Charles University

Faculty of Science

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Mgr. Lucia Kuklová (Doktorovová)

Factors influencing effectiveness of aposematic signals against avian predators

Faktory ovlivňující efektivitu aposematických signálů
vůči ptačím predátorům

Doctoral thesis

Supervisor: Doc. Mgr. Alice Exnerová, Ph.D.
Supervisor-consultant: Prof. RNDr. Pavel Štys, CSc.

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“Everyone likes birds. What wild creature is more accessible to our eyes and ears, as close to us and everyone in the world, as universal as a bird? “
David Attenborough

“The early bird gets the worm. The early worm... gets eaten. “
Norman Ralph Augustine

“The early bird gets the worm, but the second mouse gets the cheese! “
Ernst Berg

“What we see depends mainly on what we look for. “
John Lubbock

Prohlášení:

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Mgr. Lucia Kuklová

Predslov / Preface

Chcela by som sa poďakovať všetkým, ktorí sa podieľali na vzniku tejto práce, alebo boli pre mňa morálnou podporou počas skoro 9-ročného štúdia.

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ABSTRACT

This thesis focuses on various factors affecting effectiveness of aposematic signals against avian predators. Adult, wild-caught as well as hand-reared juvenile great tits (*Parus major*) were used as predators in the experiments. The thesis consists of the following four studies.

In the first study, we compared the reactions of great tits from two geographically distant populations toward aposematic firebugs (*Pyrrhocoris apterus*) and their non-aposematic artificially made colour variant. The birds from the Bohemian population mostly avoided aposematic firebugs and attacked non-aposematic variant. Finnish birds, which lacked experience with firebugs from their natural environment, were less hesitant to attack both firebug colour forms. Although the Bohemian birds avoided the aposematic prey variant, they were not more neophobic than Finnish birds. We conclude that the geographic differences in reactions of the birds to aposematic prey can be explained by a different population-specific experience of the birds with local aposematic prey communities.

In the second study, we compared effectiveness of two chemical defence strategies in leaf beetle larvae (Chrysomela lapponica) against great tits. The birds avoided larvae devoid of external secretions after the first attack, which indicates the presence of non-volatile defensive compounds within the larval body. However, survival after the first attack was greater for larvae with intact secretions than for larvae with depleted secretions. Hence, both strategies of storage of chemicals, external secretion and storage in the body, act together against bird predation. Birds learned to avoid sequestered secretions faster compared to autogenously (*de novo*) produced secretions. Nevertheless, both strategies of chemical production provided effective protection against birds. We conclude that avian predation could contribute to the evolution of both secreted and stored defensive chemicals in *C. lapponica* larvae.

In the third study, we tested the hypothesis that different aposematic colour morphs of polymorphic leaf beetle (*Chrysomela lapponica*) differ in the effectiveness of their aposematic signals against avian predators. Juvenile great tits attacked beetles of all colour morphs (red-and-black light, red-and-black dark and metallic) at the same rate, while adults attacked light beetles at the first encounter more frequently than both dark and metallic beetles. Avoidance learning was similarly fast for all three morphs; therefore, colour displays of all morphs function as effective warning signals. Morphs differed in their memorability; the dark beetles were attacked more frequently than the other two morphs during the memory test. We hypothesise that, dark colour morphs may have a selective advantage at low population densities but they lose this advantage at high population densities due to the low memorability of their display. Thus, the direction of selective bird predation on aposematic morphs may depend on prey density, contributing to cyclic shifts in the morph frequencies.

In the fourth study, we compared the performance of great tits in sequential and simultaneous prey-discrimination tasks. Colour was more effective discriminative cue than pattern for both adult and juvenile great tits. The birds performed equally well in sequential and two-choice task, but their performance in multiple–choice task was worse than in the other two tasks. Nevertheless, these differences were found only when the birds used pattern as a cue for discrimination. The birds tested with colour, more salient cue by itself, performed equally well in all three tasks. We conclude that the type of the discrimination task may affect learning performance of bird predators, but the effect also depends on effectiveness of a particular discriminative cue; if a cue is highly salient, the type of used task might not be so important.

The results of this thesis contribute to understanding variability in reactions of avian predators toward aposematic prey as well as to understanding origin of the aposematic signal.

ABSTRAKT

Tato disertační práce se zabývá různými faktory, které mohou ovlivňovat efektivitu aposematických signálů vůči ptačím predátorům. V experimentech byli testováni dospělí, v přírodě odchycení ptáci i ručně odchovaná mláďata sýkory koňadry (*Parus major*). Práci tvoří čtyři studie.

V první studii jsme srovnávali reakce dvou geograficky vzdálených populací sýkory koňadry vůči aposematické ploštici ruměnici pospolné (*Pyrrhocoris apterus*) a její neaposematické uměle vytvořené barevné variantě. Ptáci z české populace se většinou vyhýbali aposematické variantě kořisti a napadali neaposematickou variantu. U ptáků z finské populace, kterým chyběla zkušenost s ruměnicí v přírodě, byla zjištěna větší pravděpodobnost útoku na obě varianty kořisti. I když se česká populace sýkor aposematické variantě kořisti vyhýbala, ve srovnání s finskou populací nebyla více neofobní. Usuzujeme, že rozdílné reakce dvou populací sýkor na aposematickou kořist byly způsobeny rozdílnou zkušeností ptáků s lokálně se vyskytujícími společenstvími aposematické kořisti.

Ve druhé studii jsme srovnávali efektivitu dvou strategií chemické obrany larev druhu *Chrysomela lapponica* před ptačími predátory. Sýkory se naučily vyhýbat larvám zbavených možnosti vnější sekrece již po první manipulaci s kořistí, což ukazuje na přítomnost obranných chemických látek uvnitř těla larev. Nicméně larvy s úplnou sekrecí přežívali útok častěji než larvy, které byly zbaveny sekrece. Oba způsoby ukládání obranných chemických látek, vnější sekrece a ukládání látek uvnitř těla, tedy spolu působí jako obrana vůči ptačím predátorům. Sýkory se naučily vyhýbat sekvestrované sekreci larev rychleji ve srovnání s autogenní (*de novo*) sekrecí, avšak obě strategie produkce obranných chemických látek zabezpečují efektivní obranu larev vůči ptákům. Předpokládáme, že ptačí predace by se mohla podílet na evoluci vnější sekrece i na ukládání obranných chemických látek v těle larev *C. lapponica*.

Ve třetí studii jsme testovali hypotézu, že aposematické různobarevné varianty (morfy) mandelinky polymorfního druhu *Chrysomela lapponica* se liší v efektivitě varovných signálů vůči ptačím predátorům. Mláďata sýkor napadala všechny tři varianty kořisti (černo-červená světlá, černo-červená tmavá a metalická) se stejnou intenzitou. Dospělé sýkory při prvním střetnutí s kořistí častěji napadaly světlou variantu než tmavou a iridescentní (metalickou) variantu kořisti. Averzivní učení probíhalo u všech barevných variant stejně rychle, a tedy aposematický display všech tří variant je efektivním varovným signálem. Barevné varianty se lišily v efektivitě zapamatovatelnosti příslušných aposematických signálů u predátorů; sýkory napadaly tmavou variantu v paměťovém testu častěji než zbylé dvě varianty. Předpokládáme, že tmavá varianta může mít selektivní výhodu při nízké populační hustotě modelového druhu kořisti, nicméně při vysoké populační hustotě tuto výhodu ztrácí kvůli nízké zapamatovatelnosti varovných signálů. Směr selektivní ptačí predace vůči aposematickým variantám polymorfního druhu kořisti může tedy být ovlivňován hustotou kořisti, a jako důsledek mohou vznikat periodické změny ve frekvencích jednotlivých variant.

Ve čtvrté studii jsme srovnávali efektivitu diskriminačního učení u sýkor testovaných v sekvenční a simultánní diskriminační úloze. Barva byla efektivnějším diskriminačním podnětem než vzor, a to jak pro dospělé ptáky, tak pro mláďata. Simultánní úloha typu mnohonásobný výběr byla pro ptáky obtížnější než dvou-výběrová simultánní úloha a sekvenční úloha. Tyto rozdíly se ale prokázaly jenom při diskriminaci pomocí vzoru jako podnětu. Sýkory testované v diskriminaci pomocí barvy se naučily diskriminovat nezávisle na typu úlohy. Typ prezentace podnětů může tedy ovlivňovat efektivitu diskriminačního učení ptačích predátorů, ale závisí i na obtížnosti diskriminačního podnětu; při nápadnějším podnětu, který se diskriminuje snáz, nemusí mít typ diskriminační úlohy vliv na výsledky experimentu.

Výsledky této práce přispívají k porozumění variability v reakcích ptačích predátorů vůči aposematické kořisti a také k pochopení vzniku výstražných signálů.

CONTENTS

STUDY 1: Exnerová, A., Ježová, D., Štys, P., **Doktorovová, L.,** Rojas, B., & Mappes, J. (2015). Different reactions to aposematic prey in 2 geographically distant populations of great tits. *Behavioral Ecology*, 26(5), 1361-1370.

STUDY 2: Zvereva, E. L., **Doktorovová, L.,** Hotová Svádová, K., Zverev, V., Štys, P., Adamová-Ježová, D., Kozlov, M. V. & Exnerová, A. (2018). Defence strategies of *Chrysomela lapponica* (Coleoptera: Chrysomelidae) larvae: relative efficacy of secreted and stored defences against insect and avian predators. *Biological Journal of the Linnean Society*, 124(3), 533-546.

STUDY 3: Doktorovová, L., Exnerová, A., Svádová, K. H., Štys, P., Adamová-Ježová, D., Zverev, V., Kozlov, M. & Zvereva, E. L. (2019). Differential Bird Responses to Colour Morphs of an Aposematic Leaf Beetle may Affect Variation in Morph Frequencies in Polymorphic Prey Populations. *Evolutionary Biology*, 46(1), 35-46.

STUDY 4: Kuklová, L., Jůnová, L. & Exnerová, A. How type of task affects prey-discrimination learning in avian predators. (to be submitted to Ethology)

1 Introduction

Anti-predatory mechanisms have attracted the biologists since 19th century. One of the important strategies used by prey to protect them from predators is aposematism. The aposematic prey signals its unprofitability or unpalatability to potential predators to warn them and to prevent the attack (Mappes et al. 2005). Early detection of aposematic prey saves time and energy to a predator and increases prey survival (Sillén - Tullberg 1985a). Aposematism is considered a beneficial strategy for prey if its warning signals cause lower mortality than crypsis and it does not necessarily require complete avoidance of prey (Cott 1940).

The aposematic prey may use various traits from several sensory modalities when signalling its unprofitability to predator, nonetheless, not all traits are equally effective (Aronsson and Gamberale-Stille 2008). Most commonly known warning signals are visual displays, however acoustic, olfactory, gustatory or behavioural signals and their combinations (Ruxton et al. 2004) have also been described.

Aposematism is most widespread across insects (Lindstedt et al. 2008), but it was described in many other taxonomic groups too (Pekár 2014, Dumbacher et al. 2009). Whilst many studies have enhanced our knowledge on the phenomenon of aposematism (Ruxton et al. 2004), there are still puzzles that remain to be solved. One of the open topics is to understand when does a warning signal of aposematic prey effectively influence the response of the receiver, *i.e.,* predator. In this thesis, I evaluated the topic from predator´s point of view; using great tits as model avian predator species, I assessed selected factors which could potentially cause variability in reactions of avian predators towards aposematic signals of prey.

The main aims of this thesis are:

1) To test the effect of experience with local aposematic prey communities on inter-population differences in reactions of avian predators to aposematic prey.

2) To compare effectiveness of two chemical defence strategies in leaf beetle larvae (*Chrysomela lapponica*) against avian predators.

3) To test reactions of avian predators to different colour morphs of aposematic leaf beetles (*Chrysomela lapponica*).

4) To compare performance of avian predators in sequential and simultaneous prey-discrimination tasks.

2 Aposematism

The term aposematism, originated from Greek words *´apo´* – away from, and *´sémeion´* – sign, was first used by Poulton (1890), who was inspired by Wallace´s previous description of the phenomenon. The original definition applied only to visual warning signals of prey, namely colours and patterns, and even nowadays the term is most commonly known in the context of warning coloration. Nevertheless, due to numerous experimental studies on this topic, particularly during last decades, it is now known that warning signals of aposematic prey can consist apart from visual (Svádová et al. 2009, **Doktorovová et al. 2019**) also of olfactory (Marples et al. 1994, Rowe a Guilford 1996), gustatory (Brower 1984, Marples et al. 1994, **Doktorovová et al. 2019**), acoustic (Rowe and Guilford 1999a, Rowe 2002) or behavioural (Gamberale and Tullberg 1996) components.

Warning signals often consist of components signalled through more sensory modalities; in this case the aposematic signals are called multimodal (Rowe 1999). More warning signals can either reinforce in effectiveness, or interact synergistically to elicit predator's innate avoidance, improve recognition of prey, enhance avoidance learning or improve the memorability of the aposematic signal (Rowe and Guilford 1999a). There is an evidence that multimodal signals may be more effective against predators compared to unimodal signals (Marples et al. 1994, Rowe 1999, Siddall and Marples 2008).

