hemolymph (harmaline and 3-hydroxypiperidin-2-on) together with volatile methoxypyrazines (Alam et al. 2002).

To test the effect of the color signal on the ladybird protection, we altered its coloration. Besides the natural, unmodified succinea color form, we used individuals artificially painted with brown tempera paint (Koh-i-noor Hardtmuth—natural sienna) to obtain ladybirds with non-warning coloration (Figure 1). This paint is non-toxic and has been shown to have no impact on bird behavior and act as a non-warning color to passerines (Exnerová et al. 2003; Veselý et al. 2006, 2013a; Dolenská et al. 2009; Prokopová et al. 2010; Půchová et al. 2014). All ladybirds were presented alive during the experiment. Given that it takes several days to completely restore the level of toxin in the hemolymph (Holloway et al. 1991), we performed our experiments 4 days after the initial manipulation. In addition, to control for the varying levels of toxins between the brown and red ladybirds, the latter were also reflex-bled and included in the experiments after 4 days. Simultaneously, the odor of methoxypyrazines in the hemolymph adhered to the ladybird surface vanished during the first 24 h (Holloway et al. 1991).

Experiment

2–3 h before the experiment, each bird was moved into an experimental cage to get used to the new environment. The experimental cage was composed of a wooden frame (70 × 70 × 70 cm) covered with wire mesh. The front wall was composed of a one-way mirror which enabled the observer to record the bird's reactions without disturbing it. The experimental cage was illuminated by a full-spectrum light source and situated in a dark room to enhance the effect of the one-way mirror. The cage was equipped with a bowl of water and a perch situated approximately 30 cm from the prey. At the bottom of the mirror side, there was a rotating circular tray containing six cups (5.5 cm in diameter) where the prey was offered. The bottoms of the cups were white, and only one cup (in the middle) contained the prey.

Birds were deprived of food for 2 h before the experiment started, which proved to be enough to motivate the bird to forage but not enough to cause stress through starvation (Exnerová et al. 2003). Each bird was offered 5 ladybirds of the particular tested form. The repetition of presentation of the same ladybird color form was used in order to minimize the effect of neophobia in case the bird considered the offered ladybird to be a novel prey (Marples and Kelly 1999). Ladybirds were alternated with mealworms, to determine the bird's willingness to forage. If the mealworm was not eaten, we did not continue with subsequent presentation of



Figure 1. Unmodified (left) and brown-painted (right) form of the harlequin ladybird *H. axyridis f. succinea*.

ladybirds. Each presentation of a ladybird lasted, a maximum of 5 min. The reactions of the birds were recorded with camera and subsequently analyzed in Observer 3.0 ethological software (1989–1992, ©Noldus).

Ethical note

Experiments carried out in this research comply with the current laws of the Czech Republic and with the Ethical Committee of the Zoological Society of London. The authors are licensed for catching and ringing birds (Bird Ringing Centre Prague Nos. 1004 and 1159), for animal experimentation (Ministry of Agriculture, CZ 01629 and CZ 02766), and for conducting laboratory experiments with birds (Ministry of Education, Youth and Sports, licence no. 8809/2011-30). Faculty of Science of the University of South Bohemia has accredited breeding facilities for passerines (Ministry of Agriculture, licence no. 9103/2009-17210). The authors have no competing interests.

Behavior analyses

We recorded 4 behavioral displays in the tree sparrows. Attacking the prey was recorded, when the bird touched the ladybird with its beak, and this behavior is seen as a measure of the effect of ladybird colouration, which is designed to stop the attack from a distance. We used ladybird color, bird age class, and their interaction as predictive variables for the occurrence of attack as the response type (generalized linear model—GLM, binomial distribution, Likelihood ratio tests based on Chi square statistics and Fisher LSD post hoc tests with the Tukey correction).