Aposematic prey uses the ability of predators to associate warning signals with unprofitability of a prey (Cott 1940). The main function of warning (aposematic) signals is to facilitate recognition of unpalatable prey by potential predator. Thus, the effective warning signal should be as conspicuous as possible to enable fast and correct recognition by predator (Guilford and Dawkins 1991), to enhance unlearned wariness (Schuler and Hesse 1985, Mastrota and Mench 1994, Lindström et al. 1999a) or to improve discrimination learning in predators (Roper and Redstone 1987, Lindström et al. 1999b), and consecutively also to enhance memorability of defended prey (Prudic et al. 2007). Nevertheless, conspicuousness depends on the context; some warning signals are conspicuous at a close distance, but with increasing distance or against another background they become cryptic to predator (Gamberale-Stille et al. 2009; Barnett et al. 2017). Furthermore, more salient warning signal can overshadow less conspicuous one and avian predators learn to avoid aposematic prey based on more salient signal

(Gamberale-Stille and Guilford 2003). Co-occurrence of aposematic prey may result in more effective avoidance learning in avian predators (Riipi et al. 2001, but see **Kuklová et al., to be submitted**).

Early detection of aposematic prey saves time and energy to predator and increases prey survival (Sillén - Tullberg 1985a). Aposematism is considered a beneficial strategy for prey if warning signals cause lower mortality than crypsis and it does not necessarily require complete avoidance of prey; predators decide whether to avoid aposematic prey or not based on their level of hunger or the occurrence of alternative prey (Cott 1940, Barnett et al. 2007).

Aposematism has evolved independently in different taxonomic groups. It is most widespread across insects, such as Lepidoptera (Lindstedt et al. 2008, Dell'Aglio et al. 2016), Coleoptera (Zvereva et al. 2002, **Doktorovová et al. 2019**), Heteroptera (Gamberale-Stille and Tullberg 1999) and Hymenoptera (Mora and Hanson 2019). Aposematism also exists in marine invertebrates, for example in Nudibranchia (Ritson-Williams and Paul 2007). It also occurs in other taxa such as spiders (Pekár 2014), frogs (Maan and Cummings 2012), snakes (Kikuchi and Pfennig 2010), mammals (Larivière and Messier 1996) and surprisingly in birds as well (Dumbacher et al. 2009). Some plants are also known to be aposematic (Rubino et al. 2004).

3 Warning signals

3.1 Visual signals

Aposematic prey can use various traits from different sensory modalities when signalling its unpalatability to predator, nonetheless, not all traits are equally effective (Aronsson and Gamberale-Stille 2008). Even within a modality, certain types of traits may be distinguished better than others. Most commonly known warning signals of aposematic prey are visual displays. They are effective against predators who evaluate prey palatability from distance, such as birds. Since the most important sense of birds is vision (Lindström 1999), visual signals should be the most effective warning signals for them. When studying visual communication in birds, it is important to realize how birds really see the visual signals, as their visual capacities are far beyond those of mammals. Birds´ vision is tetrachromatic, which allows them to detect apart from the visible range of the spectrum also ultraviolet (UV) wavelengths in the range of 300 – 400 nm (Bennett and Cuthill 1994, Cuthill et al. 2000). Additionally, their cone photoreceptors contain oil droplets, which help them in discriminating colours (Bennett and Théry 2007).

Numerous studies have been conducted to test the role of visual aposematic signals of prey and their components (Mappes et al. 2005). Birds use visual signals to decide whether to attack aposematic prey, which depends primarily upon both unlearned avoidance and avoidance learning (Guilford 1990). Many prey species possess aposematic coloration to signal their unprofitability to potential predators. The most efficient warning colours are considered to be bright and conspicuous such as red, orange, yellow or white (Cott 1940, Gamberale-Stille and Tullberg, 1999, Svádová et al. 2009, but see Lyytinen et al. 1999 for white), often combined with black (Lindström et al. 2001, Stevens and Ruxton 2012) to achieve high internal contrast. On the other hand, blue colour is also considered aposematic according to some authors (Umbers 2013, Pegram and Rutowski 2014). The importance of iridescent colouration in aposematic signalling has also been documented; it may lead to delays of attack by avian predator and also to effective avoidance learning in birds (Fabricant et al. 2014, **Doktorovová et al. 2019**). Also, iridescence was shown to be an effective signal for remembering learned avoidance of aposematic prey in avian predators (**Doktorovová et al. 2019**). However, findings of another study of bird responses to iridescence are contradictory, as the effect of prey iridescent colouration as warning signal against avian predators was not proven (Pegram et al. 2015). UV seems to not be an efficient warning signal for birds on its own (Lyytinen et al. 2001) and can even attract birds more instead of preventing attack (Lyytinen et al. 2004).

Many studies have shown colours to be the most important warning signals for birds (Marples et al. 1994, Ham et al. 2006, Svádová et al. 2009), which supports the hypothesis that colour is likely to be the most salient discriminative cue for avian predators (Terhune 1977, Gamberale-Stille and Guilford 2003, Aronsson and Gamberale-Stille 2008, **Kuklová et al., to be submitted**).Moreover, even though bright and conspicuous colours are widely used and effective in aposematic signalling, birds can associate any colour with positive (rewarded) or negative stimulus (Goldsmith and Goldsmith 1979, Meléndez-Ackerman et al. 1997, Ham et al. 2006, Aust et al. 2008, **Exnerová et al. 2015**, **Kuklová et al., to be submitted**). It is important to take into account, that apart from signalling to a predator, coloration of prey may serve also other functions including sexual or social communication and thermoregulation (Endler 1983, Ruxton et al. 2004).

Other visual traits may also be important for birds when recognizing or learning to avoid aposematic prey, although their role is less clear (Aronsson and Gamberale-Stille 2008, 2012). Pattern is less effective cue for avian predators than colour (Terhune 1977, Aronsson and Gamberale-Stille 2008, **Kuklová et al., to be submitted**). Pattern is effective aposematic signal for birds according to some studies (Rowe at al. 2004, Wűster et al. 2004, Aronsson and Gamberale-Stille 2013). However, another study claims that pattern discrimination is difficult for birds (Lindström et al. 1999a). Possible function of very common black pattern in warning signalling is to support aposematic colouration by increasing the salience of colour (Rowe and Guilford 2000) instead of having signal value *per se*. The effect of pattern symmetry in aposematic signalling has been found in some experiments (Forsman and Merilaita 1999, Forsman a Herrström 2004), but others found no evidence for importance of this visual trait (Stevens et al. 2009, Aronsson and Gamberale-Stille 2013). Some specific patterns may be more effective warning signals than others (**Kuklová et al., to be submitted**), nevertheless, results of another study did not confirm these findings (Aronsson and Gamberale-Stille 2008). The importance of background contrast as aposematic signal for birds has also been demonstrated (Gamberale-Stille 2001, Aronsson and Gamberale-Stille 2009), on the other hand, this trait was found to be not always necessary for aposematic signalling (Sillén - Tullberg 1985b). The effect of prey body size in warning signalling has been documented (Gamberale and Tullberg 1996, Mänd et al. 2007); bigger size of aposematic prey is considered to increase effectiveness of signal.

3.1.1 Visual warning displays in polymorphic species

The selective pressure for warning colouration should favour monomorphism, a single colour form in a prey population (Mallet and Joron 1999, Lindström et al. 2001). It is easier for predators to learn to avoid highly recognizable, memorable and more common aposematic prey (Guilford and Dawkins 1991). A prey becomes more common after predators start to avoid it, which results in increasing of the fitness of prey phenotype (Joron and Mallet 1998). The difficulty for predators to learn to avoid several colour morphs (Endler and Mappes 2004) will give advantage to a more frequent form of a warning signal (Joron and Mallet 1998), which should eventually prevail and others should become extinct. Consequently, the relative fitness of a phenotype is improved proportionally with its increased frequency; a phenomenon described as a positive frequency-dependent (antiapostatic) selection of a prey. The selection for warning coloration should therefore not maintain stable colour polymorphism with variable coloration within one prey population (Mallet and Joron 1999). Nevertheless, contrary to theoretical predictions, many aposematically coloured species in a range of taxa are polymorphic (Hegna et al. 2013, Rojas and Endler 2013, Fabricant et al. 2014, **Doktorovová et al. 2019**), and this intra-population variation in warning displays poses a challenge for biologists.

Maintaining polymorphism depends on certain mechanisms that lead to similar fitness across multiple morphs (Pryke 2007). A phenomenon of a negative frequency-dependent (apostatic) selection has the opposite effect to an antiapostatic selection of prey. Under apostatic selection, the relative fitness of a phenotype is increased disproportionally with its frequency. A rarer prey phenotype has a selective advantage over more common one, because predators tend to overlook a prey more when it is rarer. Apostatic selection could therefore explain colour polymorphism (Clarke, 1979). However, the evidence shows that this type of selection can typically explain polymorphism in palatable cryptic prey (Dukas and Ellner 1993). Predators use a search image during searching for cryptic prey, which helps them to detect the prey more easily (Dukas 2002). Developing a search image for certain traits of prey allows predators to overcome their limited attention and to prevent errors in their decision making. Hence, it is assumed to be more effective for predator to focus attention on a single prey compared to dividing attention among several targets (Dukas and Kamil 2001) and consequently, apostatic selection with decreasing fitness of more common prey form can take place (Mallet and Joron 1999). Yet, even though the strategy to focus attention to one prey type is effective when searching for cryptic prey, predators may divide attention among the different prey types instead when facing conspicuous prey to learn to avoid all types of unpalatable prey (Dukas and Ellner 1993). Thus, colour polymorphism of aposematic prey species typically cannot be explained by apostatic (negative frequency-dependent) selection, because aposematic prey is in advantage when more common due to more effective avoidance learning of aposematic signals by predators (Borer et al. 2010).

Several experiments showed that avian predators can learn to avoid all colour morphs of polymorphic prey at the same rate; hence, all colour warning displays may act as effective aposematic signals (Ham et al. 2006, Svádová et al. 2009, **Doktorovová et al. 2019**). Nonetheless, the different colour morphs within population can vary in their conspicuousness for avian predators (Gamberale – Stille et al. 2009, Barnett et al. 2017, **Doktorovová et al. 2019**). This variation in conspicuousness between conspecific morphs may lead to change in direction of bird predation, resulting in shifts between positive (antiapostatic) and negative (apostatic) frequency-dependent selection by avian predators (**Doktorovová et al. 2019**). When all coexisting colour morphs are novel for avian predators, which can happen either due to low density of prey or predominance of juvenile birds, a less conspicuous morph has a selective advantage, because predators will attack it less frequently compared to a more conspicuous morph (Mallet and Joron 1999), and upon that, apostatic selection occurs. This prediction is supported by findings, that avian predators were more hesitant to attack less conspicuous morph compared to more conspicuous one when both morphs were novel for birds (**Doktorovová et al. 2019**). Contrastingly, if neither of the morphs is novel for the birds, a more conspicuous morph has a selective advantage, because predators will learn faster to avoid it´s warning signal and remember it better (Prudic et al. 2007, Borer et al. 2010), and upon that, antiapostatic selection occurs. However, a more conspicuous morph has an advantage only if the prey density is high, so that the most birds could gain an experience with warning signal (Prudic et al. 2007). In conclusion, when aposematic signals of polymorphic prey vary in their conspicuousness, the direction of avian predator selection for colour morphs may change with fluctuations of the prey populations densities. Less conspicuous morphs have a survival advantage over more conspicuous morphs at low population densities, thus the frequency of the less conspicuous morph is greater at low densities compared to high density populations. Contrastingly, when the population density is high, the less conspicuous morphs lose their selective advantage. The relative survival of different morphs may thus depend on their density in prey population, and this selective predation can contribute to cyclic shifts of low and high frequencies in coexisting morphs (discussed in **Doktorovová et al. 2019**).

Although predation is considered to be the strongest selective pressure favouring aposematic coloration (Ruxton et al. 2004), an alternative or additional selective pressure can also affect variability in within-population coloration. Sexual selection may act as an opposing force to the predator pressure, both favouring different colour morphs of an aposematic species (Crothers and Cummings, 2013, Henze et al. 2018), which can possibly cause maintenance of colour polymorphism within population. Various mating success of morphs can also act together with spatial variation in distribution of predators, resulting in opposing directions of selection and consequently preventing complete selective advantage of one morph over others (Gordon et al. 2015).

3.2 Chemical defences

Chemical defences are widespread across animal kingdom and many chemical compounds have been reported to serve a warning function or to cause either toxic or irritating effect (Ruxton et al. 2004). Predators can sense chemical signals by olfactory (smell), gustatory (taste) or chemesthetic (irritation and pain) receptors (Clark et al. 2014). The prey may use chemical defences, such as toxins, irritants or venoms, before, during or after a predator attack. The chemicals can either function in defence itself and cause unpalatability of prey, or they can act as aposematic signals or serve both functions (Ruxton et al. 2004). Even though birds use primarily visual cues when recognizing aposematic prey (Marples et al. 1994, Ham et al. 2006), chemical defences play important role in prey avoidance learning and they can provide effective protection against bird predation (Skelhorn and Rowe 2006a, **Exnerová et al. 2015**, **Zvereva et al. 2018**, **Doktorovová et al. 2019**). Birds taste and reject chemically defended prey (Skelhorn and Rowe 2006a, d), which can result in survival of the prey (Wiklund and Järvi 1982, Sillén - Tullberg 1985a, **Zvereva et al. 2018**, **Doktorovová et al. 2019**). Prey possessing better chemical defences has higher chance of survival during predator attack and it is more likely that avian predator will learn to avoid it (Skelhorn and Rowe 2006a, d). If chemical defences of prey are less effective, birds may avoid ingesting a prey based on their level of hunger (Barnett et al. 2007) or the occurrence of alternative prey (Skelhorn and Rowe 2009).

Many chemically defended prey species emit warning odour when attacked (Rothschild and Moore 1987). Volatile chemicals of prey have been found to provide a defensive effect against avian predators, which may hesitate longer before attacking the chemically defended prey (**Zvereva et al. 2018**). Odours can act as discriminative cues for taste-avoidance learning in birds and sometimes they can even overshadow visual cues (Roper and Marples 1997). Nonetheless, when testing the effect of individual components of warning signals in avian predators, it was shown that most effective aversion was caused by multimodal signals interacting together, whereas warning odour itself was the least effective signal (Marples et al. 1994). Odour itself may (Guilford et al. 1987) or may not (Marples and Roper 1996) serve as sufficient aposematic signal.

Some prey species possess bitter-tasting chemicals and predators can use warning gustatory cues to recognize that prey is unpalatable and learn to avoid ingestion of it (Skelhorn and Rowe 2006a, b, **Zvereva et al. 2018**, **Doktorovová et al. 2019**). Unlike visual, olfactory or acoustic signals, gustatory warning signals can be perceived by predator only after attack. Nevertheless, it was found that taste can be effective cue for discriminating unpalatable prey in birds, although the rate of avoidance learning is depending on level of unpalatability of prey (Skelhorn and Rowe 2006c, d). The presence of more different bitter-tasting chemicals can cause better avoidance learning in predators (Skelhorn and Rowe 2005).

Chemical signals may also interact with visual signals and trigger hidden aversion (innate avoidance) of warning colouration in avian predators (Rowe end Guilford 1996, Marples and Roper 1996, Kelly and Marples 2004, Rowe and Skelhorn 2004). The aversion can be elicited by chemicals processed by gustatory (Rowe and Skelhorn 2004) as well as olfactory (Rowe and Guilford 1996) receptors of predators. Odour novelty is considered important in eliciting colour biases (Rowe and Guilford 1999b, Jetz et al. 2001). Chemical defences may also accelerate avoidance learning of visual signals and increase memorability of avoidance in birds (Siddall and Marples 2008). When chemical signals interact together with visual signals, chemical signals may be less effective for prey recognition in avian predators and defensive chemicals acting alone may not be sufficient enough for birds to identify familiar aposematic prey.

3.2.1 Production and storage of defensive chemical compounds

Aposematic prey can acquire the defensive chemicals either by synthesizing them *de novo*, or by actively sequestering them from the environment, mainly from food plants (Bowers 1992, Nishida 2002). The costs for *de novo* synthesis are assumed to be higher compared to the costs for sequestration (Bowers 1992), on the other hand, other study did not find the difference in costs between the two strategies of defence acquisition (Zvereva et al. 2017). The results of several studies have shown that chemical secretions sequestered from host plants are more effective against avian predators than autogenously (*de novo*) produced defences (Rowell - Rahier et al. 1995, **Zvereva et al. 2018**), resulting in longer latencies of predator to attack prey or faster avoidance learning of prey with sequestered chemicals **Zvereva et al. 2018**). Nevertheless, both sequestered and autogenous chemicals may serve as effective warning signals for avian predators, resulting in decreasing the possibility of attack, increasing the chance of the prey to survive the attack, or in increasing memorability of prey by predator (**Zvereva et al. 2018**).

The defensive chemicals may be stored either internally in the prey body, or in specialized integumental organs and secreted externally during predator´s attack (Pasteels et al. 1983). These two strategies, which are both widely used by prey, have different effects on the individual survival of prey. If the prey stores the chemicals inside the body, predator can detect it only after killing or seriously injuring the prey. This type of defence cannot protect an individual possessing it; on the other hand, it may protect other individuals due to avoidance learning and generalisation of predators (Skelhorn and Rowe, 2006d, Svádová et al. 2009). Contrastingly, chemicals secreted externally onto the body surface can affect a predator during or even before attack, possibly decreasing the chance of injury or death of prey (Sillén - Tullberg 1985a, Hotová Svádová et al. 2013, **Zvereva et al. 2018**). However, some predators can overcome this type of defence by wiping the chemicals from the surface of the prey (Skelhorn a Rowe 2006b).

The two different storage strategies of prey also differ in the extent of costs for prey; external secretion of chemical defences is likely to be costlier than storing compounds internally in the body (Bowers 1992). However, the findings of more studies have shown that secretion of defensive chemicals of prey externally seem to be more effective for avoidance learning of avian predators compared to storing defensive chemicals inside the prey body (Skelhorn and

Rowe 2006b, Hotová Svádová et al. 2013, **Zvereva et al. 2018**). Thus, although external secretion is costlier for prey, at the same time it seems to be more effective storage strategy of prey against avian predators; hence, both strategies have similar cost-benefit ratios for prey (discussed in **Zvereva et al. 2018**).

Although the majority of chemically defended prey possess only one of the two above-mentioned strategies (Zvereva and Kozlov 2016), some prey species, such as the leaf beetle *Chrysomela lapponica* (Coleoptera: Chrysomelidae), use both external secretion as well as storage in the body (Hilker and Schulz 1994), both acting together against bird predation (**Zvereva et al. 2018**). As the same chemical warning signal can be effective against one predator and ineffective against another (Ruxton et al. 2004), extra costs for maintenance of both secreted and stored chemicals by one prey species can be explained as a protection against different predators (**Zvereva et al. 2018**).

3.3 Acoustic signals

Acoustic signals are most widespread in insects and some of the sounds have been reported to serve a warning function or to cause an irritating effect in avian predators (Rowe and Guilford 1999a, Rowe 2002, **Kuklová, unpublished data**). Compared to visual and chemical signalling, the role of acoustic warning signals in prey recognition and avoidance learning in birds is less clear, as the few studies that have investigated this topic, have provided conflicting results. Acoustic signals may interact with visual signals and enhance innate avoidance of visual warning signals in birds (Rowe and Guilford 1999a), nonetheless, findings of other studies did not prove the effect of sound in enhancing hidden wariness for colours in birds (Hauglund et al. 2006, Siddall and Marples 2011). The presence of acoustic signals may also increase speed of avoidance learning in birds (Rowe and Guilford 1999a, Rowe 2002, **Kuklová, unpublished data**), on the other hand some studies reported contradictory results (Hauglund et al. 2006, Siddall and Marples 2011). To sum up, acoustic warning signals may or may not protect prey effectively from avian predators, and it is assumed that their function is in supporting role of signals from other modalities, mainly visual displays.

4 Predator psychology and effectiveness of warning signals

Reaction of predators toward aposematic prey may be affected by several cognitive mechanisms, such as neophobia (Exnerová et al. 2010, but see **Exnerová et al. 2015**), dietary conservatism (Marples et al. 1998) and aversion against particular warning signals, which can be either inherited (Schuler and Hesse 1985, Mastrota and Mench 1995) or learned (Aronsson and Gamberale-Stille 2008, **Doktorovová et al. 2019**). Unlearned wariness (innate avoidance) against warning signals of aposematic prey, which can cause avoiding of attack by predator, is beneficial for predator if prey is highly dangerous and the cost of attack is high (Ruxton et al. 2004). Apart from different taxa, even closely related avian predator species may differ in level of unlearned wariness against aposematic prey (Exnerová et al. 2007). When aposematic signals do not enhance unlearned wariness, predators learn to avoid prey and its warning signals (Sillén-Tullberg 1985a, Svádová et al. 2009). A warning signal that is learned more effectively by predators, or that is more readily to elicit unlearned wariness, can be considered a more effective signal (Stevens and Ruxton 2012). The response of a predator to aposematic signals is affected by associative learning, the degree of which is influenced by memorability, prey recognition, discrimination (Gamberale-Stille and Tullberg 1999, Ham et al. 2006, Svádová et al. 2009). Once predators learn to avoid a particular warning signal, they may generalize their experience with familiar prey to a novel aposematic prey possessing similar warning signal. Generalization can vary in symmetry; predators may generalize their experience with different warning signals only in one direction, or symmetrically in both directions (Svádová et al. 2009, **Doktorovová et al. 2019**).

It is important to bear in mind that the same aposematic signal of prey can be effective against one predator species or taxon and ineffective against another predators (Exnerová et al. 2003, Endler and Mappes 2004, **Zvereva et al. 2018**). Since different predators may have different sensory and cognitive abilities (Sol et al. 2005), the form of warning signal has to be detectable by the sensory system of relevant predators (Guilford and Dawkins 1991, Endler and Mappes 2004). Level of hunger or the occurrence of alternative prey may also be responsible for the individual variation in predator reactions toward aposematic prey (Barnett et al. 2007).

Besides interspecific differences in effectiveness of a warning signal, there may be different reactions to aposematic prey even within the same predator species. Some studies have shown different reactions between sexes of the same avian predators; stronger avoidance of aposematic prey was found in females compared to males (Mastrota and Mench 1994, **Exnerová et al. 2015**). Additionally, females also seemed to remember the avoidance of warning signal better than males (**Kuklová et al., to be submitted**). Intraspecific differences in behaviour of predators toward aposematic prey may also depend on age of predators; there is an evidence that adults learn to avoid aposematic prey and remember their experience more effectively than juvenile birds (**Kuklová et al., to be submitted**, Exnerová unpublished data). These results are supported by findings that adult birds discriminate among visual cues better compared to juveniles (Mirville et al. 2016, Franks and Thorogood 2018), which can be explained by their higher neophobia (Greenberg and Mettke-Hofmann 2001, Sherratt and Morand-Ferron 2018) together with their greater previous experience with unpalatable prey (Ihalainen et al. 2008). Hence, responses to aposematic prey in birds may change during life.

Variation in predator behaviour toward aposematic prey may also be influenced by other factors, specifically by diversity and abundance of local aposematic prey and predator previous experience with it (Ruxton et al. 2004). Consecutively, there may be population-specific differences in reaction to aposematic prey between bird populations inhabiting geographically distant areas and different habitats. Individuals from different populations may have equal ability to learn to avoid noxious prey, and they may also not differ in reactions to a prey which is novel for both geographically distant populations. However, predators that lack the experience with local aposematic prey may be more willing to attack novel aposematic prey compared to conspecific individuals from other population inhabiting area with high diversity of aposematic prey. Thus, the different responses to warning signals of prey in geographically distant predator populations may not correlate with predator´s neophobia and exploration strategy, but rather with different previous experience with local aposematic prey communities from the wild (**Exnerová et al. 2015**). Alternatively, predators may react to warning signals of aposematic prey based on their level of individual exploration and neophobia (see Liebl and Martin 2014).

4.1 Discrimination learning

Discrimination learning plays an essential role in evolution and maintenance of warning signals in aposematic prey (Mappes et al. 2005). It is a process, by which animals learn to distinguish between two or more different stimuli, one usually indicating a reward (positive stimulus, S+), and the other being either unrewarded or indicating punishment (negative stimulus, S-) (Shettleworth 2009). Discrimination learning is an important cognitive process, which allows predators to distinguish between profitable and unprofitable or noxious prey (Cott 1940, Ruxton et al. 2004), and consequently prevents predators from ingesting unpalatable food. Aposematic prey relies on ability of predators to recognize warning (aposematic) signals; these signals are cues for predators by which they can learn to identify the unprofitable prey. The ability to discriminate between stimuli is expected to improve with increasing experience of predators (Ruxton et al. 2004). Discrimination between prey species can be difficult for predators, especially in distinguishing between models and their accurate Batesian mimics (Sheratt 2002).

4.1.1 Simultaneous versus sequential predator-prey encounters

During the process of learning to discriminate between palatable and unpalatable prey, predators may encounter various types of prey either simultaneously or in a sequence one at a time, depending on prey frequencies or microhabitat preferences. This aspect determines whether the predators can directly compare different prey, which they are learning to discriminate. During sequential encounters, predators meet only one prey individual at a time, which does not enable them to compare different prey directly, but instead they have to compare the presented prey with the previous experience with another prey (Shapiro et al. 2008). For this reason, it is expected to be easier for predators to learn to discriminate between prey types when encountering them simultaneously rather than sequentially (Shettleworth 2009). The results of some studies are in line with this assumption (Lionello and Urcuioli 1998, Beatty and Franks 2012). Contrastingly, another experiment showed that simultaneous presentation of prey did not result in more effective discrimination learning in birds compared to sequential encounters (**Kuklová et al., to be submitted**). However, different results between these studies may be based on differences between the designs of experiments. To reflect the different forms of predator-prey encounters in the wild, simultaneous as well as sequential presentation of stimuli are frequently used in experiments focused on predator learning in context of aposematism and mimicry (Ihalainen et al. 2007, Ihalainen et al. 2012). Differences in experimental design are often considered to be responsible for sometimes contradictory results of studies focused on prey discrimination learning by predators (Lindström et al., 1997; Lindström et al., 2004, **Kuklová et al., to be submitted**).