Eating the prey was recorded, when at least part of the ladybird body was swallowed. This behavior is seen as reflecting the effect of chemicals, either a foul smelling volatile (methoxy-pyrazines) or having a bitter taste (alkaloids), which are released after physical attack. The ladybird form, sparrow age, class, and their interaction were used as the predictor variables (GLM, binomial distribution, Likelihood ratio tests based on Chi square statistics and Fisher LSD post hoc tests with the Tukey correction). Only birds that attacked at least one of the 5 offered ladybirds were included in this analysis (18 naive, 25 immature, 9 summer adults, and 36 winter adults).

These 2 behavioral responses were coded binomially for each bird (1—the bird attacked/ate at least 1 of the 5 offered ladybirds). Almost all attacking/eating events recorded occurred in all 5 presentations. Only 2 naive attacked only 4 brown ladybirds out of 5 offered, and 1 naive sparrow attacked only 4 unmodified ladybirds. A similar trend was also seen for the number of birds eating the attacked ladybirds (we recorded only 1 immature bird that ate only 4 out of 5 offered ladybirds, other eating birds always ate all 5 ladybirds). This suggests that when a bird decides to attack/eat ladybirds, it always does so in all cases. The difference is in making the decision of whether to attack/eat ladybirds or not. Therefore, there was no variability enabling us to test for any learning progress within the 5 repetitions.

We analyzed the numbers of birds eating the ladybirds, rather than the survival rate of ladybirds, because all ladybirds that were attacked and not eaten at the same time, survived the attack (therefore, eating rate equated survival rate). We observed the attacked ladybirds 2 days after the experiment (returned to their breeding jars, equipped with water and food) and we never recorded any death. Ladybirds are quite strongly built insects and predator manipulation does not cause much harm to them (Dolenská et al. 2009). Most attacks not followed by eating just involved pecking

the ladybird and throwing it out of the offering tray. This manipulation was gentle enough to allow the ladybird to survive.

Further, we analyzed the latency to the attack of the offered ladybird. This analysis included only trials in which an attack occurred and the data followed the Gaussian distribution. A linear mixed effect model (LMM, Likelihood ratio tests based on *F*-test and Tukey HSD post hoc tests with the Tukey correction) was used to assess the effect of predictors (ladybird color, bird age class, and their interaction). The bird identity was included as the random factor, as there were usually 5 trials (pseudoreplications) for each bird.

The total numbers for the incidence of birds cleaning their beak, and the total time they spent drinking water during all 5 ladybird presentations were used as a measure of disgust shown after eating the ladybird. Passerines significantly increase the frequency of bill cleaning immediately after the manipulation or ingestion of an unpalatable insect (Exnerová et al. 2003). Similarly, birds tend to drink more water after the ingestion of chemically defended prey (Exnerová et al. 2003). Besides these two behavioral elements, we recorded no grinding during the experiment, or vomiting either during the experiment or 24 h after it. Nevertheless, none of these behaviors ever occurred, so only drinking water and bill cleaning were included in further analyses. The type of prey (mealworm or ladybird), the age class of the sparrow, the occurrence of eating at least 1 of the 5 offered ladybirds, and their interaction were used as predictor variables (GLM, Poisson distribution, Likelihood ratio tests based on *F* statistics). In these two models, sparrow responses to ladybirds as well as mealworms were included, to see if there are any differences between the disgust caused by ladybirds and mealworms. All computations were conducted using R for windows software (version R 3.1.2, R Foundation for Statistical Computing 2014; commands `glm` and `glht` in package `multcomp`).

Results

All control mealworms were always attacked and eaten within a couple of seconds after offering.

Attacking

The number of sparrows attacking at least 1 of the 5 offered ladybirds was significantly affected by the interaction of the color form of the ladybird and the age class of the sparrow (GLM, Chi square test: $\chi^2 = 5.89$, $df = 7$, $P = 0.027$). Within each age class, the attack rate on the unmodified and brown-painted form did not differ (Fischer LSD post hoc test: naive $Z = 0.898$, $P = 0.205$; immature $Z = 1.568$, $P = 0.068$; summer adult $Z = 1.190$, $P = 0.096$; winter adult $Z = 0.882$, $P = 0.262$; Figure 2). The attack rates of particular age classes on the brown-painted form did not differ either (Fischer LSD post hoc test: naive \times immature $- Z = 0.002$, $P = 0.999$; naive \times summer adult $- Z = 0.849$, $P = 0.299$; naive \times winter adult $- Z = 1.048$, $P = 0.100$; immature \times summer adult $- Z = 0.849$, $P = 0.299$; immature \times winter adult $Z = 1.048$, $P = 0.100$; summer adult \times winter adult $- Z = 0.413$, $P = 0.811$; Figure 2). This was predominantly true for the unmodified ladybirds as well (Fischer LSD post hoc test: naive \times immature $- Z = 0.758$, $P = 0.297$; naive \times summer adult $- Z = 0.999$, $P = 0.163$; naive \times winter adult $- Z = 0.617$, $P = 0.759$; immature \times summer adult $- Z = 0.534$, $P = 0.799$; immature \times winter adult $Z = 1.032$, $P = 0.104$; Figure 2), with 1 exception. Summer adults attacked the unmodified ladybirds significantly less than winter adults (Fischer LSD post hoc test: $Z = 2.072$, $P = 0.035$; Figure 2).

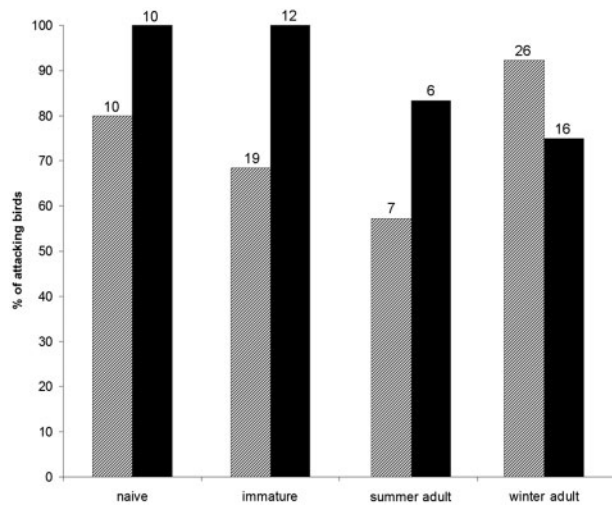


Figure 2. Proportion of tree sparrows *P. montanus* of particular age classes (for details see Methods section) that attacked at least 1 of the 5 offered unmodified (stripped bars) and brown-painted (black bars) harlequin ladybirds. Numbers of all tested birds in particular groups are mentioned above each column.

Eating

The number of tree sparrows which ate the harlequin ladybirds after an attack was significantly affected only by the age class of the sparrow (age class: GLM, Chi square test: $\chi^2 = 6.31$, $df = 3$, $P = 0.012$; ladybird color: GLM, Chi square test: $\chi^2 = 0.98$, $df = 1$, $P = 0.839$; interaction: GLM, Chi square test: $\chi^2 = 1.11$, $df = 3$, $P = 0.862$). Naïve and immature sparrows ate the ladybirds significantly less than summer and winter adults (Fisher LSD post hoc test: summer adults vs. naive $Z = 1.937$, $P = 0.033$; winter adult vs. naive $Z = 3.130$, $P = 0.009$; summer adults vs. immature $Z = 1.852$, $P = 0.047$; winter adult vs. immature $Z = 2.815$, $P = 0.024$; Figure 3). There were no differences between naïve and immature (Fisher LSD post hoc test; $Z = 0.593$, $P = 0.621$; Figure 3), nor between summer and winter adults (Fisher LSD post hoc test; $Z = 1.095$, $P = 0.851$; Figure 3).

Latency to attack

The latency to the attack of particular ladybirds (when only attacked ladybirds were included in analyses) was not affected by the ladybird color (LMM, $F_{1,436} = 0.878$, $P = 0.352$), sparrow age class (LMM, $F_{3,436} = 0.483$, $P = 0.695$), or by the interaction of these two factors (LMM, $F_{3,436} = 1.455$, $P = 0.233$).