It is important to consider the motivation of birds to handle the prey when interpreting the effectiveness of discrimination learning of avian predators during sequential and simultaneous encounter of prey, because the motivation to attack the prey may differ between sequential and simultaneous encounters. Different motivation of subjects in sequential and simultaneous encounters was assumed to affect mating preferences with stronger mate choice in simultaneous choice, which was explained by higher motivation to accept mating partners in sequential encounter because of no guarantee of another mating opportunity in the future in case of mate rejection. Thus, the cost of rejecting mating partner is lower in simultaneous encounter (Dougherty and Shuker 2015).

Analogically, there are different consequences of prey rejection depending on number of prey individuals the predator can meet at one moment. The cost associated with rejecting a particular prey may be greater in the sequential encounter compared to simultaneous encounter. Thus, predators may more likely decide to taste the prey in sequential encounter, compared to simultaneous presentation. This hypothesis is supported by the experiment, where the birds manipulated with more individuals of palatable as well as unpalatable prey in the sequential presentation compared to simultaneous encounter (Karlíková et al. 2016). By tasting more individuals of unpalatable prey, the predators encountering the prey in a sequence (one at a time) gain more experience with the prey unpalatability, and they may be more likely to develop stronger association between the discriminative stimulus and prey defences (Skelhorn and Rowe 2006b).

To sum up, when encountering prey types in a sequence, predator cannot directly compare them at the same time, however, higher motivation to attack the prey during sequential compared to simultaneous encounters may help the predator to learn to discriminate warning signals of aposematic prey, which may outweigh the disadvantage of sequential encounter of prey for discrimination learning.

Additionally, even though encountering several individuals of prey at once was assumed to result in more effective discrimination learning in predators (Tullberg al. 2000), another study has shown that multiple-choice presentation of prey may be the least effective for discrimination learning compared to sequential encounter or simultaneous encounter with two prey types (**Kuklová et al., to be submitted**). One of the mechanisms possibly contributing to the less effective discrimination learning of predators during multiple-choice encounters may be their limited attention; meeting more prey individuals at once may lead to errors in decision making due to competing of stimuli for predator´s attention (Dukas and Kamil 2001, Dukas 2002).

It may not always be important, whether predators encounter prey types simultaneously or sequentially when learning to discriminate between palatable and unpalatable prey. If the discriminative cue is highly salient, predators may learn to avoid aposematic prey equally effectively when meeting them in a sequence compared to simultaneous encounters. The dynamics of prey encounters might be more important when the discriminative cue is less salient (**Kuklová et al., to be submitted**).

Beatty and Franks (2012) hypothesised, that the aspect of sequential versus simultaneous encounter of prey might play a role in selection pressure for mimetic accuracy. More accurate Batesian mimicry was expected when models and mimics are coexisting and predators can directly compare them during simultaneous encounters, which could lead to better discrimination learning. In contrast, if the mimetic species are solitary and do not closely coexist, predators encounter models and mimics in a sequence one at a time, which could make discrimination between models and mimics more difficult and imperfect mimicry could be sufficient for mimic to survive (Stride 1956). Nonetheless, when discriminative cue is highly salient, predators may learn to discriminate between models and mimics equally effectively when encountering them simultaneously compared to sequential encounters. Moreover, if predators encounter several mimics and models at once, the multiple choice may have a confusing effect, which would decrease the effectiveness of discrimination learning (**Kuklová et al., to be submitted**).

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Contribution to the publications

I declare the following participation of **Lucia Kuklová (Doktorovová)** on the publications included in this thesis:

STUDY 1: Exnerová, A., Ježová, D., Štys, P., **Doktorovová, L.,** Rojas, B., & Mappes, J. (2015). Different reactions to aposematic prey in 2 geographically distant populations of great tits. *Behavioral Ecology*, 26(5), 1361-1370.

- performing part of the experiments, analysing data, writing and editing the manuscript

STUDY 2: Zvereva, E. L., **Doktorovová, L.,** Hotová Svádová, K., Zverev, V., Štys, P., Adamová-Ježová, D., Kozlov, M. V. & Exnerová, A. (2018). Defence strategies of *Chrysomela lapponica* (Coleoptera: Chrysomelidae) larvae: relative efficacy of secreted and stored defences against insect and avian predators. *Biological Journal of the Linnean Society*, 124(3), 533-546.

- designing and performing the experiments, analysing data, writing and editing the manuscript

STUDY 3: Doktorovová, L., Exnerová, A., Svádová, K. H., Štys, P., Adamová-Ježová, D., Zverev, V., Kozlov, M. & Zvereva, E. L. (2019). Differential Bird Responses to Colour Morphs of an Aposematic Leaf Beetle may Affect Variation in Morph Frequencies in Polymorphic Prey Populations. *Evolutionary Biology*, 46(1), 35-46.

- designing and performing the experiments, analysing data, writing the first draft of the manuscript, further manuscript editing

STUDY 4: Kuklová, L., Jůnová, L. & Exnerová, A. How type of task affects prey-discrimination learning in avian predators. (to be submitted to Ethology)

- designing and performing the experiments, analysing data, writing the first draft of the manuscript, further manuscript editing

On behalf of the co-authors, I agree with including the above-mentioned articles into the doctoral thesis of Lucia Kuklová.

alice Exner

doc. Mgr. Alice Exnerová, Ph.D.

Department of Zoology

Faculty of Science, Charles University

STUDY 1

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

Alice Exnerová, Dana Ježová, Pavel Štys,
Lucia Doktorovová, Bibiana Rojas, & Johanna Mappes (2015)

Behavioral Ecology, 26(5), 1361-1370

STUDY 2

Defence strategies of *Chrysomela lapponica* (Coleoptera: Chrysomelidae) larvae: relative efficacy of secreted and stored defences against insect and avian predators

Elena L. Zvereva, Lucia Doktorovová, Kateřina Hotová Svádová, Vitali Zverev,
Pavel Štys, Dana Adamová-Ježová, Mikhail V. Kozlov & Alice Exnerová (2018)

Biological Journal of the Linnean Society, 124(3), 533-546

STUDY 3

Differential Bird Responses to Colour Morphs of an Aposematic Leaf Beetle may Affect Variation in Morph Frequencies in Polymorphic Prey Populations

Lucia Doktorovová, Alice Exnerová, Kateřina Hotová Svádová, Pavel Štys,
Dana Adamová-Ježová, Vitali Zverev, Mikhail V. Kozlov, & Elena L. Zvereva (2019)

Evolutionary Biology, 46(1), 35-46

STUDY 4

How type of task affects prey-discrimination learning in avian predators

Lucia Kuklová, Lenka Jůnová & Alice Exnerová

to be submitted to Ethology

How type of task affects prey-discrimination learning in avian predators

Lucia Kuklová [1], Lenka Jůnová [1], Alice Exnerová [1]

[1] Department of Zoology, Faculty of Science, Charles University, Prague, Czech Republic

Abstract

When learning to discriminate between palatable and unpalatable prey, predators may encounter the prey either simultaneously or individually in a sequence. Both types of discrimination tasks are frequently used in experiments focused on predator learning in the context of aposematism and mimicry despite the fact that they may differ considerably in difficulty. Surprisingly, there are very few studies directly comparing performance of predators between sequential and simultaneous discrimination tasks using otherwise identical experimental design, same predator and prey. We tested an effect of the type of discrimination task on rate and effectiveness of discrimination learning in adult and juvenile great tits (*Parus major*). Birds were trained to discriminate between palatable and unpalatable paper "bugs" baited with mealworms soaked in either water or quinine; the shape, colours and patterns of the bugs were derived from real shield bug species). We compared the performance of birds tested with two discriminative cues, colour and pattern, using three discrimination tasks: 1) sequential - alternative presentation of one palatable and one unpalatable prey item; 2) two-choice - simultaneous presentation of one palatable and one unpalatable prey item; 3) multiple-choice - simultaneous presentation of multiple palatable and unpalatable prey items. Colour was more effective discriminative cue than pattern for both adult and juvenile birds. Adults performed better than juveniles regardless of task. The birds performed equally well in sequential and two-choice task, but their performance in multiple–choice task was worse than in the other two tasks. However, these differences were found only when the birds used pattern as a discriminative cue. The birds tested with colour, more salient cue by itself, performed equally well in all three tasks. The type of discrimination task may therefore affect learning performance of predators, but the effect also depends on effectiveness of a particular discriminative cue.

Keywords: experimental design, simultaneous task, sequential task, great tit, *Parus major*

Introduction

Discrimination learning is a process, by which animals learn to respond differently to different stimuli, one usually indicating a reward (positive stimulus, S+), and the other being either unrewarded or indicating punishment (negative stimulus, S-) (Shettleworth 2010). Since predators usually encounter prey of very different qualities, discrimination learning represents one of key predators' strategies, which allows them to distinguish between profitable and unprofitable or noxious prey (Ruxton et al. 2004). Predator discrimination learning plays an important role in evolution and maintenance of warning signals in aposematic prey (Mappes et al. 2005).

Birds are commonly used as subjects in studies involving discrimination learning, because their most important sense is vision (Lindström 1999). The majority of empirical studies were conducted under laboratory conditions using domestic pigeons (*Columba livia domestica*; Williams 1972, Lionello and Urcuioli 1998, Aust et al. 2008, Teng et al. 2015) and domestic chicks (*Gallus gallus domesticus*; Skelhorn and Rowe 2006, Aronsson and Gamberale-Stille 2008) as model avian species. However, since the experiments with natural predator species can better estimate the overall response of predators in the wild, insectivorous passerines started to be used more often in experiments, particularly in the last decades. Most frequently tested passerine species include great tits (*Parus major* L.; Lindström et al. 1997, Ihalainen et al. 2012), blue tits (*Cyanistes caeruleus*; Aronsson and Gamberale-Stille 2013), and starlings (*Sturnus vulgaris*; Skelhorn and Rowe 2009). Moreover, the experiments involve both wild-caught adults (Ham et al. 2006, Exnerová et al. 2015) and hand-reared juveniles (Lindström et al. 1999, Exnerová et al. 2010) as model avian species.

Live insects (Järvi et al. 1981, Sillén-Tullberg 1985, Svádová et al. 2009) as well as artificial prey items (Speed et al. 2000, Riipi et al. 2001, Aronsson and Gamberale-Stille 2008) are used as stimuli in studies of discrimination learning in birds. Additionally, in matching to sample tasks, subjects peck directly to response keys to obtain food (Dittrich et al. 1993, Lionello and Urcuioli 1998).

Predators can use various visual traits when learning to discriminate between palatable and unpalatable prey, however not all visual traits are equally effective (Aronsson and Gamberale-Stille 2008). Colour is likely to be the most salient cue for prey discrimination learning in bird predators (Terhune 1977, Gamberale-Stille and Guilford 2003, Aronsson and Gamberale-Stille 2008, 2012). Moreover, birds can associate any colour with positive or negative stimulus (Goldsmith and Goldsmith 1979, Meléndez-Ackerman et al. 1997, Ham et al. 2006, Aust et al. 2008). However, other visual traits may also be important for discrimination learning, although their role is less clear (Aronsson and Gamberale-Stille 2008, 2012). For instance, discrimination between patterns has been found easy for birds in some studies (e.g. Rowe at al. 2004) but difficult in others (e.g. Lyytinen et al. 1999).

Various experimental designs are used to test the effectiveness of discrimination learning. Majority of studies were conducted under laboratory conditions (Ham et a. 2006); however, some experiments were done in the field as well (Speed et al. 2000, Siddall and Marples 2011, (Dell´Aglio et al. 2016). Additionally, discrimination learning can be tested either in a natural context, where birds search for food the same way as they do in the wild (Skelhorn and Rowe 2009), or using operant conditioning chambers where birds are taught to peck to the response keys to obtain food first, and then they are trained to discriminate between different stimuli (Lionello and Urcuioli 1998).

During the process of learning to discriminate between palatable and unpalatable prey, predators may encounter various types of prey either simultaneously or in a sequence one at a time, depending on prey frequencies or microhabitat preferences. To reflect the different forms of predator-prey encounters in the wild, simultaneous as well as sequential presentation of stimuli are frequently used in experiments focused on predator learning in context of aposematism and mimicry. In the sequential task (Lindström et al 1997, Pegram and Rutowski 2014), only one prey item is offered at a time, not allowing the predator to compare different prey types directly. For this reason, sequential tasks are considered to be more difficult for predators than simultaneous tasks (Lionello and Urcuioli 1998, Shettleworth 2010, Beatty and Franks 2012), in which two or more prey items are offered at once (Lindström et al. 2006). On the other hand, sequential tasks might better reflect the situation in nature, where predators mostly encounter only one prey individual at a time. A question of differences in difficulty between sequential and simultaneous tasks might also play a role when testing resemblance between mimetic species (Stride 1956).