Measure of disgust

The sparrow age class, the occurrence of eating of the prey, the prey identity (ladybird vs. mealworm), and the interaction of these factors did not affect the number of beak-cleaning events (GLM, $F_{7,104} = 1.94$, $P = 0.071$; Figure 4A) or the total time spent drinking water (GLM, $F_{7,104} = 1.07$, $P = 0.389$; Figure 4B).

Discussion

Contrary to our first prediction, all age classes of tree sparrows attacked the unmodified and brown-painted harlequin ladybirds equally often. This suggests that the visual signal of the harlequin ladybird is not perceived as a warning by tree sparrows. This result is in concordance with Exnerová et al. (2003) which showed that

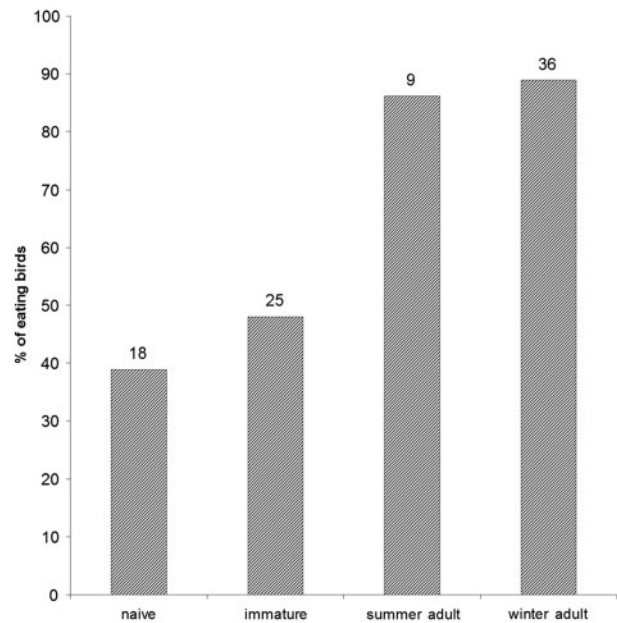


Figure 3. Proportion of tree sparrows *P. montanus* of particular age classes (for details see Methods section) that ate at least 1 of the 5 offered harlequin ladybirds. Numbers of birds that attacked the ladybirds in particular groups are mentioned above each column.

partially grani- and frugivorous passerines attacked unmodified and brown-painted red firebugs equally. In this study, the authors suggest that strong aversion toward red–orange coloration would not be beneficial for these species because it would limit the acceptance of berries and fruits in their diet (Honkavaara et al. 2004). Gamberale-Stille and Sillén-Tullberg (2001) showed domestic chicks to be able to treat aposematic insects and red fruit as different prey and not to mistake them for each other, which importantly conditions this explanation.

Contrary to our second prediction, adult tree sparrows commonly ate the already attacked ladybird. In most existing studies, birds usually hesitated to eat the already attacked defended prey (Wiklund and Järvi 1982; Brower 1988; Exnerová et al. 2003). One possible explanation may reside in the fact that the ladybirds in our design were bled-out during handling (coloration). However, we left 4 days for ladybirds to replenish their chemical protection, which should have been enough (Marples et al. 1994) so that we may suppose that ladybirds in our experiment were equipped with sufficient chemical protection.

This result suggests that the volatile substances (methoxy-pyrazines and haemolymph containing bitter alkaloids) present in the ladybirds (Alam et al. 2002) did not discourage most of the tree sparrows (see below for details) from continuing the attack. This is surprising because pyrazines (e.g., Marples and Roper 1996; Rowe and Guilford 1996; Lindström et al. 2001; Siddall and Marples 2011a), alkaloids (e.g., Marples et al. 1994; Dolenská et al. 2009; Průchová et al. 2014), and also other bitter chemicals such as quinine or Bitrex solution (e.g., Gamberale-Stille and Guilford 2004; Skelhorn and Rowe 2006a, 2006b; Halpin et al. 2008; Skelhorn et al. 2008) are generally considered to be very effective substances in repelling bird predators.