Simultaneous tasks further differ according to number of prey items presented at the same time: two-choice task (Lindström et al. 2001, Lyytinen et al. 2001) and multiple-choice task (Ihalainen et al. 2007, Hansen et al. 2010). Within sequential tasks, three types are used according to the order of presented prey types in a sequence: regular sequences (Fabricant et al. 2014), semi-randomised sequences, (Ihalainen et al. 2012, Pegram and Rutowski 2014) and fully random sequences (Lindström et al 1997, Skelhorn and Rowe 2009). Irregular types of sequential tasks are considered to better reflect the natural situation.

Besides the differences in number of presented stimuli, discrimination learning experiments also differ in the number of trials and trial durations. Discrimination tasks can also differ in the type and presence of negative stimulus. A frequently used negative stimulus is unpalatable prey (Skelhorn and Rowe 2009), which could result in more effective discrimination learning. Alternatively, a negative stimulus can be represented by no food delivered (Teng et al 2015, Gamberale-Stille et al. 2018) or by turning the light off (Lionello and Urcuioli 1998).

Differences in experimental design are often considered to be responsible for sometimes contradictory results of studies focused on prey discrimination learning by predators (Lindström et al., 1997; Lindström et al., 2004). Therefore, it is surprising that studies directly comparing performance of predators between sequential and simultaneous discrimination tasks using otherwise identical experimental design, same predator and prey species are scarce. Using human respondents and prey items displayed on a computer screen, Beatty and Franks (2012) directly compared difficulty of simultaneous and sequential tasks. Their results showed that sequential task was the more difficult one. Effect of simultaneous and sequential tasks on learning was also compared directly in pigeons. Birds learned to discriminate sooner in simultaneous task than in sequential task, but by the end of training, performance in two tasks were similar (Lionello and Urcuioli 1998).

In the present study, we tested an effect of the type of discrimination task on rate and effectiveness of prey-discrimination learning in adult and juvenile great tits. Our specific questions were as follows: 1) Is the rate and effectiveness of prey-discrimination learning affected by the type of discrimination task? 2) Does the type of discrimination task affect memorability of the learned discrimination? 3) Does the simultaneous presentation of both discriminated stimuli represent an easier discrimination task than when the stimuli are presented individually in a sequence? 4) Is colour more effective discriminative cue than pattern? 5) Does the performance in discrimination task differ between adult and juvenile birds?

Materials and Methods

Birds

Great tits (*Parus major*) are frequently used in studies involving discrimination learning because they easily learn how to handle artificial prey items (Ham et al. 2006). We used both wild-caught adults and hand-reared juveniles in our experiment. Adult birds were caught using mist nets in Prague (50.08 N, 14.24 E). The birds were then kept separately in indoor cages (50x40x50cm) under natural light and temperature conditions. Their diet consisted of mealworms (larvae of *Tenebrio molitor*), sunflower seeds and a commercial food mixture (Uni patee, Orlux); water was provided ad libitum. The birds participated in the experiment after 2 - 7 days of habituation to the laboratory conditions.

Juveniles were taken from nest boxes placed in Prague (Bohemia) when 12 - 15 days old; one or two nestlings were taken from a single brood. This way, they were naive as for any experience with unpalatable prey. Upon arrival, the birds were kept in small groups in artificial nest boxes and hand-reared. Mealworms, a commercial mixture for hand-rearing passerine birds (Handmix, Orlux) and boiled eggs were provided as food. After fledging, the birds were housed in cages under the same laboratory conditions as adults and hand-rearing continued until they were able to feed themselves. After that, they had free access to water and food (mealworms and commercial food mixtures (Oké-bird and Nutribird, Versele-Laga; Uni patee and Insect patee, Orlux). Experiments with juveniles started when they had reached a stage of full independence (after the 35th day of life).

We obtained permits to carry out the experiments from the Environmental Department of Municipality of Prague (S-MHMP-83637/2014/OZP-VII-3/R-8/F), Ministry of Agriculture (13060/2014-MZE-17214), and Ministry of the Environment of the Czech Republic (42521/ENV/14-2268/630/14). After experiments, the birds were ringed and released back to the sites of capture.

Artificial Prey

We used artificial prey items (15 x 10 mm) with visual traits based on real insect prey - shield bugs (Heteroptera: Pentatomidae). The artificial bugs (“bugs” further on) represented palatable or unpalatable prey (positive and negative stimuli) and birds were trained to discriminate between them either by colour or by pattern. The artificial prey items were designed to differ only in a single discriminative cue in order to ensure that the birds would be able to discriminate between two forms only by tested cue.

Bugs were made of cardboard and baited with halves of mealworms glued to their lower side using a non-toxic glue (Kores). Shape of the bugs was derived from *Palomena* shield bugs and they were printed with colours and/or patterns pertinent to the particular task. For a colour discrimination, we used monochromatic bugs, which differed only in their colour, either red or green (Fig. 1). For a pattern discrimination task, we used green bugs with spotted or striped black patterns (Fig. 1). The patterns were derived from those of *Eurydema* spp. (spotted) and *Graphosoma* spp. (striped) shield bugs. The colours and patterns were designed in Adobe Photoshop and printed using an RGB printer. We used following image parameters: 100% saturation and lightness and RGB parameters 0, 255, 0 for green bugs and 255, 0, 0 for red bugs.

The mealworms used for palatable prey variant were soaked in water. An unpalatable prey was made by soaking the mealworms in 6% quinine solution (chloroquine diphosphate); this concentration is known to be aversive for great tits (Ham et al. 2006). We observed bill wiping and head shaking behaviour of the birds following the contact with quinine-soaked mealworms during the experiment, confirming their unpalatability. All variants of prey were presented to the birds in glass Petri dishes against the achromatic grey (lightness 40%) background.

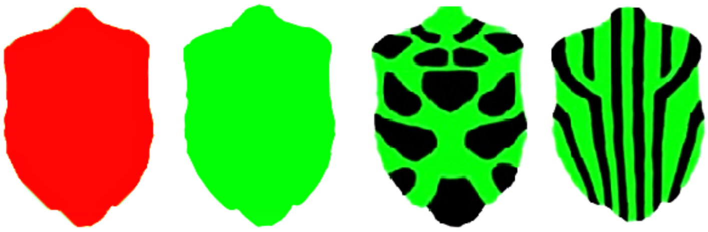


Fig. 1 Four types of silhouettes used in the experiment (from left to right): red and green (colour), spotted and striped (pattern).

Experimental Set-up

Experiments were conducted in Prague at the Faculty of Science, Charles University. All tests were performed in wooden cages (70 x 70 x 70 cm) with wire mesh walls, equipped with a perch, a water bowl and illuminated by bulbs (Biolux Combi 18W; Osram) simulating natural daylight. The front wall of the cage was made of one-way glass allowing us to observe the bird without disturbing it. The bugs were offered to the birds in glass Petri dishes placed on the rotating tray on the cage floor. During the multiple-choice experiments, Petri dishes with bugs were attached to a plate of corrugated cardboard instead of a tray. The plate was slid through a hatch into the front part of the cage floor. All trials were video-recorded, and behaviour was continuously noted using Observer XT 8.0 (Noldus). From 2 h prior to testing, the birds were familiarized with experimental cages and food deprived to increase their motivation to forage on artificial prey.

Experimental Design

The experiment was designed to test an effect of the type of discrimination task on rate and effectiveness of predator learning and memorability of aversion against aposematic prey. Birds were trained to discriminate between palatable and unpalatable prey in discrimination-learning task (training) and then re-tested in memory test.

We compared the performance of birds tested with two discriminative cues, colour and pattern, using three discrimination tasks: 1) sequential - alternative presentation of one palatable and one unpalatable prey item; 2) two-choice - simultaneous presentation of two prey items, one palatable and one unpalatable; 3) multiple-choice - simultaneous presentation of multiple items of palatable and unpalatable prey (see Scheme).

A total of 120 adult and 120 juvenile birds were used in the study. Prior to the experiment, adults as well as juveniles were divided into six experimental groups following the combinations of two discriminative cues and three tasks: colour/two-choice, colour/sequential, colour/multiple-choice, pattern/two-choice, pattern/sequential, pattern/multiple-choice, so that every experimental group included 20 adults and 20 juveniles. The birds were assigned to experimental groups randomly, but the groups of adults were balanced as for the sex and age. Each bird was tested individually and only once. Identical three-day tests were performed for both wild-caught and hand-reared birds. All parts of the experiment consisted of a series of consecutive trials.

Pre-training and Initial Preference Test (first day)

Prior to the discrimination-learning task, we pre-trained the birds to eat mealworms from the Petri dish and then to handle a white paper square with a piece of mealworm attached underneath.

Immediately after the successful pre-training, we tested whether the birds had an initial preference for one of the two prey types used in a particular task (see Scheme). The birds assigned to be tested with colour were offered red and green bugs, the birds from the group tested with pattern were offered spotted and striped bugs. Both prey types were presented simultaneously in the same Petri dish, and preferences were recorded in five successive 4-minute trials. The birds had to handle at least one prey item for the trial to be considered valid.

All mealworms offered in the preference test were palatable so that the birds learned to find food under both items. In all trials, we recorded which of the two prey types was handled as the first one, and we calculated preference scores for particular prey types.

Discrimination-Learning Task (training; second day)

The second part of the experiment consisted of training the birds to discriminate between palatable and unpalatable prey. We compared an effectiveness of two discriminative cues: 1) colour - for half of the birds, unpalatability was signalled with red colour, and for the other half with green colour, and 2) pattern: for half of the birds, spotted bugs were palatable and striped bugs unpalatable, and the reverse was true for the other half of the birds (see Scheme).

The birds were tested in three discrimination tasks using both discriminative cues. We used two types of simultaneous and one sequential presentation, in which the bird was offered one bug per trial. In all discrimination tasks, birds were offered altogether 40 palatable and 40 unpalatable prey items during the training. The three discrimination tasks were as follows:

(1) Two-choice task. Two prey items, one palatable and one unpalatable, were simultaneously offered in 40 consecutive trials, each lasting for a maximum of 4 min. Left-right positions of the two items in a Petri dish were changed across trials to prevent an association of a particular prey position with its palatability and to avoid an effect of preferred position on prey choice (this was also true for all simultaneous trials described below). The trial was considered valid if the bird handled at least one prey item. The birds had to handle at least three palatable and three unpalatable items during the first ten trials to ensure that they get an experience with both stimuli at the beginning of a training sequence (see Scheme).

(2) Sequential task. The two prey types were alternately offered in a semi-randomised order. We decided to use a semi-random sequence, because it better reflects the natural situation. The sequence, always starting with a palatable item, was generated prior to the experiment with respect to a maximum of three items of the same prey type in a row. Birds were first exposed to 15 trials of alternating presentations (in total of 30 trials). After that, five simultaneous trials followed, in which the two prey types were presented together in pairs. Consecutively, another 30 sequential and five simultaneous trials took place, identically as in the first half of the sequential task. In order to get the experience with both stimuli at the beginning of a training sequence, the birds had to handle at least three palatable and three unpalatable items during the first ten trials. At least every other bug offered during sequential series had to be handled so that the sequence could continue. Each sequential trial lasted 2 min at maximum and each simultaneous trial 4 min (see Scheme).

(3) Multiple-choice task. In the first half of the task, five palatable and five unpalatable prey items were placed in separate Petri dishes, randomly distributed on the cardboard plate and simultaneously offered to birds in three consecutive multiple-choice trials. Similarly to the sequential task, this part of training was followed by five simultaneous trials with the two prey types presented in pairs. The second half of the multiple-choice task was identical to the first one, consisting of three multiple-choice trials followed by five simultaneous trials. The birds had to handle at least three palatable and three unpalatable prey items in the first trial to get the experience with both stimuli. The rest of multiple-choice trials were considered valid if the bird handled at least five items irrespective of their palatability. The multiple-choice trials lasted a maximum of 30 min and simultaneous trials a maximum of 4 min (see Scheme).