Contrary to our third prediction, sparrows ate 5 harlequin ladybirds in a series and did not show any signs of discomfort or nausea. We observed the birds throughout the day immediately following

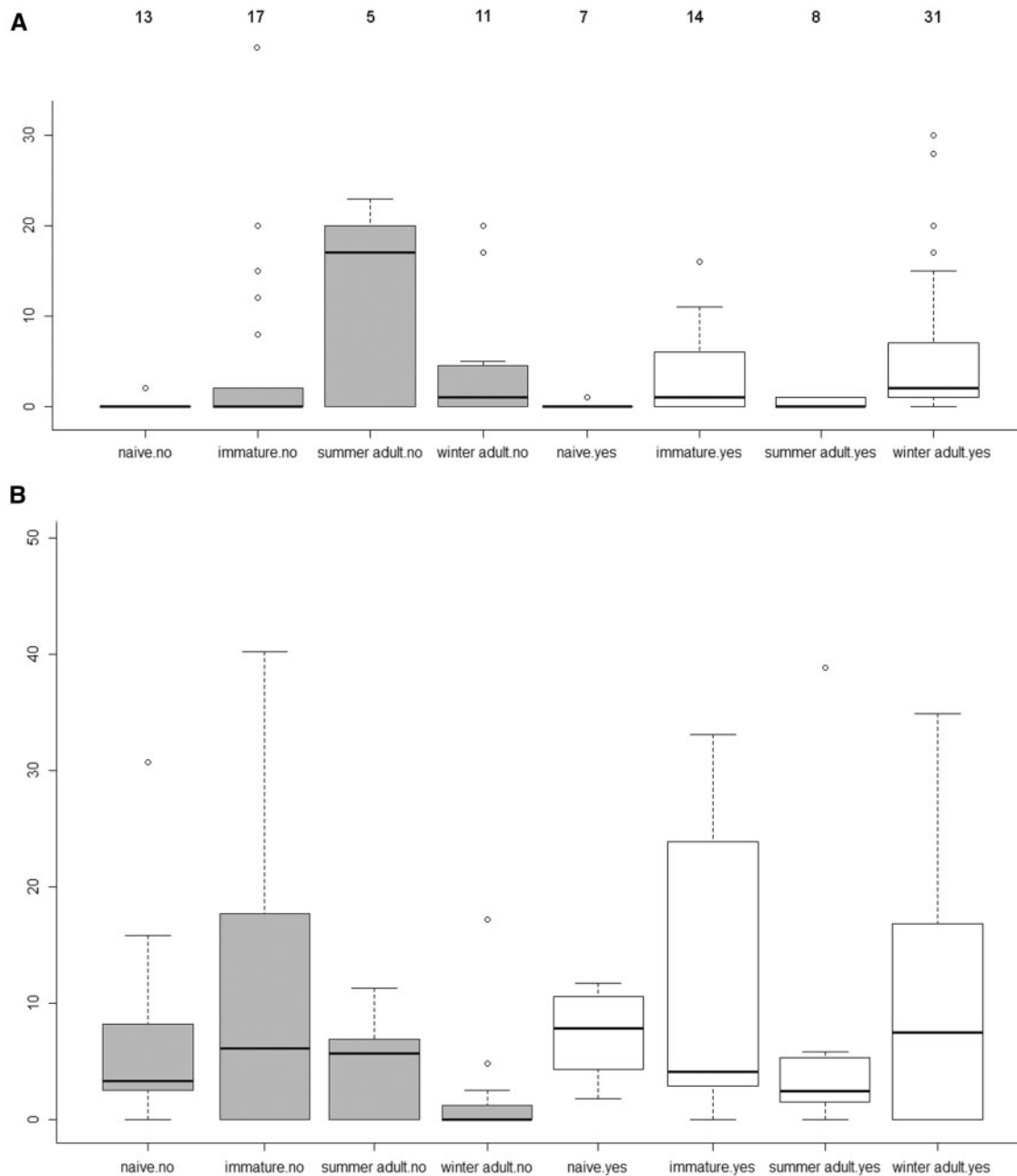


Figure 4. Number of cleaning bill events (A) and the total time spent drinking the water (B) by tree sparrows *P. montanus* of particular age classes (for details see Methods section) in all 5 trials, depending on the presence of eating the ladybird within at least 1 of the 5 trials. Columns for birds that did not eat any ladybirds are gray and x-axis labels end with “no” and columns for birds that ate at least 1 of the 5 offered ladybirds are white and the x-axis label ends with “yes”. Number of birds in each column is mentioned above the column.

the experiment, and we never recorded any marks of disgust or physiological difficulties. This is in contrast to experiments with young blue tits *Cyanistes caeruleus*, which showed slower growth rate, physiological difficulties, and sometimes even death, when provided with a diet containing the mashed bodies of the 7-spot ladybird (Marples et al. 1989). The harlequin ladybird is considered to be more toxic than the 7-spot ladybird (Nedved et al. 2010) and we could thus expect that it should have at least a similar effect on sparrows as the 7-spot ladybird on titmice.

The ability of birds to include toxic prey in their diet has been experimentally shown several times before. For example, several studies have shown that when palatable prey are relatively scarce,

the benefits of obtaining energy from defended prey increases, and predators could raise their attack rates on aposematic prey despite the costs involved with ingesting toxins (Kokko et al. 2003; Lindstrom et al. 2004). Other examples originate from experiments with tropical birds (e.g., Chai 1986; Pinheiro 2003). Most often it is the case of a predator in physiological stress due to being deprived of food (Barnett et al. 2007, 2011). Such predators attack protected prey more often, when it provides higher nutrition value, than unprotected prey (Halpin et al. 2013, 2014; Smith et al. 2014) but the level of toxicity of particular prey is also important (Barnett et al. 2011). In our experiments, sparrows were deprived of food for 2 h, which should not elicit any stress in passerines of the sparrow’s

body size (Exnerová et al. 2003). Moreover, ladybirds were presented alternately with mealworms, so the birds had an equally common, nutritionally rich, alternative edible prey.

In most studies, where bird predators attack and eat toxic prey, some form of regulation of the amount of ingested toxins is recorded (Halpin and Rowe 2010). For example, Brower (1988) showed that black-headed grosbeaks commonly ate aposematic monarch butterflies, but after the ingestion of 1 or 2 butterflies they stopped attacking them and needed some time to cope with the ingested chemicals. Our sparrows usually ate all 5 offered ladybirds, when they decided to eat at least 1. Collectively, our results suggest that the sparrows are able to cope with this amount of toxin without complications.

The question remains, if toxicity effects would have been detected if sparrows were able to eat more ladybirds in a series. Birds in the natural condition usually regulate the amount of ingested toxins by ceasing attacks on noxious prey after some individuals have already been ingested (Chai 1986; Brower 1988). We may expect that sparrows would do the same after having ingested more than 5 ladybirds. Such avoidance would then follow the visual aposematic signal of the noxious prey in the same manner as shown in jacamars preying on toxic butterflies (Chai 1986). This suggests that the aposematic coloration does protect the prey against predators adapted to ingesting toxins, and that the selection pressure represented by such predators may not differ in fact from predators sensitive to their chemical protection.

In any event, tree sparrows are probably adapted to ingesting more ladybirds and better coping with the alkaloids and pyrazines in them. Our results are in concordance with evidence obtained in the natural environment that several ladybird species, including 7-spot ladybirds, have been found to be part of the tree sparrow diet (Krištín 1984, 1986, 1988; Krištín et al. 1995; Field et al. 2008). We may hypothesize that this adaptation originally developed to cope with plant alkaloids. The ability of grani-, frugi-, and nectarivorous birds to cope with alkaloids as the secondary plant metabolites deposited in seeds or nectar has been shown several times (Díaz 1996; Levey and Cipollini 1998; Banko et al. 2002; Matson et al. 2004; Ríos et al. 2012), though various bird species may significantly differ in their ability to ingest alkaloids (Lerch-Henning and Nicolson 2013, 2015). There is no study comparing the effect of plant and insect alkaloids on the bird metabolism (see Fu et al. 2004 for review), so we may only hypothesize that the ability to deal with chemical alkaloids was predisposed by the ability to deal with plant alkaloids. Most alkaloids block specific enzymes in the liver tissue, resulting in a decreased function of the liver (Fu et al. 2004), or function as strong agonists at the acetylcholine receptor, which affects nervous activity (Ríos et al. 2012). It is widely documented that birds sometimes eat clay to minimize the effect of alkaloids on their metabolism (Brightsmith et al. 1998; Gilardi et al. 1999), which might be the case for tree sparrows as well.