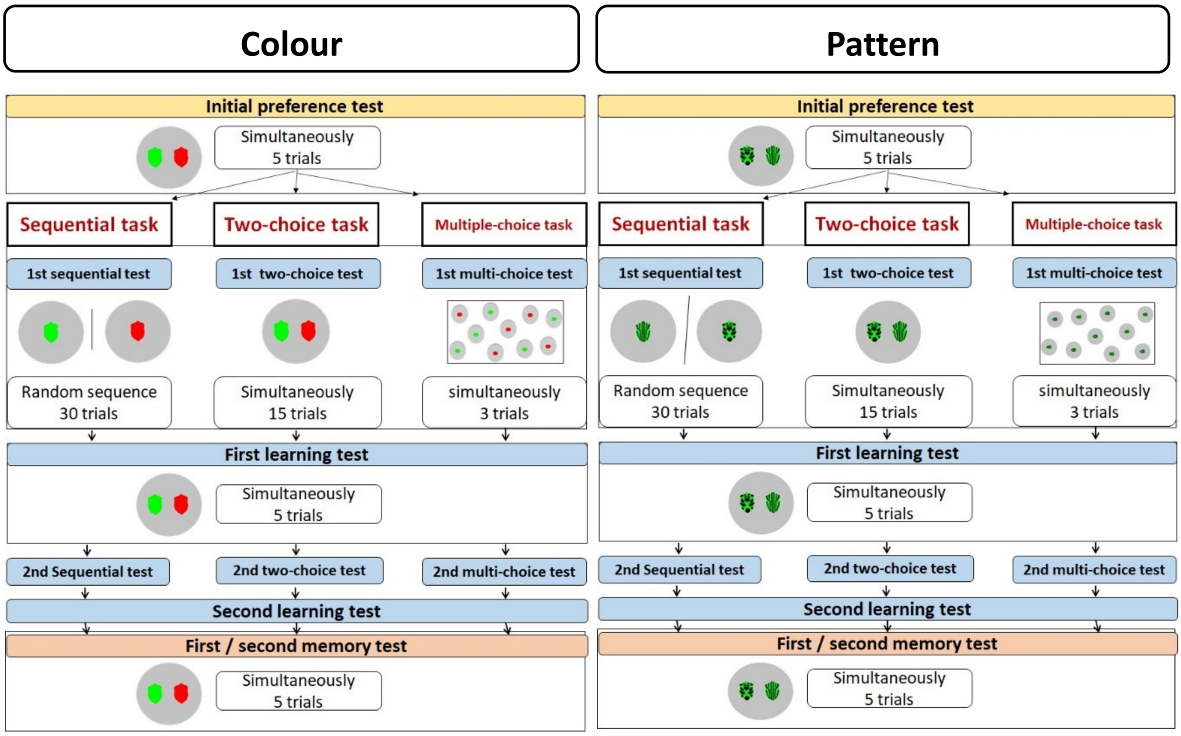
We included two blocks of five simultaneous trials (always lasting for 4 min each) in the sequential as well as the multiple-choice task to be able to compare directly the effectiveness of discrimination-learning between three tasks (see Scheme). Moreover, we examined a within-individual improvement of the bird during the experiment by comparing the performance of the same bird in simultaneous blocks in the middle and at the end of the training as well as in the first half of memory test (discrimination improvement). Whenever "first learning test" is mentioned in the following text, it refers to the first simultaneous block of five trials in training. We refer "second learning test" to the second simultaneous block of five trials in training, i.e. the last five trials of discrimination-learning task.

To measure the performance of birds in different tasks we recorded whether the bugs were attacked by the bird, and consequently we analysed the proportions of correct decisions in the first learning test and second learning test as well as in the memory test. We considered a decision correct if the bird attacked palatable prey as the first one (an attack being defined as touching the bug with the beak).

The general terms regarding validity of trials were true in all three days of the experiment as follows: Trial was always terminated earlier than maximum time if a bird ate or threw away all presented prey items. If a bird failed to attack required amount of prey, trial was repeated until the bird cooperated. This way we were ensuring of ongoing foraging motivation of the bird during the experiments.

Memory Test (Third Day)

In the memory test, carried out the following day after the training, we assessed the memorability of prey discrimination. For all the experimental groups, memory test consisted of ten simultaneous two-choice presentations using the same type of palatable and unpalatable prey as in the training (see Scheme). Each trial lasted 4 min (maximum). The bird had to choose at least one item to consider the trial valid. As in the training, the numbers of correct decisions were recorded.



Scheme: Experimental design

Statistical Analyses

A one-sample t-test was used to account for possible preferences for one of the prey types (green-red / spotted-striped) in the initial preference test. Differences between adults and juveniles in their preferences for a particular prey colour/pattern were analysed by t-test. We used Spearman rank correlations to analyse the relationship between the initial preference scores for future rewarded (positive) stimuli and the numbers of correct decisions at the end of the training sequence (second simultaneous block).

Discrimination-learning task (training) and memory test were analysed separately using the same type of models. We used analysis of variance in general linear modelling (lm anova) to compare the performance of birds in different types of discrimination tasks. Post-hoc comparisons between different groups were made by Tukey HSD test. Initial models included proportions of correct decisions in a particular learning or memory test as a response variable and the following explanatory variables: age (adults/ juveniles), cue (colour/ pattern) and task (sequential/ two-choice/ multiple-choice). When testing the performance of adults separately, sex (male/ female) and age class (yearlings/ older birds) were also included as explanatory variables in the models. When analysing differences between tasks for a particular cue separately, variant of a rewarded (positive) stimulus (red/green, spotted/striped) was also included as an explanatory variable in the models. The initial models were simplified to include only significant terms.

To compare the performance of the same bird in the first learning test and at second learning test, we applied general linear mixed-effect models (lme) with bird identity as a random factor and task, variant of a rewarded (positive) stimulus, and in case of testing adults also sex and age class as fixed factors. Proportions of correct decisions in the particular learning test was used as response variable. The initial models were simplified to include only significant terms. All statistical analyses were performed in R 2.15.1 (package nlme) with a 0.05 level of significance.

Results

1. Initial Preference Test

The birds did not show a preference to attack prey of any particular colour (one-sample t-test: $t = 0.140$, $P = 0.889$) or pattern ($t = -0.404$, $P = 0.687$); no differences between adults and juveniles were found (t-test; colour: t = -0.280, P = 0.781/ t-test; pattern: t = - 1.545, P = 0.125). In birds tested with colour as a cue, preference scores for future rewarded (positive) stimuli did not correlate with numbers of correct decisions in the second learning test (Spearman: r_s = 0.129, t = 1.411, P = 0.161). Therefore, potential preferences of birds for future palatable prey type did not help them to perform better in discrimination task. In birds tested with pattern, no correlation between preference scores for future rewarded stimuli and numbers of correct decisions in the second learning test was found in adults (r_s = -0.080, t = -0.614, P = 0.542), while this correlation was found in juvenile birds (r_s = 0.400, t = 3.325, P = 0.002).

2. Discrimination-Learning Task

a) Performance in the First Learning Test

The best fitting model explaining performance in the first learning test included only task as an explanatory variable (lm ANOVA, $F_{2,237}$ = 4.580, P = 0.011). Post-hoc comparisons showed significant differences between two-choice task and multiple-choice task (Tukey HSD, P = 0.009); therefore, in the early stage of training, birds performed better in two-choice task regardless of their age and the discriminative cue.

b) Performance it the Second Learning Test and in the Memory Test

Comparison of Adults and Juveniles

We started the models with the full dataset of 240 birds. We compared the performance of adults and juveniles in the second learning test and found significant effect of age: adults performed better than juveniles regardless of discriminative cue and task (lm ANOVA, $F_{1,235}$ = 19.153, P < 0.001). Adults also performed better than juveniles in the memory test ($F_{1,235}$ = 53.407, P < 0.001). Therefore, we split the data into subsets of adults and juveniles and analysed them separately.

Effect of Discriminative Cue

In adults, colour was more effective discriminative cue than pattern, which resulted in better performance of adults tested with colour in the second learning test (lm ANOVA, $F_{1,116}$

= 11.754, $P < 0.001$) as well as in the memory test ($F_{1,117} = 10.493$, $P = 0.002$). The effect of cue was significant also in juveniles: the birds tested with colour performed better in the second learning test ($F_{1,118} = 17.723$, $P < 0.001$) and also in the memory test ($F_{1,116} = 19.246$, $P < 0.001$). Therefore, we further analysed the effect of discrimination task in both adults and juveniles subsets separately for colour and pattern.

Effect of Discrimination Task

We compared the performance of birds in discrimination using a particular cue across three different tasks.

a) Birds tested with colour

Adult birds tested with colour as a discriminative cue performed equally well in all three types of tasks in the second learning test (lm ANOVA, $F_{2,56} = 0.128$, $P = 0.881$; Fig. 2a) as well as in the memory test ($F_{2,55} = 2.565$, $P = 0.086$). In the memory test, we found a significant effect of sex on performance of adults; females performed better than males ($F_{1,55} = 5.111$, $P = 0.028$). The task did not influence performance of juveniles tested with colour either, in the second learning test the birds performed equally well in all three types of tasks ($F_{2,56} = 0.506$, $P = 0.606$; Fig. 2b). However, a significant effect was found in the memory test ($F_{2,57} = 7.985$, $P < 0.001$), the juveniles tested in sequential task performed better than those tested in two-choice task (Tukey HSD, $P = 0.002$) or multiple-choice task ($P = 0.004$).

We examined a within-individual change in performance of birds during the experiment. Pairwise comparisons between the first and second learning test showed that adults improved their performance in colour discrimination regardless of the task (lme ANOVA, $F_{1,59} = 22.729$, $P < 0.001$; Fig. 3a). There was also a significant interaction between the age class of adults and their individual improvement ($F_{1,58} = 5.148$, $P = 0.027$); yearlings improved their performance less than older birds. When we compared individual performance of adults between the second learning test and the first memory test, birds did not further improve their discrimination; they discriminated equally well in the first memory test as they did in the second learning test ($F_{1,59} = 1.578$, $P = 0.214$). Juveniles also improved their colour discrimination regardless of the task ($F_{1,59} = 10.602$, $P = 0.002$; Fig. 3c). Individual improvement of juveniles between the second learning test and the first memory test depended on task: the birds tested in multiple-choice task continued to improve their performance ($F_{1,19} = 4.540$, $P = 0.046$), while the birds tested in two-choice ($F_{1,19} = 0.521$, $P = 0.480$) and sequential task ($F_{1,19} = 1.879$, $P = 0.186$) did not.

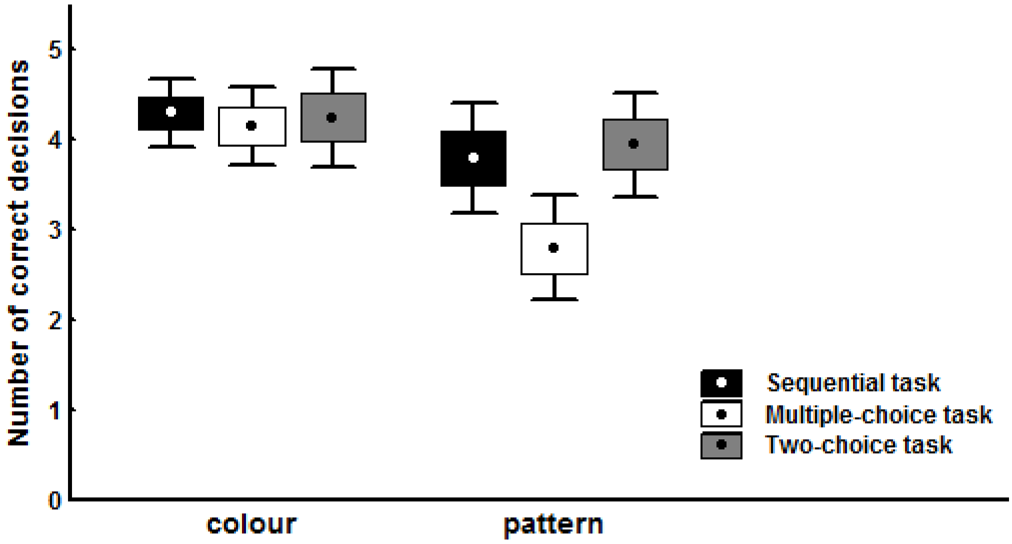


Fig. 2a Performance of adult birds in the second learning test: differences in the number of correct decisions of birds tested with either colour or pattern in three different tasks (Sequential, Multiple-choice, Two-choice). Boxes: mean +/- SE, whiskers: mean +/- 0.95 Conf. Int.