Contrary to our fourth prediction, we found that none of the classes, including the hand-reared naïve birds, considered the visual signal of the ladybird as a warning. This is in discordance with studies showing that birds may possess innate aversion toward aposematic prey (Jetz et al. 2001; Exnerová et al. 2007; Siddall and Marples 2011b). Despite the fact that the naïve sparrows did not show any aversion toward the visual signal of the ladybirds, they were significantly less willing to eat the attacked ladybirds than the adults. This 1) suggests a certain level of innate aversion to the insect defensive chemicals (e.g., Rowe and Guilford 1999) or rather 2) is a result of sparrow parents providing ladybirds for their nestlings. Tree sparrow nestlings have a significant portion of ladybirds in their diet

(Krištín 1988; Krištín et al. 1995) and we may expect that even our experimental nestlings were not fully naïve in this manner.

Body mass is another important parameter affecting the willingness to include toxic prey in a bird's diet. This works on 2 levels. First on the inter-species level, birds of a higher body weight more easily cope with ingested chemicals (Exnerová et al. 2003). Second on the intra-species level, birds of lower body weight experience more physiological stress and are then more willing to attack toxic prey (Barnett et al. 2007). In the present study, sparrows of different age classes did not differ in body weight, so we cannot suppose this to be the reason for the differing eating rates of young and adult sparrows. This still does not exclude the possibility of some sparrows (winter adults) experiencing increased energy demands.

The winter adults attacked and ate the ladybirds most often, which may suggest that the willingness to include ladybirds in their diet increases due to a significant shortage in the insect diet available during the winter. This is in concordance with studies of Barnett et al. (2007, 2011, 2014) showing starlings to be more willing to include toxic prey in their diet when their body mass and fat stores are experimentally reduced (undergoing physiological stress). Chatelain et al. (2013) showed that cold temperatures may also result in a higher willingness to include toxic prey in their diet. This result could importantly condition the conclusions of studies with aposematic insects and birds caught in the wild during the winter months (Exnerová et al. 2003, 2007; Tesařová et al. 2013; Veselý et al. 2013a, 2013b; Cibulková et al. 2014; Karlíková et al. 2016; Turini et al. 2016).

Our study may also potentially contribute to our knowledge of the invasion biology of the harlequin ladybird. This species has swiftly spread throughout Europe (Brown et al. 2008, 2011), occupying an empty niche and being dominant over other ladybird species. Nothing is known about the natural predators of harlequin ladybird in its native range in eastern Asia, but the tree sparrow's area of occurrence stretches to the far east of Russia, Japan, and China (Cramp and Perrins 1993), where they co-occur with the harlequin ladybird (Poutsma et al. 2008). Nevertheless, the potential evolutionary familiarity of this ladybird to tree sparrows is hard to corroborate. It is more probable, that tree sparrows are able to include harlequin ladybirds in their diet thanks to their ability to prey on other ladybird species.

To conclude, the ability/willingness of the tree sparrow to include ladybirds in its diet and to cope with their toxins appears rather high. Similarly to other bird species, they use this strategy more often when facing nutritional restrictions and increasing energy demands for thermoregulation (during winter). At least half of any tested age/experience class of sparrows always attacked and ate 5 offered ladybirds and showed no apparent discomfort. Nonetheless, we cannot expect that their ability is without limits, and we may expect that tree sparrows cease eating the ladybirds after some amount of toxins are ingested. This avoidance would then follow the visual aposematic signal as it works in species with strong aversive responses (Mallet and Joron 1999; Barnett et al. 2011).

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