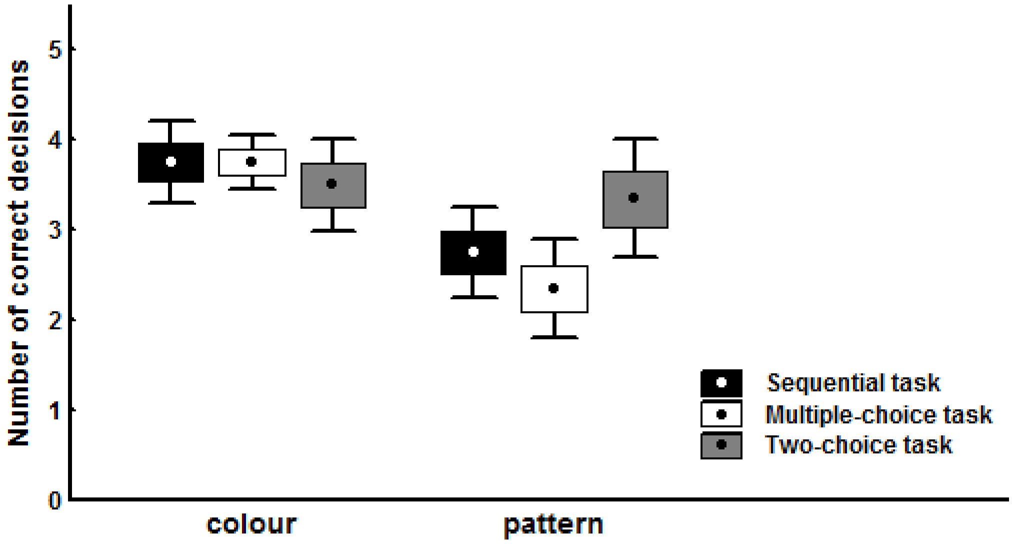
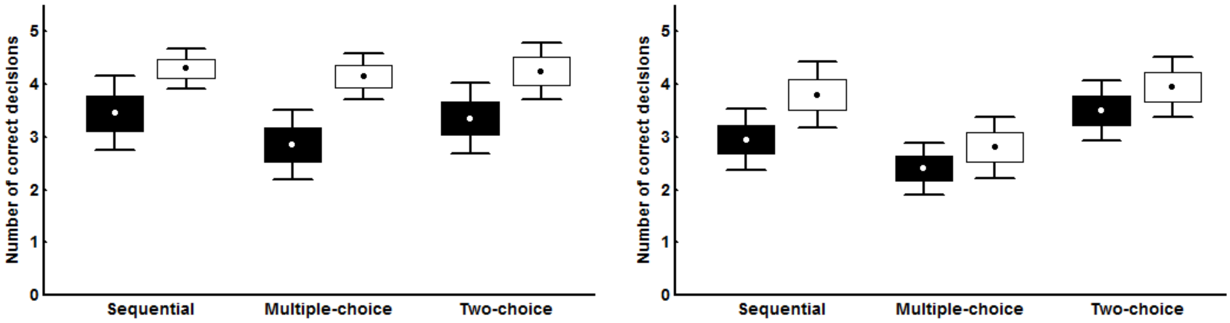


Fig. 2b Performance of juvenile birds in the second learning test: difference in the number of correct decisions of birds tested with either colour or pattern in three different tasks (Sequential, Multiple-choice, Two-choice). Boxes: mean +/- SE, whiskers: mean +/- 0.95 Conf. Int.

b) *Birds tested with pattern*

Performance of adult birds tested with pattern as a discriminative cue was affected by type of discrimination task in the second learning test (lm ANOVA, $F_{2,57} = 4.878$, $P = 0.011$; Fig. 2a); birds performed worse in multiple-choice task in comparison to both sequential task (Tukey HSD, $P = 0.040$) and two-choice task ($P = 0.016$). Similar differences between the tasks in performance of adults were found in the memory test ($F_{2,57}$ = 5.315, P = 0.008); birds performed worse in multiple-choice task than in two-choice task (P = 0.005). An effect of task on performance in the second learning test was also found in juveniles ($F_{2,56}$ = 3.599, P = 0.034; Fig. 2b); birds performed worse in multiple-choice task than in two-choice task (P = 0.032). In juveniles, there was also a significant effect of a variant of a rewarded (positive) stimulus on their performance - birds tested with striped pattern as a rewarded stimulus performed better in the second learning test than birds tested with spotted pattern ($F_{1,56}$ = 4.274, P = 0.043). In juveniles tested with pattern, performance in memory test was not affected by task ($F_{2,56}$ = 2.691, P = 0.077), but there was a significant effect of a variant of a rewarded stimulus - birds tested with striped pattern as a rewarded stimulus remembered the discrimination better than birds tested with spotted pattern ($F_{1,56}$ = 11.339, P = 0.001).

3a) adults colour **3b) adults pattern**



3c) juveniles colour **3d) juveniles pattern**

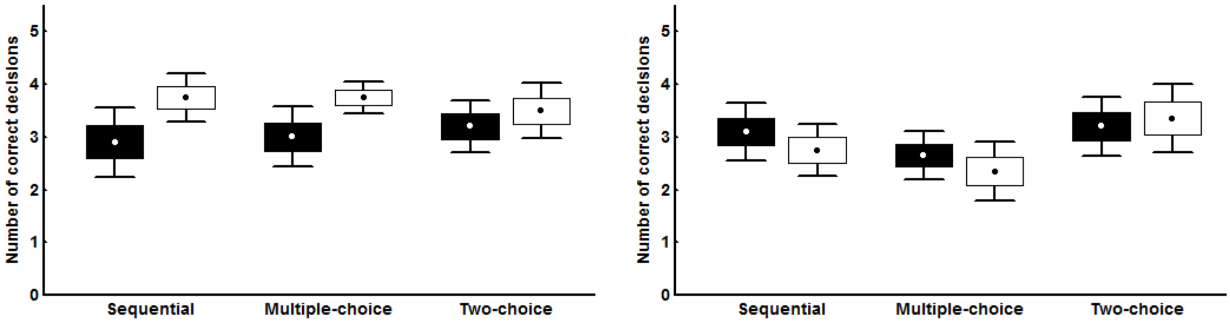


Fig. 3 Pairwise comparisons of performances of birds between first learning test (black boxes) and second learning test (white boxes): differences in the number of correct decisions of birds tested with three different tasks (Sequential, Multiple-choice, Two-choice), 3a) adults tested with colour, 3b) adults tested with pattern, 3c) juveniles tested with colour, 3d) juveniles tested with pattern. Boxes: mean +/- SE, whiskers: mean +/- 0.95 Conf. Int.

We examined a within-individual change in performance of birds during the experiment. Pairwise comparisons between the performance in the first and second learning test showed that the adults significantly improved their pattern discrimination (lme ANOVA, $F_{1,59} = 6.506$, $P = 0.013$; Fig. 3b), however there was also a significant effect of task ($F_{2,57} = 8.966$, $P < 0.001$). Adult birds improved their discrimination in sequential task ($F_{1,19} = 5.033$, $P = 0.037$), and there was also an effect of a variant of a rewarded stimulus - birds performed better when striped pattern was a rewarded stimulus ($F_{1,18} = 5.285$, $P = 0.034$). Adults did not improve their performance in multiple-choice task ($F_{1,19} = 1.070$. $P = 0.314$). For adults tested in two-choice task, comparison of first and second learning test showed no improvement ($F_{1,19} = 1,263$, $P = 0.275$). Comparison of individual performance of adults between the second learning test and the first memory test shows that the birds did not further improve their discrimination ($F_{1,59} = 3.877$, $P = 0.054$). Juveniles did not improve their pattern discrimination in any of the tasks ($F_{1,59} = 0.711$, $P = 0.403$; Fig.3d) and did not change their performance between second learning test and the first memory test either ($F_{1,59} = 0.054$, $P = 0.818$).

Discussion

Differences in experimental design are often considered to be responsible for sometimes contradictory results of studies focused on prey discrimination learning by predators (Lindström et al., 1997; Lindström et al., 2004). One of the aspects, in which experimental setups used for discrimination tasks differ, is the number of prey items that predators encounter at the same time. This aspect determines whether the predators can directly compare different prey types they are learning to discriminate.

In this study, we compared the performance of avian predators between three types of discrimination tasks using otherwise identical experimental design and artificial prey. This way, we controlled for other factors that might contribute to potential differences in performance of birds tested in different tasks.

Initial Preference Test

At the beginning of the experiment, we tested whether the birds had any initial preference for one of the two prey types, which could potentially affect their performance in discrimination-learning task. Neither adults nor juveniles showed any preference for a particular prey colour or pattern. In case of juveniles, this result is not surprising since in previous studies great tits did not show any innate avoidance of aposematic prey (Exnerová et al., 2007, but see Lindström et al. 1999). Absence of initial colour preferences in wild-caught adults indicates, that they probably did not generalise their experience with real aposematic prey from the wild towards the red artificial prey due to the absence of a contrasting black pattern. Moreover, reactions of adult birds could be influenced by positive experience with red fruits (Gamberale-Stille and Tullberg 2001, Schmidt & Schaefer 2004, Gamberale-Stille et al. 2007).

Discrimination-Learning Task

Comparison of Adults and Juveniles

Adult birds performed significantly better than juveniles in the second learning test and also in the memory test regardless of discriminative cue and type of the task. More effective discrimination of visual cues in adults than in juveniles was also found in previous experiments with great tits (Exnerová, own data) and other avian species (Mirville et al. 2016, Franks and Thorogood 2018). An effective prey discrimination learning in adults can be explained by their higher neophobia (Greenberg & Mettke-Hofmann 2001, Sherratt & Morand-Ferron 2018) and also by their previous experience with unpalatable prey from the wild; both factors could facilitate further prey-discrimination learning. In contrast, juvenile birds did not have any experience with unpalatable prey until the start of the experiment, which might have caused their lesser initial cautiousness and also the fact that they needed more experience with prey unpalatability during the experiment to learn the discrimination. Findings of other experiments also show that effectivity of prey discrimination learning depends on the previous experience of avian predators (Ihalainen et al. 2008).

Besides the differences in performance between adults and juveniles, there was also a further effect of age within the group of wild-caught adults tested with colour; yearlings improved their performance less than older birds.

Our results suggest that age of tested avian predators together with their previous experience with unpalatable prey can significantly affect the results of experiments. This should be taken into account when comparing experiments focused on discrimination learning, where predators differ in their age and history.

Effect of Discriminative Cue

We compared the performance of birds tested with two discriminative cues - colour and pattern. Our results support the hypothesis that, for avian predators, colour is more salient cue for prey discrimination than pattern (Kazemi et al. 2014), and we found this effect in both adult and juvenile birds. The birds tested with colour learned to discriminate more effectively and also performed better in the memory test. Our findings are in line with previous studies showing that colour is the most salient visual discriminative cue for avian predators (Terhune 1977, Gamberale-Stille and Guilford 2003, Aronsson and Gamberale-Stille 2012).

We found differences between males and females in their performance in colour discrimination task with females performing better than males during the memory test. Stronger avoidance of aposematic prey in females than in males was found also in another study testing great tits (Exnerová 2015). Likewise, female bobwhites *Colinus virginianus* showed stronger avoidance of red and orange food than did the males (Mastrota and Mench 1994). In addition, females in our study discriminated better than males regardless of which stimulus signalled palatable prey, thus females performed better with green as well as red colour as a rewarded (positive) stimulus. Better memory of female great tits compared to males was also found in observation learning task, where birds located food caches after observing another bird storing the food (Brodin and Urhan 2015). Similarly, faster auditory discrimination learning in females than in males was found in zebra finches *Taeniopygia guttata* (Kriengwatana et al. 2016).

Effect of Discrimination Task

We tested whether simultaneous presentation of discriminated stimuli represents easier discrimination task than when the stimuli are presented individually in a sequence. Based on previous studies (Lionello and Urcuioli 1998, Shettleworth 2009, Beatty and Franks 2012), we predicted that birds would find it easier to distinguish between the stimuli when they encounter them simultaneously rather than sequentially, because of the possibility to directly compare discriminated prey types at the same time.

The type of discrimination task did not affect discrimination learning in birds tested with colour; the birds performed equally well in all three tasks. This result suggests that if the discriminative cue is highly salient, predators perform equally well regardless of task and it may therefore not be important, which type of discrimination task is used in a particular experiment. The only significant effect of task on performance of birds tested with colour was that juveniles showed better memory in sequential task than in both two-choice and multiple-choice tasks. This result is consistent with the fact that juveniles generally performed worse than adults. The differences in task difficulty therefore affected their performance in colour discrimination despite the high salience of colour. This effect was evident only in the memory test, in which the juveniles also performed worse than the adults. The effect of age on memory was found also in another study, where yearlings of mountain chickadees *Poecile gambeli* showed worse memory for cache location than adults (Tello-Ramos et al. 2018).

Performance of adult birds tested with pattern was affected by type of discrimination task: birds generally performed worse in the multiple-choice task in comparison to other two tasks. This difference was evident in both the discrimination learning and the memory test. For adults tested in two-choice task, comparison of first and second learning test showed no improvement. However, the absence of a difference in performance of birds in the second learning test between the sequential and two-choice task supports the evidence, that adult birds improved their performance in pattern discrimination also in the simultaneous task and it is likeably, that this group improved their discrimination even earlier in the training.

Juveniles in general did not learn to discriminate as effectively as adults; they did not significantly improve their performance during the training with pattern as a discriminative cue, which also adds to the evidence that pattern was less effective than colour.

Juveniles did not learn successfully the pattern discrimination in any of the tasks and did not improve their performance between training and the first memory test, so none of particular patterns (striped or spotted) helped them to perform better in discrimination. Even though the significant correlation between initial preference for future rewarded (positive) stimulus and performance in the second learning test was found in juveniles tested with pattern, potential preference of birds for future palatable prey type did not help them to perform better, as they did not learn successfully the pattern discrimination.

Nevertheless, there was a significant effect of a variant of a rewarded (positive) stimulus on performance of juveniles - birds tested with striped pattern as the rewarded stimulus performed better than birds tested with spotted pattern in the second learning test as well as in the memory test. Effect of rewarded stimulus on performance was also found in adults tested with pattern, but only in the sequential task. Consistently with juveniles, adults also performed better when striped pattern was the rewarded stimulus. Striped pattern may therefore be more easily associated with prey palatability than spotted pattern. Alternatively, adult birds were likely to have more experience from the wild with unpalatable prey resembling the spotted experimental prey compared to the striped one, and this could help them to associate the spotted prey with unpalatability. Effectiveness of striped pattern in prey discrimination was also found in the study with blue tits, which learned to discriminate between palatable and unpalatable prey faster when the prey items were striped compared to monochromatic ones (Aronsson and Gamberale-Stille 2013). Contrastingly, domestic chicks learned to discriminate palatable and unpalatable prey equally well regardless whether the pattern consisted of stripes or spots (Aronsson and Gamberale-Stille 2008).

Sequential versus Two-choice Task

Simultaneous discrimination task should theoretically be easier for predator compared to a sequential one, in which the subjects cannot compare the two prey items directly, but instead they have to compare the presented prey with the previous experience with another prey (Shapiro et al. 2008). For this reason, we predicted that it would be more difficult for birds to discriminate between the two prey types in sequential task, without possibility of direct comparison. Surprisingly, we have found almost no differences between sequential and two-choice task in our experiment.

There are two previous studies that directly compared the difficulty of sequential and two-choice discrimination tasks (Lionello and Urcuioli 1998, Beatty and Franks 2012). Results of both of studies showed sequential task as more difficult than simultaneous task, which is in contrast to our findings. However, both previous studies used matching to sample task and comparing their results with our experiment has to be done with caution due to more differences between the designs. The main question for the subjects in the two above-mentioned studies (pigeons in the former study and humans in the latter) was to recognise whether the two stimuli were identical or not. In contrast, the two stimuli (palatable and unpalatable prey) in our study were by default always different and the subjects learned to associate them with prey palatability or unpalatability. Consequently, in matching to sample design, simultaneous task could help the subjects to better distinguish if the prey items were identical. It could be beneficial especially for discriminating between very similar items, such as in the study of Beatty and Franks (2012), who used butterfly images displayed on a computer screen. In contrast, in our study where the stimuli were never identical, simultaneous task might not bring another benefit compared to sequential task. The differences between our results and the previous two studies could also be explained by absence of unpalatable prey in previous two studies, which could cause different motivation for discrimination learning in subjects. As for the study of Lionello and Urcuioli (1998), it should also be noted, that although sequential task was identified as more difficult than simultaneous, the reason was that the subjects learned to discriminate sooner in simultaneous task. However, by the end of the training, performance in the two tasks did not differ. These results may suggest, that the subjects tested in discrimination tasks with different levels of difficulty may need various time until they learn to discriminate, and hence the duration of discrimination tasks could be one of the factors impacting whether discrimination learning will be successful.

In order to interpret our results correctly, we should not simply make a conclusion about difficulty of various discrimination tasks based on performance of birds in particular tasks; it is also important to consider the motivation of birds to handle the prey, which may differ between the tasks. Different motivation of tested subjects in sequential and simultaneous tasks was suggested as a reason for differences in mating preferences. The meta-analysis of 38 studies and 40 animal species including birds has shown that type of task affected mating preferences, with stronger mate choice found in simultaneous choice (Dougherty and Shuker 2015). The authors suggested that this difference was caused by increased cost of rejecting mating partners in a sequential task, because there is no guarantee of another mating opportunity in the future in case of mate rejection. Analogically, there are different consequences of prey rejection depending on number of prey items the predator can approach at one moment. In the sequential task, the cost associated with rejecting a particular prey may be greater compared to simultaneous task. Therefore, predators may more frequently decide to taste the prey in a sequential task, compared to simultaneous task, where they get access to more prey items at once. This hypothesis is supported by the results of Karlíková et al. (2016), who tested the ability of great tits to discriminate between palatable and unpalatable artificial prey according to subtle body-shape cues, and found that the birds handled more items of palatable as well as unpalatable prey in the sequential task compared to simultaneous task.

By tasting more items of unpalatable prey, the birds tested in the sequential task get more experience with the prey unpalatability, and they may be more likely to develop stronger association between the discriminative cue and prey defences (Skelhorn and Rowe 2006b). Therefore, even though predators cannot directly compare the discriminated prey types when encountering them in a sequence, higher motivation to attack the prey during sequential compared to simultaneous task may help them to get more information about discriminated palatable and unpalatable prey, which may outweigh the disadvantage of sequential task in discrimination learning. However, predator motivation to sample the prey during sequential encounters may also depend on the availability of alternative food in the habitat.

The predator's decision to attack prey in the sequential task can also be affected by a degree of prey unpalatability; if the prey is not unpalatable or toxic enough, predator may accept it to avoid starvation. For example, starlings tested with bitter tasting but non-toxic prey, attacked both palatable and unpalatable prey items when presented sequentially and only avoided unpalatable prey in the preference test with both prey types presented simultaneously at the end of the experiment (Skelhorn and Rowe 2009).

Using a theoretical model, Kacelnik et al. (2001) were comparing the benefits of predator adaptations to sequential or simultaneous encounters with prey. Assuming, that simultaneous encounters with several different prey types are rare in nature, and that predators often meet only a single prey at a time, they consider adaptations of predators for sequential choices evolutionary more stable compared to mechanisms of choices in simultaneous encounters. The model predicts, that even if those mechanisms impose a cost in the relatively rare simultaneous choices, animals rely on the same process when facing different prey types simultaneously, as they would during the sequential encounters. Hence, they process each prey item independently by evaluating their relative worth (Kacelnik et al. 2001). However, simultaneous encounters of predators with several prey types may not be as uncommon in nature as Kacelnik et al. (2001) assume. Predators may, for instance, encounter several prey species feeding on the same host plant, an aggregation of different life stages (or even species) or simply a high density of more than one species in suitable habitats and localities.

Effectivity of Multiple-choice Task

We predicted that the possibility to directly compare several items of both prey types would result in more effective prey discrimination in multiple-choice task. Contrary to our predictions, the birds performed worse in multiple-choice task than in other two tasks. The birds tested with colour did not learn the multiple-choice task more effectively than the other two tasks, and the performance of birds tested with pattern was even worse in the multiple-choice task compared to other two tasks. Thus, the multiple-choice task seemed to be the most difficult one for the birds to learn. This effect was evidenced in the group of birds tested with pattern as a discriminative cue, which by itself was more difficult compared to colour. Our results show that already in the early stage of discrimination training (first learning test), there were differences in learning between the tasks: the birds performed better in two-choice task than in multiple-choice task regardless of their age and discriminative cue. This result supports the view of multiple-choice task being the most difficult one, because the birds tested in other two tasks learned to discriminate considerably faster. The view that multiple-choice task might in fact be more difficult than the other two tasks is also indirectly supported by comparison of performance in the second learning test and in the first memory test. Juveniles tested with colour in multiple-choice task continued to improve their performance, which could mean that in this task the birds needed more trials to learn the discrimination compared to the other two tasks.

One of the mechanisms possibly contributing to the worse results of birds tested in multiple-choice task may be limited attention, which is known to affect the diet choice in predators (Dukas 2002). Due to the limited capacity to process information about several prey items presented simultaneously, individual items may in effect compete for predator's attention, which could lead to errors in decision making. Thus, it may be more effective to focus attention on a single prey than to divide attention among several prey items (Dukas and Kamil 2001). However, focusing attention to one prey type is known to be effective when predators form search images when search for cryptic prey. In contrast, when the prey is conspicuous, predators may divide their attention among several prey types and therefore learn to avoid unpalatable prey (Dukas and Ellner 1993).

Even though it is possible for predators to directly compare the discriminated prey types when encountering them at the same time, lower motivation to attack the prey due to smaller cost of prey rejection compared to avoiding a single prey item could result in gaining less experience with palatable and unpalatable prey, and possibly less effective discrimination learning (see analogy with Dougherty and Shuker 2015 above).

Responses of predators to palatable prey in multiple-choice task may also be affected by co-occurrence of unpalatable items, which was shown to be an advantage of gregariousness in aposematic prey (Riipi et al. 2001).

Since the multiple-choice task turned out to be the most difficult for birds in our experiment, future comparison of effectivity of more multiple-choice tasks differing in numbers of presented prey could reveal more about the reasons why this type of task seems to be so difficult.

Implications for simultaneous vs. sequential predator-prey encounters

In summary, our study has shown that the differences in experimental design and particularly in type of the discrimination task may significantly contribute to differences between the results of studies focused on discrimination learning. However, if discriminative cue is highly salient, it might not be so important which type of discrimination task is used, and using different types of task in this case would not lead to different results. Nevertheless, the type of discrimination task may affect learning performance in cases of less salient discriminative stimuli. Therefore, when testing the function of aposematic signals and assessing predator discrimination learning, both the salience of cue and the type of discrimination task should be taken into account.

Differences in effectiveness of predator learning when confronted with sequential versus simultaneous discrimination tasks might belong to important factors affecting the degree of mimetic accuracy between the species engaged in mimetic complexes (Beatty and Franks 2012). Higher degree of mimetic resemblance could be expected if the mimetic species are closely coexisting and gregarious, because predators can frequently encounter the models and mimics together and compare their appearance directly, which could increase their chance to discover the differences and learn to discriminate between them. In contrast, if the mimetic species are solitary and do not closely coexist, predators can only compare presently encountered prey with their experience with another prey in the past, which could make discrimination between models and mimics more difficult (Stride 1956). Whether predators encounter mimetic species simultaneously or sequentially may have an important effect on the evolution of imperfect mimicry and be particularly important for Batesian mimics. More accurate Batesian mimicry is usually expected when models and mimics can be encountered simultaneously, whereas the situations when predators encounter models and mimics in a sequence could favour less accurate mimicry (Beatty and Franks 2012).

Considering the findings of our study, we hypothesise, that depending on discriminative cues that predators use, it may not always be important, whether predators encounter mimetic species simultaneously or sequentially. When discriminative cues are highly salient, predators may learn to discriminate between models and mimics equally effectively when meeting them at the same time compared to sequential encounters. The dynamics of encounters might be more important when the discriminative cue is less salient. Moreover, if predators encounter a number of mimics and models at once, the multiple choice may in fact have a confusing effect which would hinder the discrimination learning.

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6 Conclusions

- Great tits (*Parus major*) from the Bohemian population mostly avoided aposematic firebugs (*Pyrrhocoris apterus*) and attacked non-aposematic variant. Finnish birds, which lacked experience with firebugs from their natural environment, were less hesitant to attack both firebug colour forms. Although the Bohemian birds avoided the aposematic prey variant, they were not more neophobic than Finnish birds. Differences between Finnish and Bohemian great tits in behaviour to aposematic variant did not correlate with the differences in exploration strategy and neophobia. We conclude that the geographic differences in reactions of the birds to aposematic prey can be explained by a different population-specific experience of the birds with local aposematic prey communities. Our findings indicate that studies on aposematism and mimicry based on geographically distant conspecific populations can be compared and generalized.

- Volatile external secretions of larvae of the leaf beetle (*Chrysomela lapponica*) provided a direct defensive effect against avian predators. Great tits also learned to avoid larvae devoid of secretions, confirming the anti-predatory function of non-volatile compounds within the larval body. However, survival after the first attack was greater for larvae with intact secretions than for larvae with depleted secretions. Hence, both strategies of storage of chemicals, external secretion and storage in the body, act together against bird predation. Secretions of *C. lapponica* larvae sequestered from host plants were more effective against avian predators than autogenously (*de novo*) produced secretions. Nevertheless, both strategies of chemical production may provide effective protection against birds. We conclude that avian predation could contribute to the evolution of both secreted and stored defensive chemicals in *C. lapponica* larvae.

- Juvenile great tits attacked all conspecific colour morphs (red-and-black light, red-and-black dark and metallic) of aposematic leaf beetles (*Chrysomela lapponica*) at the same rate, while adults attacked light beetles at first encounter more frequently than both dark and metallic beetles. The numbers of beetles that survived bird attack did not differ among morphs. Avoidance learning was similarly fast for all three morphs, hence red-and-black as well as metallic (iridescent) displays of morphs function as effective warning signals. Morphs differed in their memorability and ability to elicit generalisation in birds. We hypothesise that when aposematic signals of polymorphic prey vary in their conspicuousness for avian predators, colour morphs with less conspicuous display have a selective advantage at low population densities but they lose this advantage at high population densities due to the low memorability of their display. Thus, the direction of selective bird predation on aposematic morphs may depend on prey density, contributing to cyclic shifts in the morph frequencies.

- Colour was more effective discriminative cue than pattern for both adult and juvenile great tits. The birds performed equally well in sequential and two-choice task, but their performance in multiple–choice task was worse than in the other two tasks. However, these differences were found only when the birds used pattern as a discriminative cue. The birds tested with colour, more salient cue by itself, performed equally well in all three tasks. We conclude that the type of the discrimination task may affect the results of studies focused on discrimination learning, but the effect also depends on effectiveness of a particular discriminative cue; if a cue is highly salient, the type of used task might not be so important. Nevertheless, the type of task may affect learning performance in cases of less salient discriminative stimuli. Consequently, we hypothesise the importance of dynamics of encounters of the species engaged in mimetic complexes on the evolution of imperfect mimicry.